Factors affecting *Plasmodium falciparum* sporozoite formation in *Anopheles* mosquitoes

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Abstract

The relative contribution of different factors, including environmental and genetic variables, on the observed variation in genome numbers per oocyst was investigated. The variation in sporozoite numbers from two genetically different *Plasmodium falciparum* clones (3D7 and HB3) in *Anopheles gambiae* mosquitoes, and the pattern of inheritance of the phenotype was investigated.

Membrane-feeding of cultured *P. falciparum* parasites to laboratory-reared *Anopheles* mosquitoes produced infected midguts for dissection. Microdissection of single oocysts from the infected midguts and the employment of polymerase chain reaction techniques allowed the products of self- and cross-fertilisation to be distinguished, and the number of genomes per oocyst to be counted. Utilising these methods allowed comparison of the relative productivity of oocysts from different genetic and environmental backgrounds.

Environmental factors such as the size of the mosquito and the number of oocysts on the midgut appeared to have no effect on the number of genomes per oocyst. However the genomes per oocyst were signifcantly different when oocysts from the same clone were isolated from two different *Anopheles* mosquito species.

The two parasite clones differed significantly in the number of genomes per oocyst they produced, and this trait was inherited in a dominant fashion.

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Author's declaration

I hereby solemnly declare that the work presented in this thesis is my own, unless otherwise stated, and that it has not been submitted for any other degree previously.

Signed

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1 Chapter 1. General Introduction

1.1 Introduction

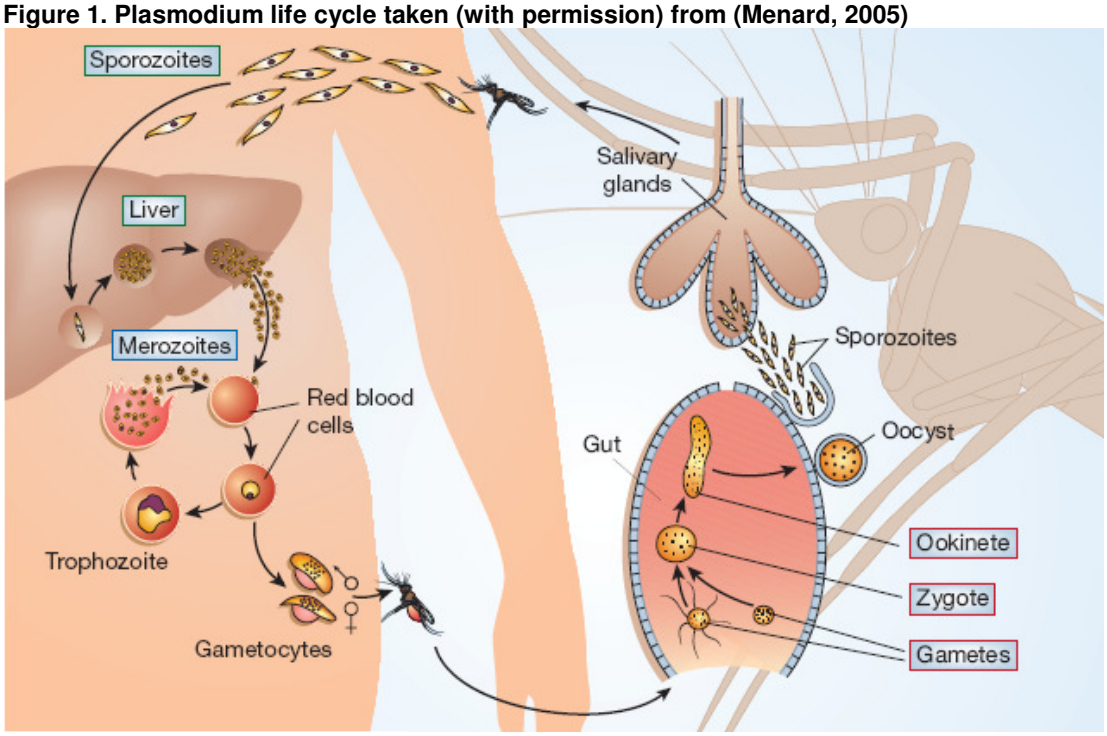
Malaria is a devastating disease which threatens nearly half the global population and holds the unenviable title of the deadliest human protozoan parasite. Malaria causes 300-500 million cases, and kills 1-2 million people every year, 90% of those in Africa (WHO, 2005). Malaria is caused by protozoa of the genus *Plasmodium.* Of around 120 species of *Plasmodium,* five can infect humans – *P. falciparum, P. vivax, P. ovale, P. malariae and P. knowlesi* (Carter and Mendis, 2002; Cox-Singh et al., 2008). Distributions of the species differs depending on the geographical location, for example in sub-Saharan Africa about 80% of cases are caused by *P. falciparum*, whereas in Asia roughly 95% of cases are diagnosed as *P. vivax* (Carter and Mendis, 2002). Of the five species that infect humans, *P. falciparum* is the most pathogenic as it is able to cause severe manifestations of malaria such as cerebral malaria and severe malarial anaemia.

The parasite adopts three particular strategies to maximise self-replication and proliferation of the population, and evade the host immune system. The first is the ability to replicate extensively through sporogony, exo-erythrocytic schizony to pre-erythrocytic schizony, and erythrocytic schizogony. The second strategy is to invade the host cells, which in the case of erythrocytes means the parasite can evade many arms of the host immune response. Thirdly, the parasite undergoes a sexual stage which can involve both cross- and self-fertilisation events which allow the parasite to mate and sustain a population even when only single genotype infections exist. (Warrell DA and Gilles HM, 2002)

1.2 Overview of the malaria parasite lifecycle

All malaria parasites require two hosts – the vertebrate host where schizogony and other events occur, and the insect vector where sporogony occurs (see Figure 1).

Figure 1. Plasmodium life cycle taken (with permission) from (Menard, 2005)



1.3 Vertebrate host stage

When a vertebrate host is bitten by an infected mosquito, sporozoites may be injected with the saliva into the bloodstream and/or the skin. These sporozoites then move quickly to the liver and invade hepatocytes; invasion has been observed as quickly as 2 minutes from inoculation (Warrell DA and Gilles HM, 2002), although a recent report suggests that injected sporozoites may also remain in the skin for hours (Amino *et al*., 2007). It is thought that about 20 sporozoites are injected each time the mosquito probes (discussed in more detail in section 1.4.2.4) but that most sporozoites (~70%) are retained in the skin from minutes to hours (evidence of up to 2 hours with *P. falciparum*) before they target the bloodstream (Amino et al., 2006; Amino et al., 2007; Ponnudurai et al., 1991).

Once inside a hepatocyte, the parasite may then initiate a vegetative growth stage which leads to primary schizogony, or can enter an arrested phase of development termed the hypnozoite. The hypnozoite may then remain dormant for days, weeks, or even months until unknown factors trigger the development cycle. However, the hypnozoite phase is only found in *P. vivax* and *P. ovale* of the human malaria parasites.

Mitotic replication of parasite within the hepatocyte takes 5-7 days and results on 30,000-50,000 merozoites in each infected hepatocyte. Once the liver schizont is mature it ruptures to release merozoites into the blood stream where they invade erythrocytes

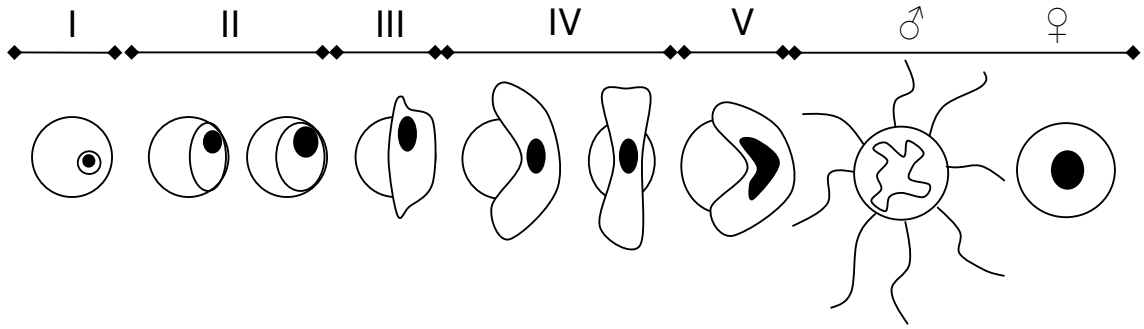
(Warrell DA and Gilles HM, 2002). Once inside the red cell, the parasite develops through the ring, trophozoite and schizont stages to eventually rupture and release 8-16 merozoites (depending on the species) which can re-invade erythrocytes. These rounds of multiplication and invasion cause the characteristic fevers observed in malaria-infected patients. Whilst the majority of merozoites invading erythrocytes are destined to continue through the asexual cycle, a small fraction can develop into gametocytes, the first sexual stage of the life cycle.

1.3.1 Gametocytogenesis

The presence of gametocytes in the peripheral blood generally arises 7-15 days after the initial asexual wave of parasitaemia, but in *P. falciparum*, gametocytes can occur at any point throughout infection, usually in very low numbers. The different sequential stages of gametocyte maturation (named stages I to V before micro- and macrogametocyte production) were first described by Hawking in 1971 (Hawking et al., 1971) and they are shown in Figure 2. Gametocytes of other human malaria species do not follow the same sequence nor do they take as long to mature as *P. falciparum*, and the mature gametocytes are not crescent shaped but round. *P. falciparum* gametocytes take between 14-16 days to complete the maturation process (Kariuki *et al*., 1998).

The gametocytes are sequestered in deep tissue capillaries, particularly in the bone marrow and maybe the spleen pulp, until stage V when they then emerge to circulate in the peripheral blood for several days and possibly weeks (Nacher, 2004; Smalley and Sinden, 1977). There are two sexes of gametocytes (micro- and macro) and they are distinguishable at maturity (stage V) on the basic of morphology on Giemsa-stained slides.

Figure 2. The sequential stages of *P. falciparum* gametocyte maturation, redrawn after (Sinden 1983b)

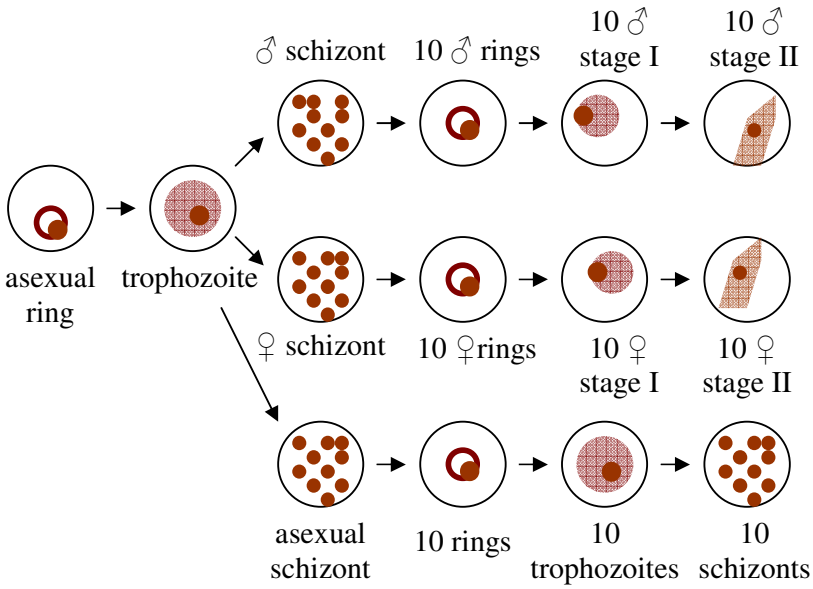


The trigger for conversion from an asexual parasite to a gametocyte is not fully understood but it does seem that environmental factors such as anaemia and high parasitaemia do play

a role (Bruce et al., 1990; Carter and Miller, 1979). Amongst other factors host immune response factors such as tumour necrosis factor α (TNF- α) and interleukin 1 (IL-1), as well as antifolate drugs such as pyrimethamine, and host hormones such as insulin and progesterone have all been described as affecting the rate of conversion (reviewed in (Dyer and Day, 2000)).

It was proposed, among other possibilities, by Carter and Miller (Carter and Miller, 1979) that merozoites of an individual schizont might already be committed either to become asexual parasites or to develop into gametocytes. This suggestion was demonstrated by Bruce and co-workers (Bruce et al., 1990) who showed that all the merozoites of a particular schizont were destined to become gametocytes. They also provided evidence to show the gametocyte conversion rate varies greatly under different growth conditions; at higher parasitaemias when asexual growth is slow, the proportion of schizonts giving rise to gametocytes can increase to as much as 70% (shown by (Carter and Miller, 1979)) .

Figure 3. Model of sexual differentiation of *P. falciparum* (redrawn from Smith et al(Smith et al., 2000). Ten merozoites are shown per schizont as an illustration only, and maturation of the gametocytes is only shown to stage II.



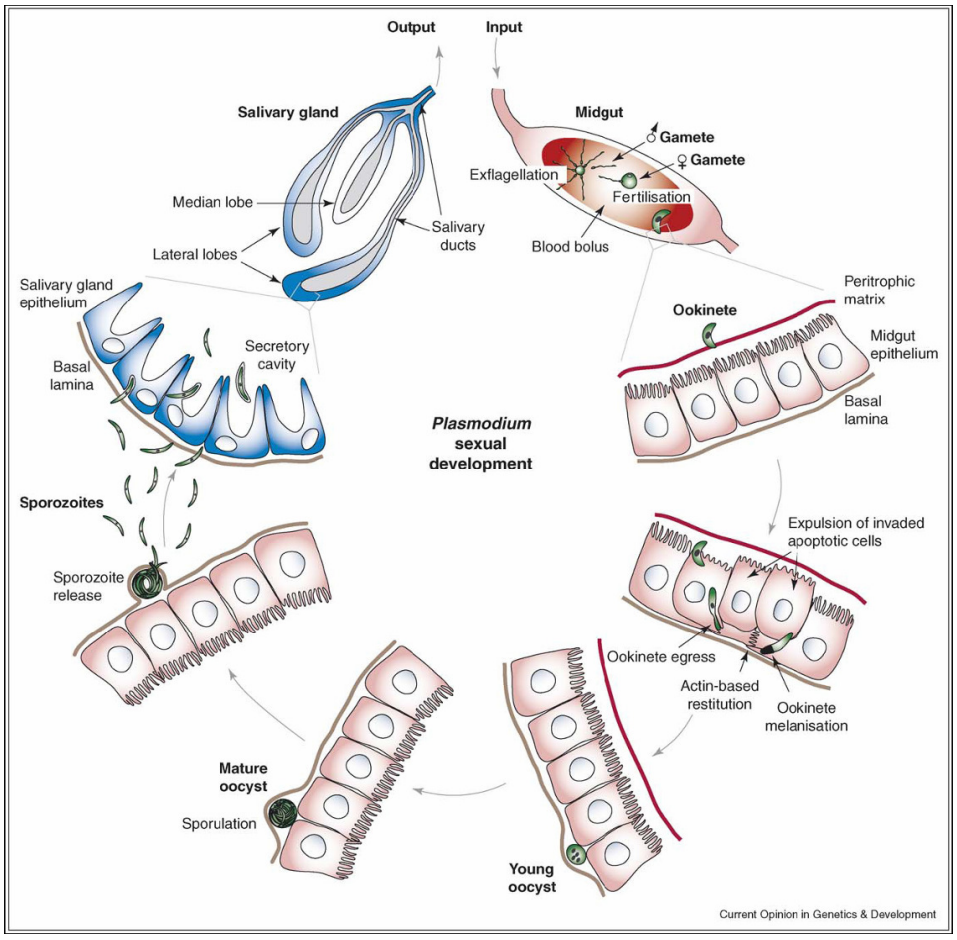
However, it was not until Smith's paper of 2000 (Smith *et al*., 2000; Warrell DA and Gilles HM, 2002) that the differentiation into male and female gametocytes was defined. Smith *et al* (Smith et al., 2000) investigated sex differentiation utilising antibodies specific for different sexes of gametocytes to identify the point in the life-cycle at which parasites commit to developing into either male or female gametocytes, concluding that the model shown in Figure 3 is correct for *P. falciparum.* Within this model all the merozoites within

a single schizont are committed to the same sex, implying that the commitment to sexual differentiation is likely to occur in the preceding ring or trophozoite stage (Smith *et al.*, 2000).

1.4 Mosquito host stage

The female *Anopheles* mosquito must take regular bloodmeals to support the development of her eggs. Whilst biting an infective vertebrate host, the mosquito can pick up parasitised erythrocytes, which may include the gametocyte stage, which can then undergo the sexual stage of development in the midgut of the mosquito (see Figure 3).

Figure 3. Plasmodium migration in the mosquito host (taken, with permission, from Vlachou 2006 (Vlachou et al., 2006)).



1.4.1 Fertilisation, zygote formation and ookinete migration

Following uptake into the mosquito midgut, the gametocytes escape the host erythrocytes. The macrogametocyte emerges from the erythrocyte to produce a single extracellular and non-motile macrogamete (Sinden, 1983b). Evidence suggests that osmiophilic bodies are involved in the emergence of macrogametocytes from the erythrocyte by disrupting the erythrocyte plasmalemma (Koning-Ward *et al*., 2008).

The microgametocyte undergoes an amazing transformation termed exflagellation. Exflagellation was first observed by Laveran in 1881 in the blood of an infected patient; this was also the first observation of the causative agent of malaria. Exflagellation eventually produces up to eight haploid microgametes from each microgametocyte. These motile gametes can then move around until they find a non-motile female gamete, at which point they may fuse and form a diploid zygote.

In the mosquito midgut gametocyte maturation into gametes is triggered by changes in the environment such as a drop in temperature (which can be as much as 12^{o}C), an increase in pH, and exposure to xanthurenic acid (XA) (Billker et al., 1998; Sinden, 1983a; Sinden et al., 1996a). Initially a mosquito exflagellation factor were identified, which later was described as XA; the natural chemical signal, produced by mosquitoes, that induces exflagellation of the male gametocyte in the midgut (Billker et al., 1998; Garcia et al., 1998; Nijhout, 1979). A Ca^{2+} signalling pathway was found to be necessary for exflagellation, as XA triggers a rise in cytosolic calcium, which is essential for differentiation of gametocytes into gametes (Billker *et al*., 2004).

At fertilization, membranes of the macro- and microgametocytes fuse and the male axoneme swims in to the cytoplasm of the female taking the nucleus with it (Sinden *et al*., 1996b). Conventional two-stage meiosis occurs following fusion of the small male nucleus and the large female nucleus producing a single, approximately tetraploid zygote nucleus (Sinden and Hartley, 1985). By 18-24 hours post-blood feed, the zygote transforms into a mature, motile, banana-shaped ookinete that is about 2-4μm by 12-18μm (Meis et al., 1992)}.

The ookinete's principal role is to escape from the relatively hostile environment of the bloodmeal (due to the parasite susceptibility to both vertebrate and mosquito-derived components of the bloodmeal e.g. lactic acid, anti-parasitic antibody, and proteolytic

enzymes) by invading the gut wall tissues (Gass, 1977). The ookinete actively migrates through the bloodmeal and invades the midgut epithelium of the mosquito (Baton and Ranford-Cartwright, 2005). There is convincing evidence (Baton and Ranford-Cartwright, 2004) that *P. falciparum* follows the 'time bomb' model, which was first introduced in 2000 from studies with *P. berghei* in *An. stephensi* (Han *et al*., 2000) of ookinete invasion of the midgut epithethial cells. The model describes the parasites invading the midgut epithelial cells and passing through them to the basal side. Ookinete invasion causes significant morphological changes in the mosquito epithelial cells that result in cell death and ejection of invaded cells from the midgut wall into the lumen, but rarely in time to also kill the ookinete. Ookinetes which have left the invaded epithelial cell before the extrusion of the cell come to rest on the basal lamina where they start to transform into an oocyst, some 24-36 hours post-blood feed.

1.4.2 Oocyst formation, growth and differentiation

Oocysts are formed from the development of ookinetes that have traversed the midgut epithelial cells and made contact with the basal lamina of the mosquito (reviewed in (Beier, 1998). Many of the ookinetes which are formed within the midgut lumen do not reach the basal lamina to form oocysts. Vaughan and co-workers observed a logarithmic-type relationship between cultured *P. falciparum* ookinete and oocyst densities in lab-infected *An. gambiae* which dictates a threshold density of about 30 ookinetes per mosquito to be required before oocyst development occurs, and a 100-fold loss of conversion from ookinete to oocyst (Vaughan et al., 1994b). In other parasite-vector combinations the picture is mixed; the conversion efficiency of *P. vivax* zygotes/ookinetes to oocysts was independent of zygote/ookinete density in *An. dirus* mosquitoes (Zollner *et al* 2005). Whereas the number of *P. berghei* oocysts was found to correlate with the number of ookinetes in a non-linear fashion in *An. stephensi* mosquitoes, such that a minimum number of ookinetes was needed to produce one oocyst but as the number of ookinetes increased further the transformation became constrained at a plateau of oocysts per mosquito (Sinden et al., 2007; Zollner et al., 2005).

The establishment of oocysts appears to be a true bottleneck in the parasite lifecycle with total parasite losses during the establishment of an oocyst from gametocytes having been shown to be very large, although the figures vary greatly between vector-parasite species combinations (3,000-160,000-fold) (Alavi et al., 2003; Vaughan et al., 1994b). Parasite losses within the early sporogonic cycle (from 3h post-infection to day 7 with *P.*

falciparum parasites in *An. gambiae* mosquitoes) may be as much as 75% (Gouagna et al., 1998).

Oocyst formation begins ~24-36 hours after mosquito blood feeding. Oocyst maturation (between 10-22 days) is asynchronous and oocysts of various different stages of maturity and size can be found when dissecting an experimentally infected mosquito (Huff, 1940; Ponnudurai et al., 1989b; Sinden and Strong, 1978). Evidence from experimental infections suggests that once established, oocysts usually survive the remaining period of sporogony (Gouagna et al., 1998).

Early microscopy work on the development of *P. falciparum* in experimentally infected *A. gambiae* showed that oocysts were of uniform size in low density infections, but at high densities, sizes varied widely (Sinden and Strong, 1978). One hypothesis was that this variation results from competition for a limited supply of nutrients.

Oocysts obtain nutrients that they require for development, including DNA replication, from the mosquito haemolymph. However there is an inherent difficulty in learning more about this stage as although *P. falciparum* oocysts have been grown *in vitro* (Warburg and Schneider, 1993), the method has been difficult to replicate. Evidence from the asexual stages of the parasite lifecycle showed that no more than 2% of the purine required by *P. falciparum* could be met by the intracellular stores of purine nucleotides, suggesting that most of these essential nutrients need to come from an exogenous source (Berman et al., 1991). Clearly, the intracellular store for an asexual parasite (the cytoplasm of an erythrocyte) is completely different for an ookinete or oocyst (bloodmeal or haemolymph), so drawing paralleles between the two environments should be done with caution. It would be an assumption that purines will be just as necessary for parasite development in mosquitoes, as in the asexual stages. However, there is evidence that the developing oocyst takes up certain amino acids from the haemolymph, including valine, histidine, methionine and leucine (Mack *et al*., 1979b; Mack and Vanderberg, 1978; Vanderberg *et al*., 1967).

1.4.2.1Intensity of infection

Oocyst numbers on the mosquito midgut can vary widely depending on the density, stage and potentially on the mixture of gametocyte genotypes in the blood feed.

Individual parasite clones produce different numbers of gametocytes both *in vitro* and *in vivo* (Diebner *et al*., 2000; Graves *et al*., 1984). Gametocyte densities in natural infections of *P. falciparum* are generally low (Abdel-Wahab *et al*., 2002), as are oocyst densities in field-caught mosquitoes (Lyimo and Koella, 1992). Mixed genotype *P. falciparum* infections are common in areas of high transmission (Taylor, 1999), but there is little evidence that this influences oocyst density in naturally infected mosquitoes.

Experimental infections with *P. falciparum* involving mixtures of gametocytes of two different genotypes do not produce higher oocyst numbers than single clone infections (Ranford-Cartwright et al., 1993; Walliker et al., 1987), unless there is a defect in production of one of the sexes of gametocytes as in the case of parasite clone Dd2 (Vaidya et al., 1995a). However, studies with the rodent malaria *P. chabaudi* showed higher oocyst burdens from mixed-genotype infections compared to either clone infected alone (Taylor, 1997), although in this case no investigation was made regarding functionality of the different sexes of the gametes.

A predictable positive relationship between intensity (number of oocysts on the midgut) and prevalence of infection was found with laboratory studies with *P. berghei* in *An. stephensi* mosquitoes, although sample size critically affected the relationship, suggesting that without a big enough sample the association would not be clearly seen (Medley et al., 1993). Generally this relationship is seen with experimental infections of *P. falciparum* in *An. gambiae*: mosquitoes with a low prevalence of infection often have low numbers of oocysts, although this is not always the case (Mwangi pers comm). Reported infection intensities in experimental infections of *P. falciparum* isolate NF54 in *An. gambiae* mosquitoes ranged between 0 to 56 oocysts per midgut, and distributions for oocysts were significantly skewed to the left (Vaughan et al., 1992).

Oocysts are not distributed randomly among mosquitoes. Even within a population of mosquitoes feeding on the same blood simultaneously, most mosquitoes might contain few or no oocysts, whereas a minority contain many oocysts. This pattern of distribution has been described as fitting a negative binomial frequency distribution (Medley et al., 1993) and this appears consistent across a range of vector-parasite combinations (Vaughan et al., 1994b; Vaughan et al., 1994a). A negative binomial distribution may arise from two sources of heterogeneity: 1) the number of infective gametocytes ingested by mosquitoes; and 2) the mosquito susceptibility to the subsequent development of those gametocytes (Vaughan, 2007). The use of the membrane-feeder and thorough mixing of the infectious

bloodmeal should eliminate the first option of heterogeneity, leaving the second as the explanation for the aggregated distribution found in laboratory-infected mosquitoes.

Although the very high intensities of infection sometimes seen experimentally (>100 oocysts/midgut) are rarely reported in wild-caught infected mosquitoes, the negative binomial distribution found in laboratory studies appears to be maintained in the field (Billingsley et al., 1994; Paul et al., 2007). In a study in Tanzania 18% of wild-caught *An. gambiae* mosquitoes were carrying *P. falciparum* oocysts with a range of 1 to 49 oocysts per midgut (Lyimo and Koella, 1992) with the majority (82%) of mosquitoes with no infection. The median of the infection intensity data was 2.5, with nearly 40% of those infected having only 1 oocyst on the midgut. Another study, also conducted in Tanzania, looked at *P. falciparum* infection rates in *An. gambiae* and *An. funestus.* They reported about 10% prevalence and a range up to 43 oocysts/midgut, with 95% of the infected mosquitoes holding fewer than 10 oocysts per midgut (Taylor, 1999).

1.4.2.2The effect of intensity of infection on the number of sporozoites per oocyst

The only factor that has been specifically studied for its association with sporozoite production is the oocyst burden (intensity) on the midgut. A few studies have looked at the oocyst intensity, reasoning that competition for nutrients in the midgut might have an effect on sporozoite numbers. It is not possible to examine oocyst intensity and subsequent sporozoite number within the same mosquito, and so these studies rely on average counts of oocyst numbers and sporozoite intensity. Two reports found an overall positive linear relationship between oocyst density and sporozoite density; as the mean number of oocysts on the midguts increased, the mean sporozoite burden, as measured by counting the number of sporozoites present in the salivary gland, also increased. The number of sporozoites did not reach a plateau, suggesting that nutrients for further parasite population growth within mosquitoes are essentially unlimited and there is no 'carrying capacity' imposed on developing oocysts by their 'habitat'(Vaughan et al., 1992; Zollner et al., 2006). Additionally, one of these studies took an average sporozoite production per oocyst, by dividing the mean number of sporozoites by the mean number of oocysts on the midgut. There was no association between sporozoites per oocyst and oocyst density on the midgut using this approach suggesting that there was no limiting factor on sporozoite production from competition for nutrients (Vaughan *et al.* 1992).

The relationship between the number of sporozoites per oocyst and the intensity of infection was also investigated by Bell and Ranford-Cartwright using the 3D7 clone of *P. falciparum* in *A. stephensi* mosquitoes (unpublished). In this study, sporozoite numbers were estimated using quantitative real time PCR (Bell and Ranford-Cartwright, 2004) in single oocysts removed from infected mosquitoes, and oocyst fecundity (number of genomes per oocyst) was not found to be correlated with the intensity of infection (unpublished).

1.4.2.3 Sporozoite formation within the developing oocyst

Within the developing oocyst, the genome undergoes multiple rounds of mitotic replication which are marked by the appearance of one or more spindles in each nuclear lobe, of which there are many (Sinden and Strong, 1978). Mitotic replication begins immediately after oocyst formation and continues throughout the period of sporogony. As cytokinesis does not occur at this point the multinucleated parasite gradually grows in size. The oocyst plasma membrane becomes folded inwards and forms crevices which extend across the oocyst cytoplasm, partitioning it into compartments called sporoblasts (see Figure 4).

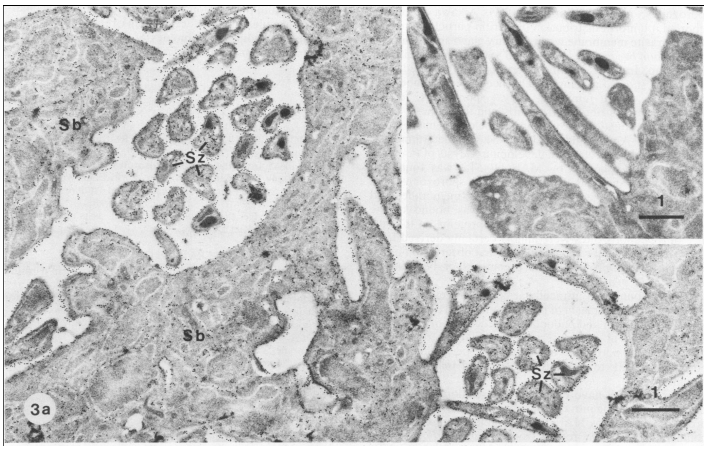
Sporoblast formation begins around 5 days after establishment of the oocyst and continues until around day ten (Howells and Davies, 1971b). The developing sporozoites then bud off from the sporoblasts in a process that involves mobilisation of the nucleus and other cellular organelles into each budding sporozoite. Cytokinesis of the budding sporozoites results in the formation of a mature oocyst that contains haploid sporozoites. Sporozoite budding occurs synchronously throughout a single oocyst

Before their escape from the oocyst, the sporozoites are relatively straight cells with an elliptical cross section with variable diameters from between 1.8μm in the central region to 1.2 μm at the pole (Meis et al., 1992; Sinden and Strong, 1978).

After around 10-14 days oocyst development and sporogony are complete. The haploid invasive sporozoite parasites are packed within a single oocyst capsule and a small residue of vacuolated sporoblast cytoplasm containing the remains of nuclear material and membrane-bound haemozoin retained from the intraerythrocytic macrogametocyte stage (see Figure 4) (Howells and Davies, 1971a; Vanderberg.J *et al.*, 1967). Although the oocyst sporozoites are morphologically identical to salivary gland sporozoites, they are

phenotypically distinct, oocyst-derived sporozoites are virtually non-infective(Vanderberg, 1975).

Figure 4. Taken from Nagasawa *et al* (Nagasawa *et al*., 1987) (a) Mature oocyst of P. ovale labelled with Mab and gold particles. Sporoblast (Sb) and Sporozoites (Sz). Inset: Mature oocyst incubated without any gold labelling as control. Bars indicate 1µm.



Release of sporozoites by oocysts from a single infectious feed is probably not synchronous. In a study using *P. berghei* in *An. stephensi* mosquitoes, the decline in oocyst numbers appeared to be gradual and evenly spread over time suggesting that sporozoites were released over time asynchronously and that some oocysts failed to release sporozoites at all (Dawes et al., 2009). There is evidence from a *P. vivax* study that not every oocyst achieves its full potential, and that some oocysts contribute fewer sporozoites to the total crop, and some contribute none at all. Apparently healthy oocysts were seen up to 22 days p.i., but why some oocysts fail to release sporozoites is still not clear (Zollner et al., 2006).

The availability of nutrients may affect the synchronicity of oocyst development. Subsequent uninfected bloodmeals after the initial infected one are thought to synchronise the oocyst development, rather than increase either the infection intensity or the number of sporozoites per oocyst (Ponnudurai et al., 1989b; Ponnudurai et al., 1990; Rosenberg and Rungsiwongse, 1991).

The efficiency of the parasite development in the mosquito can be evaluated by counting the number of sporozoites produced by single oocysts. Due to the technical demands of this type of study, very little data is available on the numbers of sporozoites found in single oocysts of *P. falciparum* infection, and the numbers that are available show wide variation. A few studies have concentrated on microscopically counting sporozoites from single

lysed oocysts whereas others have counted sporozoites in the salivary gland (plus the haemolymph in some cases) using microscopy and then divided that number by the mean of the oocysts counted in the midgut, or performed linear regression analyses to obtain this number.

Direct counts of sporozoites within single oocysts ranged from 1000 to 9555 for *P. falciparum* oocysts (Pringle, 1965; Rosenberg and Rungsiwongse, 1991; Ross R, 1910) and 508 to 3688 for *P. vivax* oocysts (Rosenberg and Rungsiwongse, 1991; Zollner et al., 2006). Analysis by linear regression of oocyst densities plotted against salivary gland sporozoites gave estimates of 850 gland sporozoites per oocyst for *P. vivax* in *An. dirus* (Sattabongkot *et al*., 1991), and 663 gland sporozoites per oocyst for *P. falciparum* in *An. gambiae* (Vaughan et al., 1992).

1.4.2.4 Salivary gland sporozoites

Once the sporozoites are fully developed and are released from the oocyst, they make their way in the haemolymph to the salivary glands. Some evidence suggests that sporozoites actively exit the oocyst with the use of a parasite-derived cysteine protease, and secretory factors (Aly and Matuschewski, 2005; Engelmann et al., 2009), although other evidence supports passive liberation of the sporozoites as a result of breakdown of the oocyst wall (Chen et al., 1984; Meis et al., 1992). Little is currently understood about the migration of sporozoites to the salivary glands, and it is unclear whether chemotaxis or a passive/random process is involved (Akaki and Dvorak, 2005; Rodriguez and Hernandez-Hernandez, 2004).

A linear relationship between oocyst and sporozoite densities has been reported from experimentally infected mosquitoes (Vaughan et al., 1992).Virtually all experimental oocyst infections produce salivary gland infections (Vaughan et al., 1992). Only about 10-20% of oocyst sporozoites will successfully attach to and invade salivary glands, and the rest may be recognised and destroyed by the immune system of the mosquito (Barreau et al., 1995; Myung et al., 2004; Natarajan et al., 2001). In wild-caught *An. gambiae,* sporozoites failed to enter the salivary glands in 43% and 10% of infected mosquitoes, respectively, from Burkino-Faso (Lombardi *et al*., 1987) and western Kenya (Beier *et al*., 1990). Vaughan and colleagues reported that 3 to 18% of the sporozoites produced by oocysts remained in the hemolymph (Vaughan et al., 1992). Sporozoites have been

detected in the legs, wings and heads of mosquitoes (Robert *et al*., 1988) suggesting that many do not reach the salivary gland once released into the haemolymph.

Invasion of salivary glands takes place around 14 days from the infected blood feed for *P. falciparum* depending on the temperature of the mosquito, and few sporozoites appear to penetrate the salivary glands after 18 days p.i. (Meis et al., 1992; Rosenberg and Rungsiwongse, 1991).

Sporozoites cross the basal lamina of the salivary gland, and invade the secretory cells and the salivary duct through the formation of transient vacuoles (Pimenta et al., 1994). The sporozoites then escape from the vacuoles and form large bundles of parasites arranged in parallel lines inside the duct (Pimenta et al., 1994)

Several sporozoite surface proteins that are thought to be important in salivary gland invasion have been described including, for example, TRAP, circumsporozoite protein (CSP) and MAEBL. TRAP is a transmembrane protein that mediates sporozoite gliding motility and is important in salivary gland invasion (Sultan *et al*., 1997) and evidence suggests that CSP (the major surface protein on sporozoites) binds specifically to median and distal lobes of the salivary glands (Sidjanski *et al*., 1997). MAEBL is a single transmembrane-like protein which appears to have no role in gliding motility but is important in salivary gland invasion(Kariu *et al*., 2002).

Median salivary gland counts of 8170, and median number of sporozoites ejected of 15 (range 0-978) were reported for *P. falciparum* in *An. stephensi* experimental infections (Rosenberg *et al*., 1990). A geometric mean of 4.5 injected sporozoites (range 1-136) was obtained for experimentally infected *An. gambiae* mosquitoes with *P. falciparum* (Beier *et al*., 1991a). A different approach utilising GFP-tagged *P. berghei* parasites in *An. stephensi* mosquitoes allowed the number of sporozoites injected into a mouse to be established (Amino et al., 2006). Twenty sporozoites could be observed after one minute of probing, and most mosquitoes ejected sporozoites only at the beginning of salivation, suggesting that only those parasites in the common and secondary salivary ducts at the time of feeding can be ejected.

Sporozoite load and transmission potential varies between different *Anopheles* mosquito species. The geometric mean number of sporozoites in the salivary gland following

experimental infection with P. *falciparum* was slightly higher in *An. stephensi* (5.4) than in *An. gambiae* (4.5) (Beier et al., 1991b).

There is conflicting evidence as to whether the number of sporozoites in the salivary glands is a useful predictor of either the probability of transmission or the number of sporozoites transmitted. For experimental infections of *P. falciparum* in *A. gambiae*, there was no good correlation between sporozoite loads and the number transmitted; over 86% of mosquitoes transmitted between one and 25 sporozoites regardless of sporozoite load, and the number transmitted represented less than 1% of the sporozoites in the salivary glands (Beier et al., 1991a). However, Ponnudurai found that the number of sporozoites transmitted was broadly correlated to the density of sporozoites in the salivary glands (Ponnudurai et al., 1989b). Also, a recent study of *P. berghei* in *An. stephensi* found the number of sporozoites injected was correlated to the time spent feeding (~1-2.5 per second), the site being fed upon, and the numbers of sporozoites in the salivary gland (Jinj *et al*., 2007) . Evidence to date suggests that very few mosquitoes transmit hundreds of sporozoites, and that most infective mosquitoes transmit relatively few sporozoites (Beier et al., 1991a).

The number of sporozoites injected has been shown to determine not only the chance of the parasite surviving in the vertebrate host, but also the course and severity of the resultant disease (James *et al*., 1936). A single sporozoite successfully invading a hepatocyte can produce up to 30,000 merozoites, which then go on to divide into up to 16 new merozoites each time a red blood cell is invaded. Even a small increase in the initial number of invading sporozoites can dramatically reduce the time to reach the critical density required for human disease (and in the case of *P. falciparum* the greater chance of death) (Rosenberg *et al*., 1990).

A recent study on *P. berghei* sporozoites found that the number of sporozoites injected by the mosquito may be affected by the immune response of the mammalian host. Fewer parasites were injected into mice that had previously been immunised against sporozoites, and evidence was presented for an immune complex formation (with circumsporozoite protein) forming a partial obstruction at the proboscis end as the explanation for this finding (Kebaier *et al*., 2009).

Relatively little is known about the sporozoite migration after inoculation into the mammalian skin. Recent work with fluorescently-labelled parasites and confocal

microscopy has provided fresh insights into this stage of the parasite lifecycle (Amino *et al*., 2007). A recent paper by Kebaier and Vanderberg presented studies on *P. berghei.* The results showed that mosquitoes injecting sporozoites into mice re-ingest some of the sporozoites. This observation could explain why some sporozoite-infected mosquitoes fail to induce a blood infection (Kebaier and Vanderberg, 2006). So far, studies on sporozoite migration have mainly been limited to rodent parasites (*P. berghei* and *P. yoelli*) so it is not yet clear whether *P. falciparum* sporozoite migration is the same (Chen and Wang, 2008).

Most sporozoites (~70%) are retained in the skin from minutes to hours (evidence of up to 2 hours with *P. falciparum*) before they target the bloodstream (Amino et al., 2007; Ponnudurai et al., 1991).

1.5 Mosquito factors affecting sporozoite development

The complex interactions between parasite and host, and the mosquito factors that might have an effect on *Plasmodium* development, have been considered for many years (Beier, 1998; Simonetti, 1996; Vaughan, 2007). However, there are still many questions left unanswered, and areas where evidence is conflicting.

1.5.1 Mosquito size

Variation in body size is a regular feature of natural mosquito populations and may be one of the factors that contribute to transmission of malaria. There is some debate as to the most suitable marker to use to best represent mosquito size. The commonly used parameter is wing length, as it is technically easy to measure, and evidence suggests it is a good marker of body size (Lyimo and Koella, 1992). Other studies have used bloodmeal size or weight as a measure of mosquito size. Bloodmeal size can be measured either by the amount of haemoglobin ingested (Hurd *et al*., 1995) or by the amount of digested product of haemoglobin, haematin (Briegel, 1980). Measurements of the haemoglobin ingested require killing the mosquito, and further analysis of infections cannot then be performed for the same mosquito; such studies rely on population averages. Haematin can be measured without killing the mosquito. The cubed wing length value can be used to obtain a rough three dimensional estimate of body size, which was shown to correlate with bloodmeal size (Briegel, 1990).

Many of the studies on mosquito size and its possible association with *Plasmodium* infection have been reported from field-based samples and not from laboratory-reared mosquitoes in controlled environments (Hogg and Hurd, 1997; Lyimo and Koella, 1992). Adult size in mosquitoes is determined by conditions in the larval habitat, where under optimal temperatures, higher larval densities may negatively affect body size because of competition for nutrients (Lyimo and Koella, 1992). The average size of newly emerged females of *An. gambiae* s.l. was significantly less than that of the host-seeking population (collected when actively seeking a bloodmeal), suggesting the survival rate of adult mosquitoes is positively correlated with body size (Lyimo and Takken, 1993). Large *An. gambiae* Giles s.s females survived longer and contained significantly more protein, glycogen, and lipid at emergence than did small females (Takken *et al*., 1998). Evidence suggests that smaller mosquitoes have to search for a bloodmeal sooner after emergence than larger mosquitoes who can survive on their stores of nutrients for longer.

The relationship between mosquito size and amount of blood ingested is complicated by the ability of some mosquito species to concentrate the bloodmeal through a process of prediuresis (the anal excretion of fluid during feeding). Many Anopheline mosquito species are known to concentrate blood proteins during the act of feeding (Briegel and Rezzonico, 1985), and different species vary in their ability to concentrate erythrocytes in the bloodmeal taken (Vaughan *et al*., 1991). For example ,*An. gambiae* Giles concentrated erythrocytes by a factor of 1.8 and *An. arabiensis* Patton by a factor of 1.4. The degree to which the mosquitoes concentrated erythrocytes was related to the frequency and time spent undergoing prediuresis . These differences observed in erythrocyte concentration may also correlate with host choice which also differs between these species; *An. arabiensis* is partially zoophilic, whereas *An. gambiae* prefers to feed on humans (Garrett-Jones *et al*., 1980).

The effect of mosquito size on the prevalence and intensity of parasite infection is currently unclear. In one study, four size-classes of laboratory-reared *An. dirus* mosquitoes were fed with cultured *P. falciparum*. The prevalence of infection was found to be independent of size (as measured by wing length), but there was an association between intensity of infection and mosquito size (with larger mosquitoes having significantly more oocysts per midgut than smaller mosquitoes) (Kitthawee et al., 1990). The conditions in which the mosquito larvae develop also seem to influence infection of the adults. In experimental infections with *P. falciparum* a larger numbers of oocysts developed in mosquitoes that had been reared from larvae held at low densities but this was not

explained by differences in adult size at the time of the infectious feed (Lensen, 1996). A study of wild-caught *An. gambiae s.l.* mosquitoes from Tanzania found no difference in the size of mosquitoes (as measured by wing length) infected or uninfected with *P. falciparum*, using oocysts as the marker for infection (Hogg and Hurd, 1997), but sporozoite-carrying mosquitoes had significantly larger wing lengths than uninfected mosquitoes. However, this finding was not replicated in a Kenyan study, which found no significant difference in wing lengths between sporozoite-infected and uninfected wild-caught *An. gambiae s.s.* in rural coastal sites (Mwangangi et al., 2004). In another Tanzanian study, the prevalence of infection in bloodfed wild-caught mosquitoes was independent of the size of mosquito (measured by wing length) (Lyimo and Koella, 1992). However, a correlation was observed between the size of mosquito and number of oocysts on the midgut (intensity of infection). The proportion of mosquitoes with sporozoites was highest in the intermediate-sized mosquitoes, which the authors suggested may be explained by low survival rate of mosquitoes with heavily infected midguts in the field (Lyimo and Koella, 1992).

Studies using other parasite species have not found a correlation between mosquito size and parasite infection. There was no correlation between the unfed weight of *Aedes aegypti* and the probability of infection by the avian malaria *P. gallinaceum* (Hovanitz, 1947), or between wing length of *An. stephensi* and the number of oocysts of the rodent malaria *P. yoelii nigeriensis* (Ichimori, 1989).

1.5.2 Mosquito species

Differences in *Plasmodium* infections in different mosquito species have also been considered. *P. falciparum* infections in six different *Anopheles* spp. revealed that overall susceptibilities to infection (in order) were *An. freeborni* > *An. gambiae*, *An. arabiensis, An. dirus* > *An. stephensi, An albimanus* (Vaughan et al., 1994b). Chege and Beier confirmed Vaughan's findings when they described *P. falciparum* infections in three *Anopheles* species; *An. freeborni* Aitken*, gambiae* Giles and *albimanus* Weidemann and found excellent, good and poor susceptibility to infection respectively (Chege and Beier, 1998). Another study took a strain of *P. falciparum* from an infected patient, established infection in *Aotus* monkeys, and then experimentally infected six different species of *Anopheles* mosquitoes, the most susceptible being *An. freeborni* and the least susceptible *An. gambiae* (Collins et al., 1981).

Other investigators have studied the infectivity of non human malaria species of *Plasmodium*. *P. yoelii yoelii* infection was studied in five species of Anopheles mosquitoes; *An. albimanus, An. dirus, An. freeborni, An. gambiae* and *An. stephensi*. On day 16 after the infectious feed, only *An. freeborni, An. gambiae* and *An. stephensi* mosquitoes had sporozoites present in the salivary glands but only half of the mosquitoes with mature oocysts also had salivary gland infections, showing a loss in conversion from oocysts to salivary-gland sporozoites (Vaughan et al., 1994a). Alavi *et al* also studied the infectivity of two species of *Plasmodium* (*P. berghei* and *P. gallinaceum*) in *An. stephensi, An. gambiae* and *Ae. Aegypti* and found that although *P. gallinaeceum* was transmitted by all three mosquito species, *P. berghei* was only transmitted by *Anopheles* spp. (Alavi et al., 2003).

1.5.3 Mosquito immunity

The importance of mosquito immunity in determining the infectivity of *Plasmodium* in the mosquito has been recognised for a long time (Huff, 1927). Current evidence supports the hypothesis that the presence of malaria parasites in the mosquito imposes a high cost on the mosquito, through reduced fecundity and increased mortality (Anderson et al., 2000a; Hogg and Hurd, 1995), and therefore the evolution of immune responses to reduce or prevent infection is likely to be selectively advantageous. Mosquitoes have clearly evolved a wide range of behavioural, genetic, and immune mutants that will suppress infections (Collins et al., 1986). Mosquitoes possess innate immune effectors equivalent to the cellular and humoral arms of vertebrate immunity. Cellular responses rely on immune effector cells known as hemocytes, which are responsible for phagocytosis and melotic encapsulation of foreign agents (Castillo et al., 2006).

In *Anopheles* mosquitoes, susceptibility to *Plasmodium* malaria parasites varies both between and within different species. However observations from studies on the development of two model malaria parasites (*P. berghei* and *P. gallinaceum*) in three mosquito species (*An. gambiae, An. stephensi* and *Ae, aegypti*) suggested that the mechanisms of parasite killing are conserved between different mosquito species (Alavi et al., 2003).

The mechanisms (discussed below) of two genetically inbred laboratory lines, selected for their refractoriness to malaria parasite infection, have been clearly described; a strain of *An. gambiae* which melanises *P. berghei* and *P. falciparum* ookinetes emerging from the

midgut epithelium (Collins et al., 1986), and a strain of *An. gambiae* which lyses *P. gallinaceum* ookinetes (Vernick et al., 1995).

Mosquito nitric oxide synthase (NOS) activity has also been suggested as a limiting factor for parasite development, as dietary provision of additional NOS reduced the prevalence of *P. falciparum* oocyst infection in *An. gambiae* by 28% (Luckhart et al., 1998).

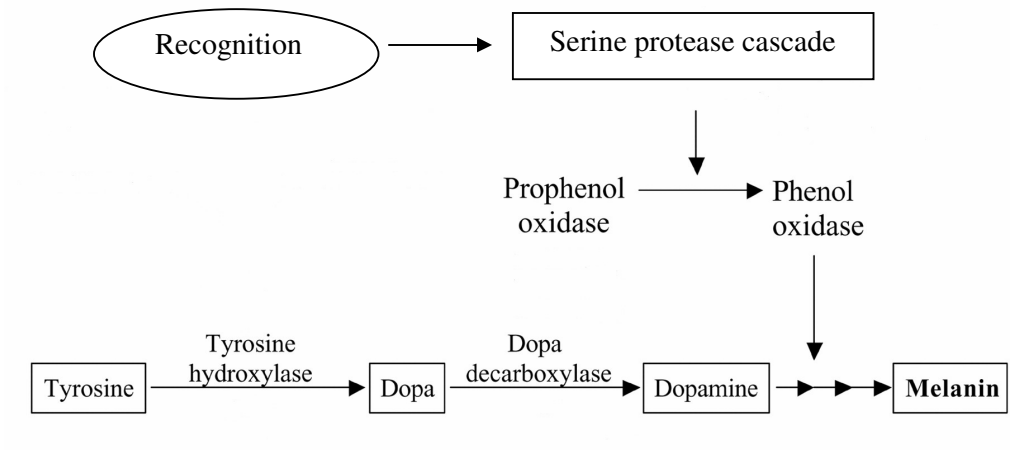
1.5.3.1 Melanisation

Melanisation is a common defence mechanism found in many insect species, and encapsulation of *Plasmodium* ookinetes after they have traversed the midgut wall has been reported in *Anopheles* mosquitoes (Paskewitz et al., 1988), although it appears to be rare in natural populations of mosquitoes (Riehle et al., 2006; Schwartz and Koella, 2002).

Recognition of potential pathogens is thought to occur through conserved pathogen-associated molecular patterns (PAMPs) but the nature of molecules that are recognised on the surface of *Plasmodium* parasites remains to be identified. PAMPs are thought to be recognised by host pattern recognition receptors (PRRs) and several classes of potential PRRs have been found in the genomes of *An. gambiae* (Christophides et al., 2002).

The recognition of microbial or parasite invaders is followed by melanin, and associated toxic metabolic by-products, being deposited on the surface of the pathogen, preventing growth and development (Nappi and Vass, 1993). The molecular events leading to melanisation are known to involve activation of a pro-phenoloxidase cascade, which is carefully controlled by both positive and negative regulators (Figure 5).

Figure 5. Schematic representation of the melanisation cascade in insects, re-drawn from (De Gregorio et al., 2001)



1.5.3.2 Lytic mechanism

The details of the lytic mechanism process are not yet clearly understood but the end result is killing of ookinetes within the midgut epithelium of mosquitoes (Vernick et al., 1995). The data from the *An. gambiae* strain that is refractory to *P. gallinaceum* shows the mechanism is controlled by a single dominant locus in the mosquito genome, which results in ookinete death within 27 hrs of midgut invasion (Vernick et al., 1995).

1.5.3.3 Haemocytes

In adult mosquitoes, haemocytes are involved in both cellular and humoral responses, however no direct interaction between ookinetes or oocysts and mosquito haemocytes has been reported to date. However haemocytes also secrete molecules, and one molecule produced by haemocytes, thioester-containing protein 1 (TEP1), has been described as playing an essential role in the mosquito antiparasitic response (Blandin et al., 2004). Although the mechanism by which it works is unclear, this complement-like protein has been described in *An. gambiae* which binds to, and mediates killing of, midgut stages of the rodent malaria *P. berghei*. In susceptible mosquitoes, the knockout of TEP1 results in a fivefold increase in the number of oocysts development in the midgut, suggesting that parasite killing in mosquitoes is mediated by TEP1 (Blandin et al., 2004).

1.5.3.4 Mosquito size and immunity

The size of the mosquito has been previously shown to be positively associated with the mosquito immune response, such that as the wing length increase so does the level of immunocompetence (Boete et al., 2002; Suwanchaichinda and Paskewitz, 1998). It was hypothesised that due to competition among larvae, individuals with sufficient resources develop into large adults which then have enough stored resources to mount an effective immune response (Suwanchaichinda and Paskewitz, 1998).

1.6 Parasite factors affecting sporozoite development

The environment that the parasite has experienced, both prior to uptake into the mosquito, and then in the mosquito itself, is likely to have an effect on the parasite infectivity and development. Factors that may affect the infectivity (prevalence and intensity) and development of the parasite in the mosquito include the gametocytaemia, gametocyte sex

ratio, and, for *in vitro* grown parasites, the length of time the parasite culture has been in continuous culture. However, these factors have not previously been considered specifically in association to sporozoite production.

For experimental infections, the gametocytaemia and age of cultures (time in continuous culture) have both been previously suggested as variables that might affect parasite development in the mosquito. For experimental infections of *P. falciparum*, the gametocytaemia, and age of gametocytes in the infectious bloodmeal has been shown to affect both prevalence and intensity of infection (Hogh *et al*., 1998; Lensen *et al*., 1999). The time that a parasite line has spent in continuous culture is inversely related to the number of gametocytes produced (Ponnudurai *et al*., 1982). In some cases the reduction in gametocyte production is associated with the loss of large pieces of the parasite genome (Day et al., 1993; Kemp et al., 1992), presumably containing genes important for gametocyte production and function.

Boyd suggested that the number of male gametes determines infection success and *in vitro* experiments supported this hypothesis by finding that clones with a higher male sex ratio were more infectious to mosquitoes (Boyd, 1935; Burkot *et al*., 1984). A study of natural infections revealed the same tendency for male bias in the sex ratio of gametocytes to be associated with transmission success (Robert et al., 1996b).

Several reports have found associations between the gametocyte density and the prevalence and intensity of infection in mosquitoes (Carter and Graves, 1988). Generally transmission in the field seems to be based on 'quality' rather than quantity of gametocytes, but there does seem to be a minimum threshold of gametocyte density required for infectivity, although that may differ depending on transmission intensity and seasonality (Bousema et al., 2006; Sutherland et al., 2005).

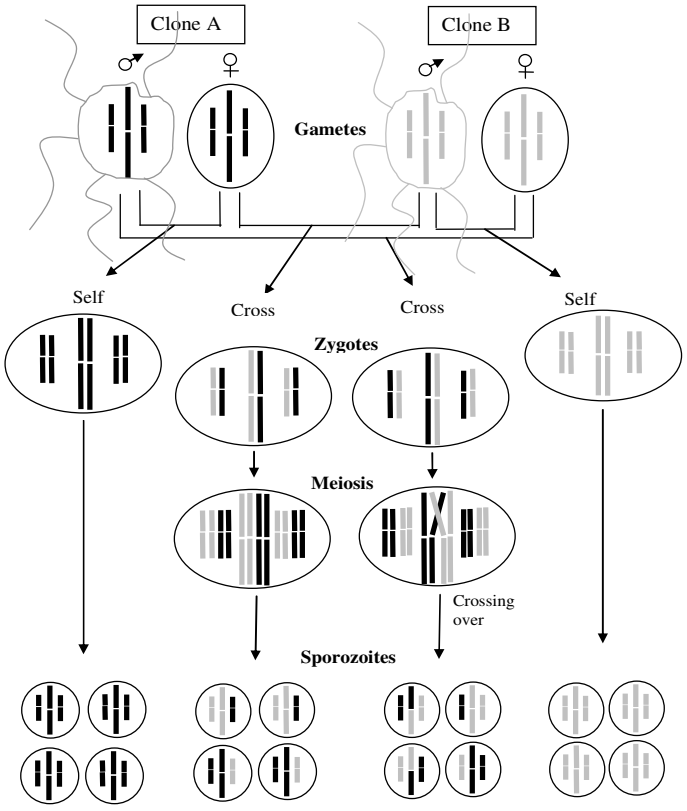
1.7 Genetics of *P. falciparum*

The malaria parasite spends most of its life cycle haploid, with a brief diploid stage when gametes fuse to form a zygote which then goes on to produce haploid sporozoites following meiosis. *Plasmodium* has a eukaryotic type genome, which is organised into chromosomes that undergo segregation, re-assortment and crossing-over at meiosis (Mable and Otto, 1998; Walliker, 1989). The *P. falciparum* genome consists of 14 chromosomes which range in size from 643 kilobases (chromosome 1) to 3290 kb (chromosome 14), in

addition to the mitochondrial and apicoplast genomes (Walliker, 1989; Walliker, 2005; Wellems *et al*., 1987). The genome of *P. falciparum* is highly polymorphic and considerable genetic diversity exists in malaria parasite populations as shown by the diverse forms of many individual characteristics such as drug resistance, and variation in sizes of chromosomes (Walliker, 1989).

During meiotic division of zygotes, each member of a given pair of chromosomes segregates randomly into haploid progeny. Crossing-over events can also take place between homologous chromosomes (Walliker et al., 1971; Walliker et al., 1987). Both self- and cross-fertilisation between genetically distinct gametes can take place in the mosquito, and the mating between different clones is random (Ranford-Cartwright *et al*., 1993). Natural infections often have many mixed genotypes (Carter and McGregor, 1973; Rosario, 1981; Thaithong et al., 1984) which therefore has important implications for the generation of novel genotypes by recombination (see Figure 6).

Figure 6. Crossing and selfing in Plasmodium



1.7.1 Laboratory crossing studies

To date, three laboratory crosses have been carried out using different parasite clones of *P. falciparum*.

The first laboratory cross of *P. falciparum* clones was carried out by Walliker *et al* in 1985 (Walliker et al., 1987). Two clones of *P. falciparum* that differed in enzymes, drug sensitivity, antigens, and chromosome patterns were used as parent lines: 3D7 (derived from the Dutch isolate NF54 (Walliker et al., 1987)) and HB3 (derived from isolate H1 from Honduras (Bhasin and Trager, 1984)). Gametocytes were produced from single clone cultures, and gametocytes of each clone were mixed in equal proportions and fed via membrane feeders to mosquitoes. Splenectomised chimpanzees were infected with the resulting sporozoites. Parasites were isolated from each animal, placed into *in vitro* culture and clones made. Recombinant forms among the progeny of the cross were identified in two different ways; (i) by treating the uncloned progeny with pyrimethamine and examining surviving parasites for enzyme and antigen markers, and (ii) by isolating clones from the progeny and characterising them for parent line markers (Walliker et al., 1987). They found a higher than expected proportion of non-parental forms in the progeny of the cross with only three out of 22 progeny clones analysed being indistinguishable from a parental type (where you would expect about five clones of each parental type) (Walliker, 1989).

The second cross to be carried out was by Wellems and colleagues in 1990 (Wellems et al., 1990). *P. falciparum* clones Dd2 (from the W2-MEF line of the Indochina III isolate) (Oduola *et al*., 1988) and HB3 were crossed in order to identify and map genes responsible for chloroquine resistance. However, there are difficulties with interpreting the results of this cross as Dd2 has subsequently proven to have a problem with self-fertilisation due to the male gametogenesis defect (Vaidya et al., 1995b), and so Dd2-parental clones are expected to be rare.

The third cross is a study involving clones Gb4 (from Ghana) and 7G8 (isolated from Brazil) that has been carried out by Hayton and co-workers (Hayton et al., 2008). The cross was established between two clones that differed particularly in their virulence to *Aotus* monkeys. 7G8 cannot infect *Aotus* erythrocytes, whereas Gb4 can. They found that in the mosquito midgut, cross-fertilisation appeared to be favoured as more than expected numbers of hybrid oocysts were identified, plus there was strong selection of progeny

clones in the chimp (171 out of 200 typed were found to be recombinant) (K Hayton per. comm.).

1.7.2 Mating patterns

An understanding of the extent of naturally occurring cross-mating among parasites is important to determine the control of malaria by chemotherapy or vaccination. Its frequency will dictate how often parasites with novel genotypes, for example those that confer multi-drug resistance, will be generated in the population (Babiker *et al*., 1994). It has been clearly demonstrated that crossing of gametes in the mosquito occurs readily. This was first shown with *P. gallinaceum* (Greenberg and Trembley, 1954) and subsequently with the rodent malaria species *P. yoelii* and *P. chabaudi* (Walliker *et al*., 1973; Walliker *et al*., 1975) and then in *P. falciparum* in 1987 (Walliker et al., 1987).

Assuming that gametes undergo classic Mendelian fertilisation, one would expect a ratio of 1:2:1 of parent1: hybrid: parent2 progeny types. However, in all three experimental crosses achieved so far an excessive number of recombinants were consistently found in the progeny collected from the chimpanzee blood. The cause of the disproportionate percentage of recombinant progeny remains unclear. Evidence from the 3D7 x HB3 cross suggests that the mating between gametes is random (Ranford-Cartwright *et al*., 1993), resulting in an approximate ratio of 1:2:1 parent: hybrid: parent oocysts. Therefore favoured cross-fertilisation in the mosquito cannot be the explanation for the excess of recombinant progeny seen in the chimp in both the 3D7xHB3 and the Gb4x7G8 crosses, since these are produced only from hybrid oocysts (from cross-fertilisation events). The failure to find Dd2 parentals in the Dd2 x HB3 cross is explained by the defect in male gametes, but more HB3 parentals were expected than were actually found, and this is still unexplained.

Interbreeding between different parasite clones may influence many factors including sex ratio (Read et al., 1992), the maintenance of distinct ‘strains’ (Gupta *et al*., 1996), and the spread of drug resistance (Hastings and Mackinnon, 1998). Interbreeding can be measured within the malaria lifecycle by genotyping the oocysts from mosquito midguts and examining the level of heterozygosity. This technique has been used to study *P. falciparum* population inbreeding in Tanzania (Babiker *et al*., 1994) and Papua New Guinea (PNG) (Paul *et al*., 1995). The reported inbreeding coefficients (*F,* probability of having the same gene from both parents) were estimated at 0.34 and 0.9 in Tanzania and

PNG respectively, differences in the observed levels may be explained by different transmission levels. If transmission levels are low, such as in PNG, then the opportunities for cross breeding are low, and hence a high inbreeding coefficient is found.

Random mating was also demonstrated in *P. vivax* oocysts dissected from mosquitoes fed directly on patients naturally infected with parasites containing two variants of the circumsporozoite gene (Rosenberg *et al*., 1992). However, Razakandrainibe concluded from a study on 613 oocysts, from 145 mosquitoes, that there was significantly fewer heterozygotes than expected, which demonstrates deviation from panmixia in this population in Kenya (Razakandrainibe *et al*., 2005).

In addition to studying the inheritance of the nuclear genome, some research on the mitochondrial and apicolplast genomes has also been carried out. Each *P. falciparum* parasite contains not only its nuclear genome but two additional extranuclear genomes – a 35kb, apicoplast, circular double-stranded DNA molecule, and a 6kb mitochondrial DNA element. The apicoplast genome primarily encodes components of the transcription and translation machinery of the organelle (Chaubey et al., 2005). Using fluorescent labelling, recent live cell imaging of *P. falciparum* during gametocytogenesis showed that neither the apicoplast nor the mitochondrion are present in male gametes, suggesting both organelles are maternally inherited (Okamoto et al., 2009).

Evidence suggests that the 6kb mitochondrial genome is inherited uniparentally in the female gamete (Creasey et al., 1993; Vaidya et al., 1993b), so by employing a marker that distinguishes the genotype of the 6kb element, hybrid oocysts from mixed clone infections can be typed as products of particular fertilisation events. Previous work on the hybrid oocysts formed from mixed clone infections with the 3D7 and HB3 *P. falciparum* clones, have identified a strong bias for female 3D7 and male HB3 fertilisation events (Creasey et al., 1993; Vaidya et al., 1993b). If random mating is assumed, two explanations for this observation are that the fitness of the gametes of the two clones are not equal, and/or that the fitness of the two types of heterozygotes are not equal (Ranford-Cartwright, 1995).

1.7.3 Recombination in malaria populations

Propagation of mutations and production of new combinations of genes are potentiated by chromosome re-assortment and crossover events that follow formation of *P. falciparum* zygotes in the mosquito. Low levels of recombination can result in linkage disequilibrium

within the parasite genome. Conway *et al* report high recombination rates of natural populations of *P. falciparum* by looking at the linkage disequilibrium rates in asexual parasites sample from six African populations (Conway *et al*., 1999).

From laboratory crossing experiments the frequency with which recombination occurs in *P. falciparum* has been calculated as 15-30 kb per cM, although there are always variations depending on hot spots, for example (Petes, 2001; Su and Wootton, 2004). However, this figure is likely to be an underestimate as more recent unpublished work using a larger number of genetic markers suggests that recombination occurred much more frequently (unpublished pers comm. Su). As a comparison the 1cM (which equals the distance between markers that have a 1% chance of being separated by recombination in a single generation) for humans is ~103 kb and ~3kb for yeast *S. cerevisiae* (Petes, 2001; Walker-Jonah et al., 1992).

The Gb4 x 7G8 cross appears to have a lower recombination rate (36kb per cM) (Hayton *et al*., 2008), suggested to be due partly to genetic heterozygosity between two clones, and a high number of chromosomal inversions, both of which mean that there are fewer areas where the two genomes will be able to complement each other.

1.7.4 Genetic mapping

A high-resolution genetic map with more than 800 microsatellite markers covering all 14 nuclear chromosomes at an average spacing of <30kb has been published for the HB3 x Dd2 cross (Su *et al*., 1999). A genetic linkage map of the *P. falciparum* genome has also been produced using the inheritance patterns of nearly 90 RFLP markers, again for the HB3 x Dd2 cross (Walker-Jonah et al., 1992; Wellems et al., 1990). A map of the 3D7 x HB3 cross progeny has been produced using 282 microsatellite markers (Vaidya, Ranford-Cartwright *et al*, unpublished), and a further map is being produced using SNP markers (Su *et al* ,unpublished). The Gb4 x 7G8 cross has also been mapped using both microsatellites and SNP markers (Hayton unpublished).

1.8 Multiple clone infections and competition

A genetically diverse parasite population (co-occurrence of >1 species or genotype in the human host) is usually found within a single host living in a malaria-endemic region (Arez *et al*., 1997; Beier *et al*., 1988; Snounou *et al*., 1993). This kind of mixed-genotype

infection, at least in rodent malaria infections in mice, has been shown to increase the length of infection and the density of parasites found (Taylor, 1997). There is also evidence from rodent malaria (*P. chabaudi*) that mixed genotype infections may increase the evolution of more virulent parasites (de Roode et al., 2005b). The presence of mixed genotype infections has stimulated the question of how much competition may exist between the clones in the same host.

With regards to the relationship between the multiplicity of infection (number of concurrent clonal infections per carrier) and morbidity and mortality the picture is quite complex. It appears that in infants both parasite densities and morbidity rates are proportional to multiplicity of infection, but in older children the relationships change (Smith et al., 1999). Among older age groups, evidence from Tanzania, Senegal, and Papua New Guinea shows that patients with clinical malaria have a *lower* average multiplicity of infection than asymptomatic children from the same community (Beck et al., 1997b; Engelbrecht et al., 1995; Farnert et al., 1997; Robert et al., 1996a). However, Ranjit *et al* found that the multiplicity of infection, as identified by *msp2* allele typing, is significantly *higher* in severe cases than in mild cases of malaria in a cross-sectional study in India (Ranjit *et al*., 2005).

It appears therefore, that the patients' previous exposure to infection affects whether multiplicity protects against, or increases the chance of, subsequent clinical attacks of malaria. Those individuals in low transmission settings (such as Sudan and Mozambique) who had little previous exposure show an increased risk of clinical malaria attacks with multiple infections (Mayor *et al*., 2003; Roper *et al*., 1998) and the converse is found in those individuals who have been more regularly exposed and may be partially immune (Beck et al., 1997a; Farnert et al., 1999).

There are few published studies on how mixed infections may affect transmission. Transmission of the rodent malaria *Plasmodium chabaudi* has been investigated following mixed infections of different clones in mice (Taylor, 1997). The relative frequency of two competing *P. chabaudi* clones altered dramatically over the course of an infection, and the frequency of transmitted genotypes could be very different from that at the start of an infection. Clones were inoculated into the mouse at ratios of 9:1 or a 1:9, and infection of mosquitoes was analysed. The initially rare clone dramatically increased its representation in the oocyst population relative to its frequency in the asexual population four days

earlier. In all the experiments the clone introduced as the minority of a mixed infection produced as many oocysts or more than it did as a single-clone infection (Taylor, 1997).

These findings were supported by de Roode and colleagues who found strong competitive interactions between genetically different clones of *P. chabaudi.* It appears that the extent to which clones suffer from competition, at least in the rodent malaria asexual stages, depends strongly on the clone genotype, whether the clone was resident before a second clone is introduced, and the duration of prior residency (de Roode *et al*., 2005a). Another paper by de Roode *et al* found a strong relationship between parasite virulence and competitive ability, so that more virulent strains have a competitive advantage in mixed-strain infections (de Roode *et al*., 2005b). Although a different study with *P. chabaudi adami* found that a more virulent strain was significantly less infective to mosquitoes than a less pathogenic strain (Gadsby et al., 2009).

1.8.1 Mating and transmission of natural infections of P. falciparum

The oocyst provides the only practical opportunity in the life-cycle of *P. falciparum* to study the products of individual mating events. The first report on cross-mating rates in natural populations of *P. falciparum* found that 63% of oocysts collected in the Tanzanian village were heterozygous for at least one of the two highly polymorphic antigen genes studied, and were therefore the products of cross-fertilisation events (Babiker *et al*., 1994). A similar study in Papua New Guinea found only 15% of oocysts were heterozygous, suggesting a greater degree of inbreeding in the New Guinean population (Paul *et al*., 1995). This difference was suggested to reflect the different transmission rates in the two areas; in a low transmission area, such as Papua New Guinea, there are fewer mixed genotype infections and hence a higher rate of self-fertilisation. Overall, therefore, evidence suggests that cross mating does occur in the wild populations, although prevalence may depend on transmission rates and the synchrony of gametocyte development of different circulating clones.

1.9 Objectives of the project

Very little is known about either the environmental or genetic factors that affect the development of *Plasmodium* sporozoites on the mosquito host midgut. With the availability of techniques such as quantitative PCR it is now possible to observe the relative differences in genomes numbers in single oocysts dissected from individual mosquitoes. This allows comparisons to be made between the development of oocysts from different environmental and genetic backgrounds, to identify the factors affecting the multiplication of parasites within the developing oocyst. The key objectives of this project were;

- to establish the variation in genome numbers in single oocysts from single clone infections with either 3D7 or HB3 *P. falciparum* in *An. gambiae* mosquitoes

- to compare the genomes per oocyst between 3D7 and HB3 single cone infections and identify any statistically significant difference

- to compare the genomes per oocyst in oocysts formed from self-fertilisation events in the presence and absence of another genetically distinct clone and identify any significant differences

- to investigate the inheritance of the oocyst replication phenotype but analysing the genomes per oocyst in the hybrid oocysts, formed from cross-fertilisation events in mixed genotype infectious feeds.

2 Chapter 2. Materials and Methods

2.1 In vitro culture of *P. falciparum*

All materials and methods followed established standard protocols for malaria parasite gametocyte culture and mosquito infection (Carter *et al*., 1993). Full details of the various solutions and media used for malaria parasite culture are given in Appendix I.

2.1.1 P. falciparum *parasite clones*

Two clones of *P. falciparum* were used in this work. The *P. falciparum* 3D7A clone was previously derived from the Dutch isolate NF54 by limiting dilution (Walliker *et al*., 1987), the origin of NF54 is described in Delemarre & Van der Kaay (1979). The HB3 clone was derived from the isolate Honduras 1/CDC (H1) (Bhasin and Trager, 1984; Trager *et al*., 1981).

2.1.2 Long-term storage of parasite stocks

Asexual erythrocytic stage malaria parasite stocks were stored in liquid nitrogen and thawed/frozen as required, according to previously published protocols which are briefly described below (Aley *et al*., 1984).

2.1.2.1 Thawing of malaria parasites

Ampoules of malaria parasites were removed from liquid nitrogen storage and slowly mixed at a 4:1 ratio with thawing solution I (Appendix I), and left to stand for 5 minutes at room temperature. Nine times the original pellet volume of thawing solution II was then added, mixed, and centrifuged at 290 x *g* for 5 minutes. After removal of the supernatant, the pellet was resuspended at a 1:9 ratio with thawing solution III, followed by centrifugation at the same speed, and removal of the supernatant.The final pellet was then resuspended in 3 ml of complete RPMI 1640 medium (see Section 2.1.3), transferred to a culture flask, gassed with a mixture of 96% N_2, 3% CO_2 and 1% O_2, and maintained in an incubator at 37^{o}C. The following day 0.1 ml of fresh washed human erythrocytes was added and the culture medium changed daily. Once the parasitaemia reached greater than

1%, the culture volume was increased to 5ml and then maintained as described below (Section 2.1.3).

2.1.2.2 Cryopreservation of malaria parasites

Asexual erythrocytic parasite stages were cryopreserved according to standard protocols (Aley *et al*., 1984) as follows: parasite cultures at a reasonably high parasitaemia (>5%), and consisting mainly of ring stages, were centrifuged at 290 x *g* for 5 minutes, the supernatant was removed and the pellet was resuspended at a 1:1 ratio with freezing solution. 0.4 ml of the mixture was aliquoted into 1.2 ml cryovials (Bibby Sterilin Ltd, UK) and immediately placed into liquid nitrogen.

2.1.3 In vitro *culture of asexual erythrocyte stage parasites*

Asexual erythrocytic parasite stages were cultured according to previously published protocols (Haynes *et al*., 1976; Trager and Jensen, 1976) with minor modifications. Five millilitre volume stock cultures at 5% haematocrit in complete RPMI 1640 medium were maintained in 25 cm^2 plug-seal tissue culture flasks (Corning Inc., NY, USA) and kept in an incubator at 37^oC. Culture medium was changed daily with complete RPMI 1640 medium, and after the medium change the flasks were individually gassed with a mixture of 1% O_2, 3% CO_2, and 96% N_2 (BOC Ltd. UK). Parasitaemia was monitored using Giemsa-stained thin smears and was maintained between 0.2 and 8% by diluting the cultures approximately every other day with washed human erythrocytes suspended in complete RPMI 1640 medium to give a final haematocrit in the culture of 5% (v/v). Human erythrocytes (any blood group) were obtained from the Glasgow and West of Scotland Blood Transfusion Service as whole blood in citrate-phosphate-dextrose-adenine (CPD-A) preservative on a weekly basis. Aliquots of blood were washed with incomplete RPMI 1640 medium to remove the preservative and any white blood cells, and were resuspended in complete RPMI 1640 before use. Human serum (group AB) was also obtained from the Glasgow and West of Scotland Blood Transfusion Service as frozen serum (off the clot). Serum from 3-6 donors was pooled, heat inactivated at 56^oC for one hour, and stored frozen at -80^oC until use.

2.1.4 In vitro *culture of sexual erythrocyte-stage parasites*

Two gametocyte-producing *P. falciparum* clones, 3D7A (Walliker *et al.*, 1987) and HB3 (Bhasin and Trager, 1984), have been used in all mosquito infection experiments. New samples were thawed every two months to ensure good gametocyte producing ability of the cultures (and infectiousness of gametocytes) (Ponnudurai *et al*., 1982).

Gametocytes were cultured according to previously published protocols (Carter *et al*., 1993; Ifediba and Vanderberg, 1981; Ponnudurai *et al*., 1982) with minor modifications.

Cultures were set up at 0.5-0.7% parasitaemia, 6% haematocrit in complete RPMI medium in a total volume of 15ml. If the culture was mainly rings, it was set up at 0.7%; if mainly schizonts at 0.5%; and if mixed at 0.6%.

All cultures were kept under a gas mixture of 1% O_2, 3% CO_2, 96% N_2 and in a reliable incubator set at 37^{o}C, with the flasks lying down. The medium was changed every day, generally as close as possible to the same time every day, taking care not to remove any of the black pigment. Once the culture reached a parasitaemia of at least 4% and was showing signs of stress as determined by slightly abnormal morphology, e.g. triangular ring-stage parasites, the culture was 'bulked up' (the volume of medium added was increased to 25ml without the addition of more blood cells). The culture was then maintained at this volume, without the addition of erythrocytes, until harvesting of mature gametocytes for mosquito infection 14-17 days later.

2.2 Mosquito colonies

2.2.1 Insectary conditions and rearing of mosquitoes

Anopheles gambiae sensu strictu Keele strain (Hurd *et al*., 2005) and *An. arabiensis* (I Lyimo, unpublished) were maintained under standard insectary conditions. The mosquitoes were maintained in a temperature- and humidity- controlled insectary at 26 ± 1^{0}C and 70-80% relative humidity with a 12:12 hour light/dark cycle. Larvae were maintained in plastic trays 5 x 16 x 16 cm, in distilled water. $NaHCO_3$ was added to the water to reduce acidity. Larvae were fed ground Tetramin® once per day. Pupae were collected daily and placed into mesh cages, 30 x 30 x 30 cm, for adult emergence. Adult mosquitoes were provided *ad libitum* with a solution of 5% glucose/0.05% para-amino-benzoic acid

(PABA). For egg production, mosquitoes were membrane-fed fresh washed human erythrocytes resuspended to 40% haematocrit in heat-inactivated pooled human AB serum once or twice per week. Small plastic bowls were left in the stock cages two days after the bloodfeed for oviposition. Eggs bowls were removed from the stock cages and hatched larvae transferred to plastic trays and reared as described above. All plastics used for rearing mosquitoes were cleaned with Bactosol detergent (Johnson Wax Professional Ltd, Surrey, England).

2.3 Infection of mosquitoes with malaria parasites

2.3.1 Collection of adult female mosquitoes

Two to three days before the feed, mosquitoes were collected from the colony and put into the secure insectary to adjust to a different room. These mosquitoes were between 3 and 5 days post emergence on this day (so they would be between 5 and 7 days post emergence on the day of feed). Female mosquitoes were collected by using an aspirator (pooter) and transferred to sealed waxed cartons covered with mesh (Carter *et al*., 1993). The mosquitoes were provided *ad libitum* with a solution of 5% glucose/0.05% PABA throughout the duration of the experiment, except for the 24 hours prior to infection when distilled water only was given to encourage engorgement from the membrane-feeders.

2.3.2 Membrane feeder set up

The day before the feed, baudruche membranes (Joseph Long Inc., Belleville, NJ) were attached to glass membrane feeders (Rutledge *et al*., 1964). The feeders were connected together using tubing, and then attached to a circulating waterbath set at 37^{o}C.

2.3.3 Preparing the infectious feed

A mixture of gametocytes aged between 15 and 17 days old have been found to give the best results in mosquito infections (Carter *et al*., 1993). Therefore two flasks of gametocytes, of day 14 and day 17 from initial set up, were grown and mixed together for the bloodmeal. Giemsa-stained thin smears were made of the cultures before mixing the day 14 and day 17 cultures to evaluate gametocytaemia. The supernatant was removed and the cultures were shaken gently to re-suspend the cells, then the contents of the day 14 and day 17 flasks were mixed and transferred to 15ml tubes in a waterbath at 37^{o}C. The mixed

cultures were centrifuged for 5 minutes at 250 x *g*. The supernatant was removed and an equal volume of serum was added to the pellet and mixed well. The parasite/serum mix was then diluted, to varying degrees as required for each experiment, with a mixture of fresh, washed uninfected blood and human serum at 40 % haematocrit. At least an equal volume of blood/serum mix was added to allow enough fresh erythrocytes for the mosquito feed. The exact amount of uninfected blood varied between experiments and was usually adjusted to give a final gametocytaemia in the bloodmeal of ≥ 0.5%. A smear of each bloodmeal tube was made for Giemsa staining, and then the tubes taken to the secure insectary in a beaker of warmed water at 37^{o}C. When mixed clone feeds (containing gametocyte cultures of both 3D7 and HB3) were carried out, the volumes of culture and uninfected blood used were calculated to give equal number of gametocytes from each clone.

2.3.4 Membrane-feeding mosquitoes cultured gametocytes

Approximately 1.5ml of the bloodmeal mixture (as described in section 2.3.3 above) was placed carefully into each membrane feeder. The membrane was moistened with saliva, a mosquito pot was placed underneath each feeder, and the mosquitoes were blown on (which increases their attraction to the feeders). To check for exflagellation, a small drop of the bloodmeal was placed on a slide, the coverslip breathed on and placed over the drop of blood. The slide was examined for the presence of exflagellation (under x 400 magnification) whilst the mosquitoes were feeding.

The mosquitoes were allowed to feed through baudruche membranes for 20-30 minutes. , Unfed mosquitoes were removed 2-3 hours later and killed by freezing. The mosquitoes which had taken a bloodmeal were maintained as described above (2.3.1).

2.3.5 Mosquito dissection and microscopic determination of oocysts infection

Dissections for oocyst enumeration were carried out ten days after the infectious bloodmeal (p.i.) at which point they were clearly visible, but not so fully mature that they were liable to burst. Day ten was taken as the most appropriate point to dissect mosquitoes for this study as previous evidence showed that most DNA replication is complete by this point but the sporozoites have not started to migrate to the salivary glands yet (Bell & Ranford-Cartwright, unpublished). Mosquitoes for dissection were exposed to fatal doses

of chloroform vapour in batches of 4, then submerged briefly in 70% ethanol, transferred to a 5ml screw cap tube containing sterile phosphate buffered saline (PBS) and kept on ice until dissection. Individual dissections were performed with mounted 26 gauge Microlance syringe needles under a dissecting microscope (initial dissection at x 180, then single oocyst dissection at x 750) in sterile PBS on a clean microscope slide. The mosquito midgut was removed from each mosquito and placed under a coverslip for oocyst counting. A wing was removed from the mosquito and kept for wing length measurement, as an indicator of mosquito body size (Lyimo and Koella, 1992). Wings were measured using a dissecting microscope fitted with a Moticam (2300) microscope camera and calibrated software.

Oocyst numbers were counted under a x 40 objective (x 400 magnification) on a Leitz compound microscope.

2.3.6 Dissection and storage of single oocysts

Single oocysts were removed from the midgut by cutting around the oocyst with 2 µl glass micro-pipettes which had been elongated by heating over a Bunsen and pulling-out to a point (CamLab Ltd). The micropipettes were discarded after each oocyst isolation, to avoid cross-contamination.

Where only one oocyst was present, the whole excised midgut was placed into an individual 1.5ml micro-centrifuge tube containing 500µl of lysis buffer (part of the ChargeSwitch® gDNA micro tissue kit, Invitrogen, UK) and proteinase K to a final concentration of 1mg/ml. Individual oocysts removed from a midgut with multiple oocysts were placed into the lysis buffer mixture with an uninfected midgut, so that there would be an equal amount of mosquito DNA in each sample (to act as a carrier for the lower amounts of parasite DNA). The midgut and oocyst material was incubated overnight at 56^{o}C, and then stored at -80^{o}C until DNA extraction.

2.4 DNA extraction from dissected material

The DNA extraction technique was crucial to the success of this project as potentially very small amounts of parasite DNA would be present. Therefore, a technique which lost the least amount of DNA through the process was preferable. Previous studies on infected midguts used phenol/chloroform DNA extraction procedure (Ranford-Cartwright *et al*.,

1991). A newer method was compared to the standard phenol/chloroform method. Of particular interest was the efficiency of the technique to isolate small amounts of parasite DNA expected from single oocysts, and eluting larger final volumes for use in multiple PCR assays.

The new method chosen for comparison to the phenol/chloroform method was the ChargeSwitch® gDNA micro tissue kit (Invitrogen, UK). This method makes use of magnetic beads to which DNA adheres under conditions of low pH; the beads are then immobilised on the side of the tube with a magnet, and cellular debris and other contaminating material is removed by washing. The DNA is then eluted with the use of a wash buffer at pH 8.5. Magnetic purification is reported to allow high yield and purity, and is 100% water-based so avoids the use of hazardous organic materials such as phenol, but is however relatively expensive.

It was impossible to use the same oocyst/midgut sample for the different extraction methods, so different samples with similar oocysts numbers over a wide range (from 1 oocyst to 100 oocysts per midgut) were selected for this trial. Non-stick sterile tubes (Alpha laboratories, UK) were used for all extractions to reduce the loss of DNA through the extraction process by adhesion to the sides of tubes.

Samples of mosquito midguts that had been dissected from 3D7 single clone infections were selected for the comparison experiments. Each sample came from a midgut where the oocyst burden had been counted by light microscopy, and the whole midgut was then incubated in lysis buffer (either from kit or for phenol/chloroform method as previously described (Ranford-Cartwright *et al.*, 1991)) and proteinase K (final concentration of 1mg/ml) overnight at 56^{o}C and then stored at -80^{o}C. Table 1 shows the samples used for the comparison studies.

Table 1. Information on the samples used for DNA extraction comparison experiments. The samples were dissected 10 days post infection and taken from different experimental feeds, with varying infection intensities (number of oocysts present on the mosquito midgut).

DNA extraction technique	Date of Feed	Infection intensity
ChargeSwitch (Invitrogen)	5.02.07	1
	5.02.07	5
	5.02.07	10
	18.05.07	105
Phenol/Chloroform	5.02.07	1
	18.05.07	5
	19.04.07	10
	18.05.07	92

2.4.1.1 ChargeSwitch® gDNA micro tissue kit (Invitrogen, UK)

DNA was extracted according to the manufacturer's protocols with minor modifications. Briefly, 5µl of RNase A (5mg/ml in 10mM Tris-HCl, pH8.5, 10mM EDTA) was added to each DNA sample and incubated at room temp for 5 minutes. Each sample then had 100µl of purification buffer (ChargeSwitch® gDNA micro tissue kit, Invitrogen, UK) added to it and 20µl of ChargeSwitch® magnetic beads (Invitrogen as above), and was incubated for 1 minute at room temperature. The tubes were then placed in the MagnaRack® for 1 minute, which pulls the magnetic beads to the magnet and allowed the removal of the supernatant. The beads were then washed twice with 0.5ml wash buffer and the supernatant discarded. The DNA was eluted from the beads with 75µl of elution buffer (Invitrogen), and the DNA samples stored at -80 ^{o}C.

2.4.1.2 Phenol/Chloroform extraction

The standard procedure (Ranford-Cartwright *et al*., 1991) was followed. 40µl of phenol and 40µl of chloroform was added to each midgut sample, vortexed and centrifuged at 10 000 x *g* for 10 minutes. The aqueous layer was removed and placed in a fresh sterile non-stick tube (Alpha laboratories, UK) and 40µl of chloroform added. The tube was vortexed and centrifuged for 2 minutes at 10 000 x *g*, and the aqueous layer was removed again to a fresh tube. An equal volume (40µl) of isopropanol at -20^{o}C was added, vortexed and incubated at -20 ^{o}C for 20 minutes to precipitate the DNA. The samples were then centrifuged for 30 minutes at 10 000 x *g* at 4 ^{o}C and the isopropanol/water mix was then aspirated off. The DNA pellet was washed by adding 70% ethanol to each tube, vortexing, centrifuging for 5 secs at 10 000 x *g*, and then aspirating off the ethanol. This washing step was repeated once and the DNA allowed to dry at room temperature, to remove all ethanol. 10µl of DNAse and RNase-free deionised, filtered H_2O (MP Biomedicals) was added to each tube to resuspend the DNA, the tubes were then incubated at 100 ^{o}C for 5 minutes, centrifuged briefly at 10 000 x *g*, and stored at -80 ^{o}C.

2.4.1.3 Comparison of two extraction methods

Table 2. Table of primer sequences and PCR conditions for *mdr1* nested PCR

Locus	Primer sequences (5'-3')	Amplicon size	PCR conditions	Reagent conc
mdr1	O1: TGT TGA AAG ATG GGT AAA GAG CAG AAA GAG	660bp	94°C/3m; 94°C/1m, 45°C/1m, 72°C/1m (x35) 72 °C/3m	dNTPs = 200µM Primer (each) = 100nM
	O2: TAC TTT CTT ATT ACA TAT GAC ACC ACA AAC			
	N1: GTC AAA CGT GCA TTT TTT ATT AAT GAC CAT TTA	560bp		
	N2: AAA GAT GGT AAC CTC AGT ATC AAA GAA GAG			

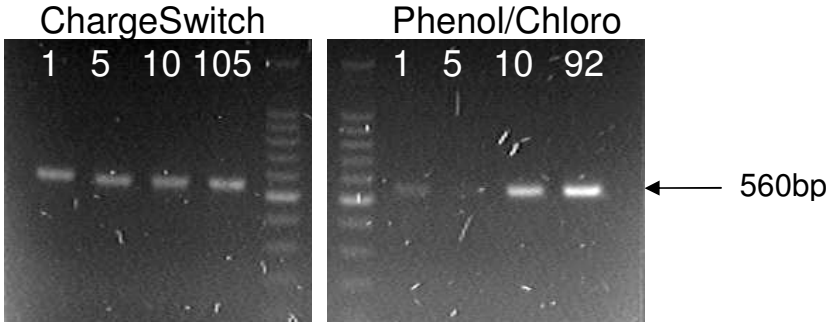
The sensitivity of the techniques was compared by amplifying a 560bp section of the *Pfmdr1* gene using nested PCR (details shown in Table 2). The expected DNA yield is very low and mosquito DNA will predominate so to distinguish the efficient of parasite DNA extraction a PCR-based functional assay was used. *Pfmdr1* is a single copy gene, which has a robust PCR amplification and a known sensitivity equivalent to 2-10 asexual (ring stage) parasites per reaction (Bell and Ranford-Cartwright, 2002).

Although the different extraction methods produced different final elution buffer volumes (75µl ChargeSwitch and 10µl phenol/chloroform), the same amount of sample was added to each PCR reaction (2µl). All PCR reactions were in the same volume (20 µl) and the same volume of nested product was added to the gel (4 µl). Amplification reactions were set up with final concentrations of 1 x PCR buffer (Roche: 100mM Tris-HCl, 15mM $MgCl_2$, 500mM KCl, pH 8.3), 100nM each primer, 200µM each dNTP, 1 unit Taq DNA polymerase (Roche). The nested PCR reaction contained the same concentrations of reagents but two different nested primers. Reactions for the outer PCR were 35 cycles of 94°C/1m: 45°C/1m: 72 °C/1m, followed by 35 cycles of the nested PCR: 94°C/ 30s: 45°C/1m: 72 °C/1m.

5ul of the nested PCR product was loaded onto a 1.5% agarose gel containing ethidium bromide (0.5mg/ml), and run at 50V for 1 hour, followed by UV illumination to visualise the bands.

Strong PCR products were obtained from DNA extracted by ChargeSwitch® from midguts containing 1 to ~100 oocysts (Figure 7). The phenol/chloroform method lacked sensitivity with midguts with fewer oocysts present (as shown by fainter bands in the 1 and 5 oocysts samples).

Figure 7. Agarose gel showing *Pfmdr1* PCR products from three different DNA extraction techniques. The numbers at the top of the columns are the number of oocysts found on each gut sample.



Although both techniques were able to amplify all samples, the ChargeSwitch technique seemed to be more sensitive, bearing in mind that the total volume of DNA solution obtained is 75µl, compared to 10µl, and so 2µl of DNA used in the PCR represented 20% of the total DNA extracted using the Phenol/chloroform method, but only ~3% of that from the ChargeSwitch method. The greater volume of DNA obtained from the ChargeSwitch method allowed amplification of more loci and for more replicates, and the technique avoids the use of toxic chemicals. Therefore the ChargeSwitch® method was used for all subsequent DNA extractions.

2.5 Genotyping single oocysts from mixed infections

Oocysts from mosquitoes offered a mixture of 3D7 and HB3 gametocytes could be the results of self-fertilisation events between gametes of the same clone, or hybrids as a result of cross-fertilisation events. To distinguish the three types of oocysts, two polymorphic genes, *msp1* and *msp2,* were amplified using nested PCR protocol (Ranford-Cartwright *et al*., 1993). The PCR products of each locus differ in size between 3D7 and HB3, with the PCR product obtained from 3D7 being larger than for HB3 in both cases (Table 3 and Table 4). If the oocyst was the result of a self-fertilisation reaction between two 3D7 gametes, then only a single PCR product would be seen for both loci (*msp1*: 534 bp.; *msp2*: 565 bp.*).* For oocysts resulting from self-fertilisation events between HB3 gametes, the single PCR product would be 486 bp.(*msp1*) or 472 bp. (*msp2*). Where cross-fertilisation events occurred, two bands would be seen, one representing each parental allele. Only samples with concurring results from both amplifications were used in the analyses.

The amplification reactions were set up with final concentrations of 1 x PCR buffer (Roche: 100mM Tris-HCl, 15mM $MgCl_2$, 500mM KCl, pH 8.3), 100nM each primer,

200µM each dNTP, 1 unit Taq DNA polymerase (Roche). 2µl of oocyst/midgut DNA was added as template to the outer PCR, and 1µl of the outer PCR was used as template for the nested reaction. The nested PCR reaction contained the same concentrations of reagents but two different nested primers. Reaction conditions for the both outer and nested PCRs are shown in Table 3 and Table 4.

4µl of the nested PCR product was loaded onto a 1.8% agarose gel containing ethidium bromide (0.5mg/ml), and run at 50V for 1 hour, followed by UV illumination to visualise the bands.

Table 3. Table of primer sequences and PCR conditions for *msp1* PCR

Locus	Primer sequences (5'-3')	Amplicon size		PCR conditions
		3D7	HB3	
Outer msp1	O1: CACATGAAAGTTATCAAGAACTTGTC	726 bp.	678 bp.	94°C/3m; 94°C/25s, 50°C/35s, 68°C/2.5m (x30) 72 °C/3m
	O2: GTACGTCTAATTCATTTGCACG			
Nested msp1	N1: GCAGTATTGACAGGTTATGG	534 bp.	486 bp.	
	N2: GATTGAAAGGTATTTGAC			

Table 4. Table of primer sequences and PCR conditions for *msp2* PCR

Locus	Primer sequences (5'-3')	Amplicon size		PCR conditions outer	PCR conditions nested
		3D7	HB3		
Outer msp2	S3: GAAGGTAATTAAAACATTGTC	645bp.	552 bp.	94°C/3m; 94°C/25s, 42°C/1m, 65°C/2m (x30) 72 °C/3m	94°C/3m; 94°C/25s, 50°C/1m, 70°C/2m (x30) 72 °C/3m
	S2: GAGGGATGTTGCTGCTCCACAG				
Nested msp2	S1: GAGTATAAGGAGAAGTATG	565 bp.	472 bp.		
	S4: CTAGAACCATGCATATGTCC				

2.6 Absolute quantitative PCR assay

Real-time quantitative PCR assays amplifying the *SSU rRNA* gene were performed to measure the amount of DNA (and by implication, parasite genomes) present within the oocyst samples (Bell and Ranford-Cartwright, 2004). Reactions were performed on a Roche LightCycler™ using the non-specific dsDNA-binding fluorescent molecule SYBR Green I as detection system for quantification. The assay utilised primers (F: 5' TCTAGGGGAACTATTTTAGCTT 3' and R: 5' CACAGTAAATGCTTTAACTGTT 3') specific to the asexually preferentially-expressed form of the small subunit of the *P. falciparum rRNA* gene, as previously described (Bell and Ranford-Cartwright, 2004).

Optimal reaction conditions employ 5mM magnesium chloride and 300nM of each primer with a cycling profile comprising an initial denaturation (hot start) 95ºC for 60 s and 48 amplification cycles of denaturation 95ºC for 15 s, annealing 60ºC for 5 s and extension 72ºC for 20 s. All assays used the Roche LightCycler FastStart DNA Master SYBR Green I hot start reaction mix in a total reaction volume of 20µl incorporating 4µl of sample DNA. Each sample was run in quadruplicate within a single run. Each assay included three DNA standards of 5, 10, and 100 parasites (see Section 2.6.1), run in duplicate. LightCycler data were analysed using the Fit Points method to obtain a (mean) genome number for each oocyst by comparison with the standard curve drawn from the DNA standards. Under these conditions the assay amplified only the 180 bp target amplicon with a characteristic melting temperature of 78.5ºC. There was no amplification of human or mosquito DNA, or primer-dimer formation, even at extremely low target template concentrations (Bell and Ranford-Cartwright, 2004).

2.6.1 Creation of qPCR DNA standards

DNA standards containing known numbers of parasites were generated as previously described for use in the quantitative PCR (qPCR) analysis of genome numbers (Bell and Ranford-Cartwright, 2004). Briefly, asexual parasite development of parasite clone 3D7 was synchronised using two repeated sorbitol treatments to provide a culture comprising only ring-stages (Lambros and Vanderberg, 1979); each ring-stage parasite has a single genome. The number of parasites (genomes) present per microlitre of culture was calculated from parasitaemia counts taken from Giemsa-stained slides and from red cell densities obtained from haemocytometer counts. Samples containing parasite concentrations that produced final counts of 2.5, 5, 10, 100, and 1000 parasites per microlitre (after DNA extractions) were prepared. The DNA extraction method itself altered the parasite concentrations by a factor of 0.133 (20µl sample ended up in 150µl of elution buffer) so dilutions were calculated to create the desired parasite concentrations after DNA extraction.

20µl of synchronised, and diluted culture was used for each DNA extraction, using the ChargeSwitch gDNA whole blood kit (Invitrogen, UK). DNA was extracted according to the manufacturer's protocols with minor modifications. Briefly, 5µl of RNase A (5mg/ml in 10mM Tris-HCl, pH 8.5, 10mM EDTA) was added to each DNA sample and incubated at room temp for 5 minutes. Each sample then had 100µl of purification buffer (ChargeSwitch® gDNA whole blood kit, Invitrogen, UK) added to it and 20µl of

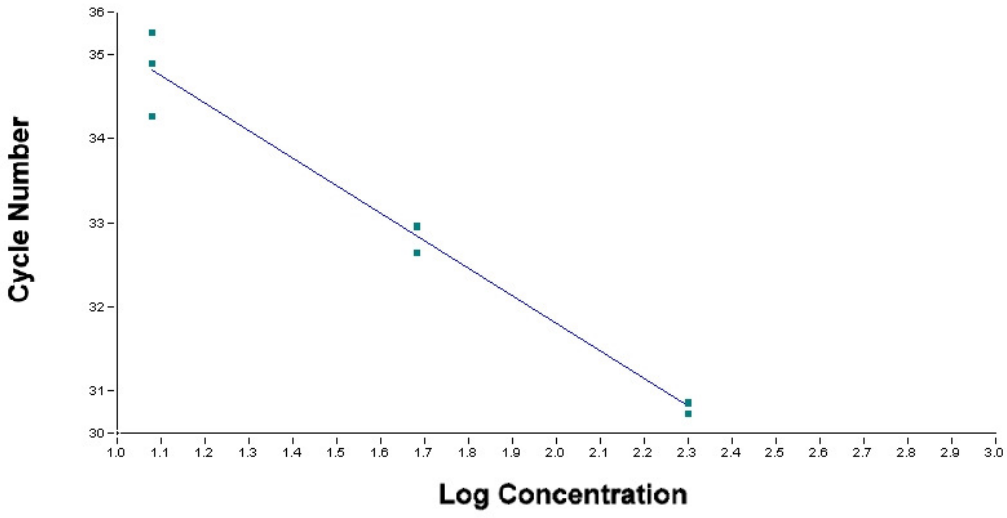
ChargeSwitch® magnetic beads (Invitrogen as above), and was incubated for 1 minute at room temperature. The tubes were then placed in the MagnaRack® for 1 minute, which pulls the magnetic beads to the magnet and allowed the removal of the supernatant. The beads were then washed twice with 0.5ml wash buffer and the supernatant discarded. The DNA was eluted from the beads with 150µl of elution buffer (Invitrogen as above), and the DNA samples stored at -80 ^{o}C.

Although it is likely that some DNA was lost in the DNA extraction procedure, because the same method was used for both mosquito/oocyst and asexual parasites, we assumed the same percentage loss, and did not adjust the genomes/µl DNA to account for any inefficiency of extraction. DNA was extracted into 150µl volumes. 4µl of each was used in the assay to generate the standard curve, thus representing the addition of the equivalent of 10, 20, 40, 400 and 4000 parasites per reaction. Previous evidence showed that the presence of host DNA (mosquito midguts) was not found to affect parasite DNA extraction or subsequent PCR efficiencies (Bell and Ranford-Cartwright, 2004)

2.6.2 Standard curve production

A standard curve was generated for every assay run, using the created standard samples of varying concentrations. An example standard curve, with three standard samples, run in triplicate, is shown in Figure 8. Efficiency of the amplification reaction is 100% when the slope of the standard curve equals -3.321. In this example, the slope of -3.264 indicates that the PCR is very efficient.

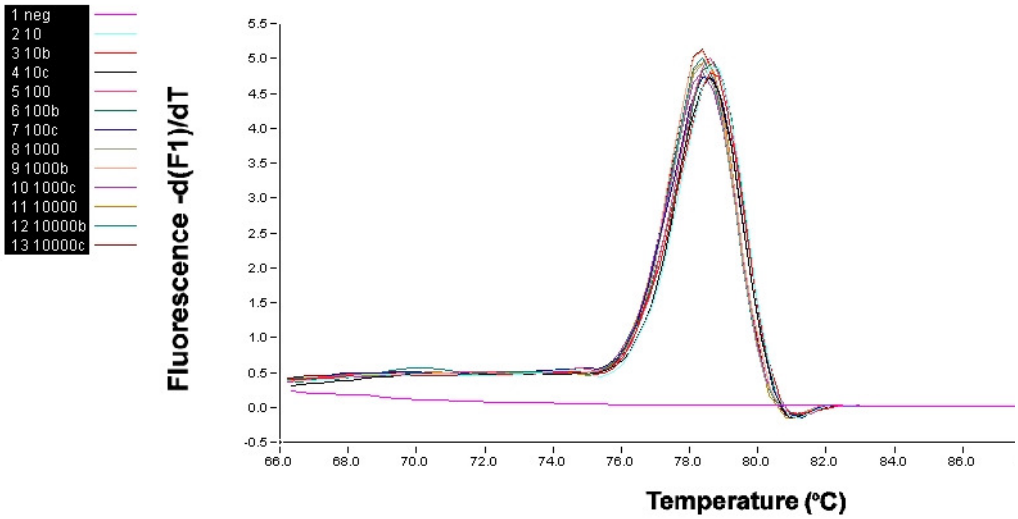
Figure 8. Standard curve for three standard samples, run in triplicate. Samples were 10, 50, and 200 parasites per reaction. Slope=-3.264, r^2=0.99



2.6.3 Melting curve analysis

At the end of every qPCR run a melting curve analysis was carried out (64 ^{o}C to 95 ^{o}C, with 0.1 ^{o}C/sec steps). This allowed confirmation of a single PCR product which melted at about 78.5 ^{o}C (an example melting curve is shown in Figure 9).

Figure 9. Melting curve from qPCR assay carried out on standard samples (run in triplicate), and including a negative control



2.7 Statistical tests

Specific statistical analyses used within each chapter are summarised within the results section of that chapter.

In general, statistical tests were performed using either SAS version 9.1 or R open software version 2.9.2. Differences were considered significant at a probability of $P<0.05$. Graphical representations of data used median values rather than means wherever the data was not normally distributed (as tested by Shapiro-Wilks test).

Both parametric (Student's t, Fisher's Exact) and non-parametric (Mann-Whitney *U*, Kruskal-Wallis, and Wilcoxon Rank Sum) analyses were employed, depending on the sample sizes and the distribution of the data used.

The Mann-Whitney *U* test is a non-parametric test for assessing whether two independent samples of observations come from the same distribution. The Kruskal-Wallis test is a one-way analysis of variance by ranks and is a non-parametric method for testing equality

of population medians among groups. The Wilcoxon signed-rank test is a non-parametric test used as an alternative to the paired Student's t-test when the population cannot be assumed to be normally distributed.

Both general and generalised linear modelling was employed depending on the distribution of the data being analysed. The general linear model is a statistical linear model based on the equation $y = b_0 + b_1x$ (where y = the dependent variable, x = the independent variable, b_0 = the intercept, and b_1 = the slope of the regression line). The generalised linear model is a flexible generalisation of ordinary least squares regression which differs from the general linear model in two major respects; firstly, the distribution of the variables can be non-normal and does not have to be continuous; secondly the model involves a link function which can allow nonlinear relationships between dependent and independent variables.

Results from all general linear models reported met the assumptions listed below. Linear regression (Model I) analysis assumptions:

1. the values of the dependent variable (*Y*) are assumed to be from a population of values normally distributed about the regression line
2. the independent variable (*X*) is measured without error
3. the dependent variable is determined by the independent variable
4. the relationship between *X* and *Y* is linear (sometimes the data are transformed to produce a linear relationship)
5. the variance of the *Y* values is also assumed to be the same whatever the value of *X*.

Logistic regression (a specific type of generalised linear model) was used for analyses where the dependent variable was binary (such as whether the mosquito was infected or not, which is considered in Chpt 3). By employing the use of the logistic function (as the link function), logistic regression provides a useful tool to describe the relationship between one or more independent variables (categorical or continuous) and a dependent variable which has only two possible outcomes.

Random effects (mixed) modelling was used when multiple samples were taken from either the same experiment and/or the same mosquito. It is possible to compare the

relationship between variables such as infection intensity and mosquito wing length with data collected from different experiments by including experiment as a random (rather than fixed) effects variable. A model that includes both fixed and random effects is usually called a mixed model, and linear mixed models were used throughout the analyses.

3 Chapter 3. Mosquito factors that influence *P. falciparum* infection

3.1 Introduction

There are many complex interactions between the parasite and the vector host and a long list of potential mosquito factors that might influence the infection. Two particular factors that have been previously studied with respect to *Plasmodium* infection are the mosquito size, and the mosquito species, and these are the two factors considered in this chapter.

Variation in body size (thought to be caused by larval habitat and nutrition levels) is a common feature of natural mosquito populations and may be one of the factors that contribute to transmission of malaria (Okech et al., 2007; Suwanchaichinda and Paskewitz, 1998), although there is conflicting evidence from laboratory and field based studies as to the extent of this association between mosquito size and infection with *Plasmodium* (as discussed in Chapter 1). There are a range of reported methods for recording mosquito size; the results presented in this thesis are all based on the wing length variable as representing mosquito size.

This chapter not only considers mosquito size as an influence on infection, but also reports a pilot study on the differences in infection prevalence, intensity and multiplication in the oocyst in two different mosquito species - *An. gambiae* and *An. arabiensis.* Other studies that looked at *Plasmodium* infections in different mosquitoes include a comprehensive look at *P. falciparum* infections in six different *Anopheles* spp. (Vaughan et al., 1994b).

The main objectives of this chapter were to investigate the influence of mosquito body size on infection prevalence and intensity and to run a pilot experiment to investigate the susceptibility of *An. arabiensis* to 3D7 *P. falciparum* infections.

If mosquito body size or species are associated with any parameter of infection (prevalence and/or intensity) this could be of epidemiological importance, as mosquito population dynamics affect transmission dynamics.

3.2 Results

A brief summary of the data collected is presented in Appendix II.

3.2.1 Size of mosquito

As the prevalence of infection varies substantially between the 3D7 and HB3 clones, the data sets from infections with each clone are considered separately. The genetic factors influencing infection are considered in Chapter 5.

3.2.1.1 The effect of mosquito size on prevalence of infection of An. gambiae s.s. *with* P. falciparum *3D7 clone*

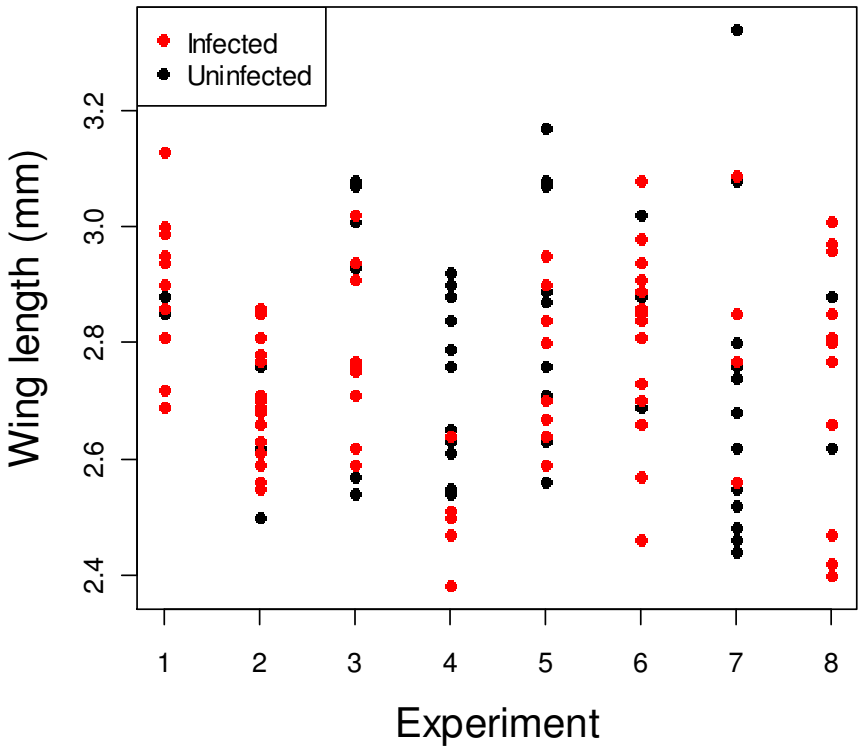
To assess the effect of the size of a mosquito on the probability of infection with *P. falciparum* (clone 3D7), data from 8 different experimental feeds were analysed (Table 5). The sample sizes within each experiment are fairly low and also variable between experiments. This just reflects a variation in the number of mosquitoes that fed, not a difference in the numbers of mosquitoes selected for each experiment. The raw data are shown in Figure 10 and summarised in Table 5.

Table 5. Size of *An. gambiae s.s.* mosquitoes and prevalence of *P. falciparum* 3D7 infection. Data are shown for 8 experimental feeds (Expt), gametocytaemia (% rbc infected with gametocytes) of the infectious bloodmeal (Gams), prevalence of infection (Prev), mean wing length of infected and uninfected mosquitoes with standard errors of these means (SEM) and numbers of mosquitoes in each group (n). Infection was defined as the presence of at least one oocyst on the midgut, 10 days post-infectious feed.

Expt	Gams	Prev	Infected			Uninfected		
			Mean wing size (mm)	sem	n	Mean wing size (mm)	sem	n
1	0.28%	73%	2.9	0.134	10	2.84	0.11	4
2	0.34%	62%	2.69	0.09	22	2.65	0.087	9
3	0.40%	56%	2.78	0.137	10	2.87	0.247	6
4	0.80%	22%	2.50	0.085	6	2.73	0.108	15
5	1.20%	50%	2.75	0.132	12	2.80	0.136	13
6	1.20%	81%	2.83	0.161	17	2.82	0.161	4
7	1.20%	17%	2.82	0.219	4	2.68	0.242	16
8	2%	88%	2.74	0.222	11	2.75	0.184	2
Totals					92			69

Figure 10 displays the wing length data for all mosquitoes dissected from eight different experiments. Mosquitoes that were infected are distinguished from those that were uninfected by the colour of the dot (red=infected, black=uninfected).

Figure 10. Mosquito wing lengths of 3D7 infected and uninfected *An. gambiae* mosquitoes, divided on the x-axis by experiment. Red dots are infected and black dots are uninfected mosquitoes, each dot represents one dissected mosquito, n=161.



The distribution of wing lengths of all mosquitoes within an experiment was examined for normality using a Shapiro-Wilks test. The wing lengths of mosquitoes in experiment 7 was found to differ from normality (p=0.00611). Wing lengths in the remaining 7 experiments conformed to a normal distribution and could therefore be analysed using parametric methods. The mean wing length of infected mosquitoes in each of these experiments was compared to that of the uninfected mosquitoes using a Student's t-test. The results are shown in Table 6. Experiment 7 which had a non-normal distribution of mosquito wing lengths was analysed using Mann-Whitney U test and the results are also shown in Table 6.

Overall, the distribution of wing lengths of all mosquitoes pooled from all 7 experiments was not significantly different from normal (Shapiro-Wilks test p=0.0953).

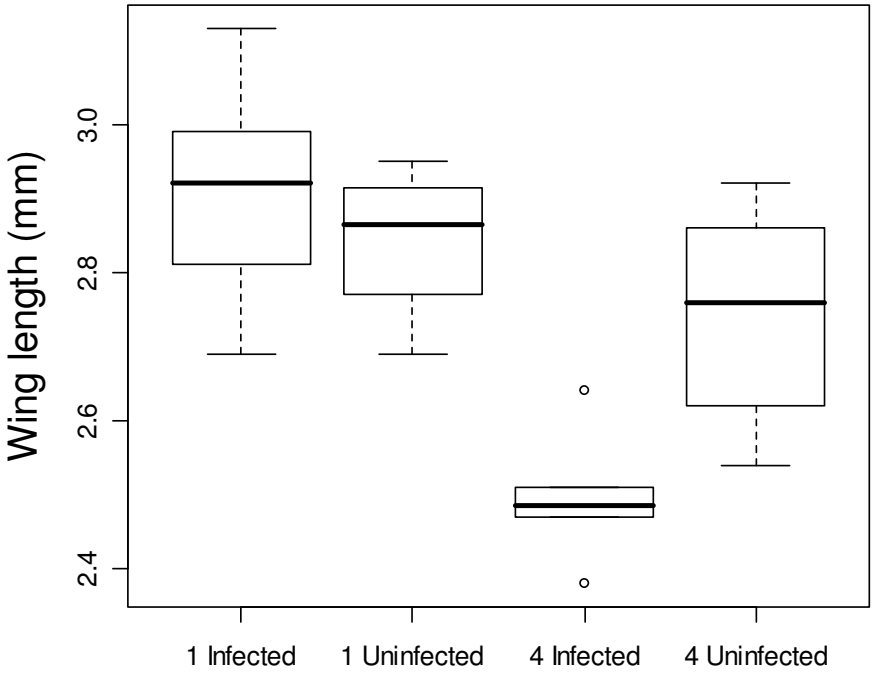
Table 6. Statistical analysis of the difference in mosquito wing lengths between 3D7 infected and uninfected mosquitoes from 8 different experiments (Expt). P values are shown for t-tests where the wing lengths conformed to a normal distribution or for Mann-Whitney U tests where they did not. The power of each test to detect the actual difference in the mean wing length observed was calculated using two-sample t test power calculation in SAS.

Expt	Power	T test p value	Mann-Whitney U p value
1	0.08	0.4430	--
2	0.165	0.2219	--
3	0.114	0.4768	--
4	0.997	**0.0002**	**--**
5	0.149	0.4481	--
6	0.05	0.9453	--
7	0.107	--	0.1184
8	0.05	0.9446	--
Totals			

There was no statistically significant difference in the wing length of infected and uninfected mosquitoes in seven of the eight experiments. In one experiment (expt 4), infected mosquitoes were significantly smaller than uninfected mosquitoes (p=0.0002). However the lack of significance for the seven other experiments may be due to small sample sizes. Power calculations (Table 6) indicate that for most experiments, the power to detect a difference in the mean wing lengths of that observed or larger was poor (range of 8 - 16.5% power), whereas for experiment 4, the difference in the mean wing length between infected and uninfected mosquitoes was relatively larger (0.23mm), and the power to detect this difference was 99%. This is illustrated in Figure 11, taking as an example data from experiment 1 to compare with experiment 4. The graph clearly displays the significant difference in mean wing lengths between infected and uninfected mosquitoes in experiment 4, but not in experiment 1.

Infected mosquitoes in experiment 4 were also statistically smaller (range 2.38-2.51mm) and less variable in size (smaller s.e.m: 0.085) than those used in five of the other seven experiments (Table 5).

Figure 11. Wing lengths of infected and uninfected mosquitoes in experiment 1 (non-significant difference in mean wing length) and experiment 4 (significant difference in mean wing length). Heavy bars indicate medians, boxes show the lower and upper quartile and whiskers show the maximum and minimum. Circles are those data points considered outliers.



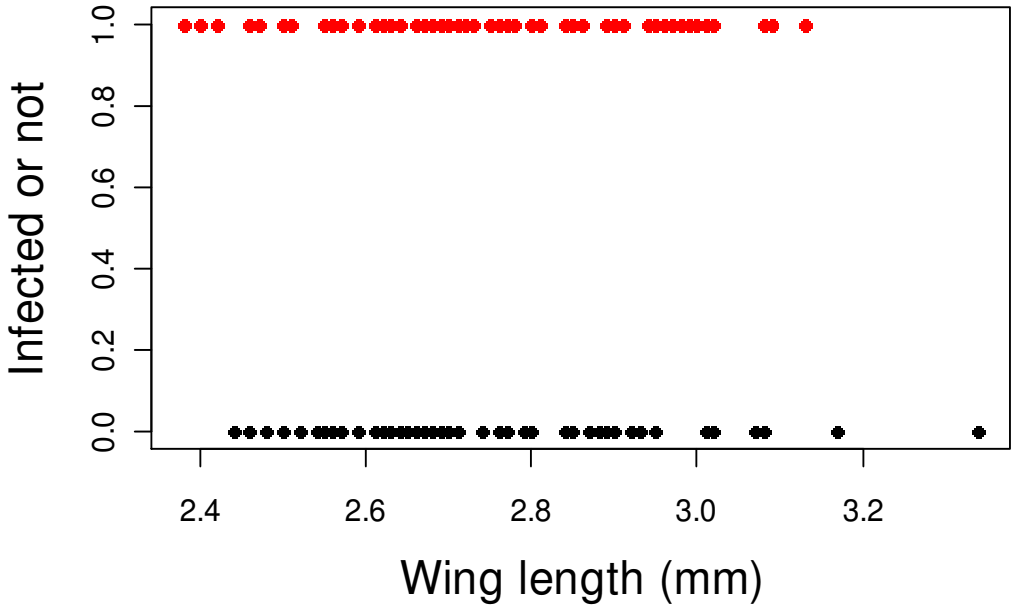
Pair-wise t-tests, with Bonferroni correction, between infected mosquito wing lengths from every experiment (except 7 which shows a non-normal distribution) shows that the wing lengths of infected mosquitoes are significantly different between experiment 4 and experiments 1,2,3,5 and 6, as well as between experiments 1 and 2 (Table 7). The results from experiment 4 may be suggestive of an effect which is only observed within small mosquitoes. The infection rate of all mosquitoes with a wing length of 2.5mm or less (across all eight experiments) was 67%, whereas the infection rate in mosquitoes larger than 2.5mm was 56%, but this difference was not statistically significant (Fisher's Exact test p=0.558). The sample size of small-sized mosquitoes (n=12) makes further analysis of the hypothesis (that smaller mosquitoes have a higher chance of getting infected) difficult to test within this data set.

Table 7. Pairwise t-tests on mean wing length of infected mosquitoes in 7 experiments. The significance value was adjusted to 0.00179 by Bonferonni's method (α' = α / κ, where α = normal significance level (0.05) and κ = number of comparisons (28). P values reaching significant are highlighted in bold.

Expt	1	2	3	4	5	6	8
1		**0.0007**	0.0734	**0.0000**	0.0162	0.2211	0.0586
2			0.0751	**0.0009**	0.1994	0.0051	0.5227
3				**0.0001**	0.552	0.4736	0.5729
4					**0.0002**	**0.0000**	0.0060
5						0.1674	0.8883
6							0.2706

There is no obvious threshold in size for infection with 3D7 (Figure 12).

Figure 12. Wing size of *An. gambiae s.s.* mosquitoes and whether they were infected (red) or not (black) with *P. falciparum* clone 3D7, 10 days after taking an infected bloodmeal.



It was possible to analyse the data from all eight experiments with a mixed effects generalised linear model. A generalised model considering infection as the outcome variable and wing length as an explanatory variable, whilst also including experiment as a random effect variable, provided no evidence for an association between wing length and infection with 3D7 infections (Table 8).

Table 8. Results from a mixed effects linear model analysis using 3D7 infections in *An. gambiae s.s* mosquitoes. Numbers of mosquitoes and experiments are shown, along with the p value for the association of wing length and probability of infection.

Explanatory variable	n (mosquitoes)	n (experiments)	p value
Wing length	161	8	0.6023

3.2.1.2The effect of mosquito size on prevalence of infection of An. gambiae s.s. *with* P. falciparum *HB3 clone*

To assess the effect of the size of a mosquito on the likelihood of infection with *P. falciparum* clone HB3, data from 124 mosquitoes (26 infected and 98 uninfected) isolated from 7 different experimental feeds were analysed. The data are summarised in Table 9.

Table 9. Size of *An. gambiae s.s* mosquitoes and prevalence of *P. falciparum* HB3 infection. Data are shown for 7 experimental feeds (Expt), gametocytaemia of the infectious bloodmeal (Gams), prevalence of infection (Prev), wing length of infected and uninfected mosquitoes with standard errors of these means (SEM) and numbers of mosquitoes in each group (n). Infection was defined as the presence of at least one oocyst on the midgut, 10 days post-infectious feed.

Expt	Gams	Prev	Infected			Uninfected		
			Mean wing size (mm)	sem	n	Mean wing size (mm)	sem	N
1	3%	30%	2.69	0.204	3	2.746	0.186	17
2	1.3%	30%	2.75	0.144	7	2.813	0.171	16
3	1.7%	20%	2.573	0.120	3	2.853	0.169	11
4	1.3%	10%	2.675	0.120	2	2.816	0.168	16
5	3%	13%	2.655	0.134	2	2.783	0.186	10
6	1.6%	33%	2.691	0.138	7	2.816	0.202	13
7	1.4%	10%	2.81	0.198	2	2.853	0.153	15
Totals					26			98

The distribution of wing lengths of all mosquitoes within an experiment was examined for normality using a Shapiro-Wilks test. The wing lengths of mosquitoes in all experiments conformed to a normal distribution and could therefore be analysed using parametric methods. The mean wing length of infected mosquitoes in each of these experiments was compared to that of the uninfected mosquitoes using a Student's t-test. The results are shown in Table 10.

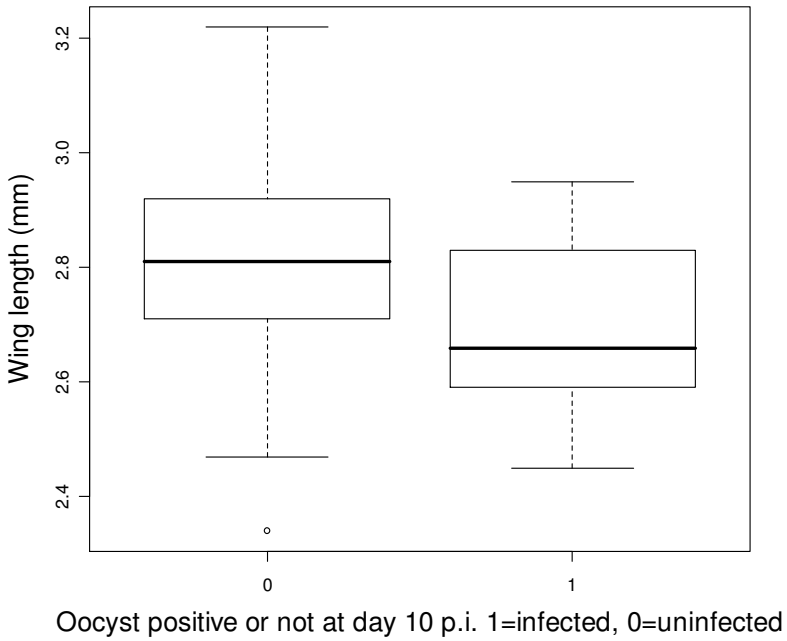
Table 10. Statistical analysis of the difference in mosquito wing lengths between infected and uninfected mosquitoes. P values are shown for t-tests. The power of each test to detect the actual difference in the mean wing length observed was calculated using two-sample t test.

Expt	Power	t test p value
1	0.060	0.6721
2	0.092	0.3835
3	0.370	**0.0267**
4	0.083	0.3097
5	0.072	0.3768
6	0.222	0.1212
7	0.053	0.8101

There was no statistically significant difference in the mean wing length of infected and uninfected mosquitoes in six of the seven experiments, although the mean wing lengths in all experiments were lower in the infected than uninfected groups. In one experiment (expt 3) infected mosquitoes were significantly smaller than uninfected mosquitoes (p=0.0267). However the lack of significance for the six other experiments may be due to small sample sizes. Power calculations (Table 10) indicate that for all of the experiments, the power to detect a difference in the means of that observed or larger was poor (range of 6 - 37% power).

When the mosquitoes from all experiments were pooled for analysis the mean wing length of the infected mosquitoes was 2.698 (n=26), and the mean of the uninfected mosquitoes was 2.810 (n=98). The distributions of these two groups (Figure 13) were found to be normal using a Shaprio-Wilks test (infected p= 0.249, uninfected p=0.549).

Figure 13. Wing lengths of infected and uninfected mosquitoes pooled from all 7 HB3 experiments. Heavy bars indicate medians, boxes show the lower and upper quartile and whiskers show the maximum and minimum. Circles are those data points considered outliers (outside 1.5 x interquartile range).



A mixed effects generalised linear model, using infection as the outcome variable and wing length as the explanatory variable, whilst also including experiment as a random effects variable, provides strong evidence for an association between wing length and infection

(results shown in Table 11). The model parameters were an intercept of 9.886 and a variable coefficient of -4.074.

Table 11. Results from a mixed effects generalised linear model analysis using HB3 infections in *An. gambiae s.s* mosquitoes. Numbers of mosquitoes and experiments are shown, along with the p value for the association of wing length and infection.

Explanatory variable	N (mosquitoes)	n (experiments)	Variable coefficient	P
Wing length	124	7	-4.074	**0.0028**

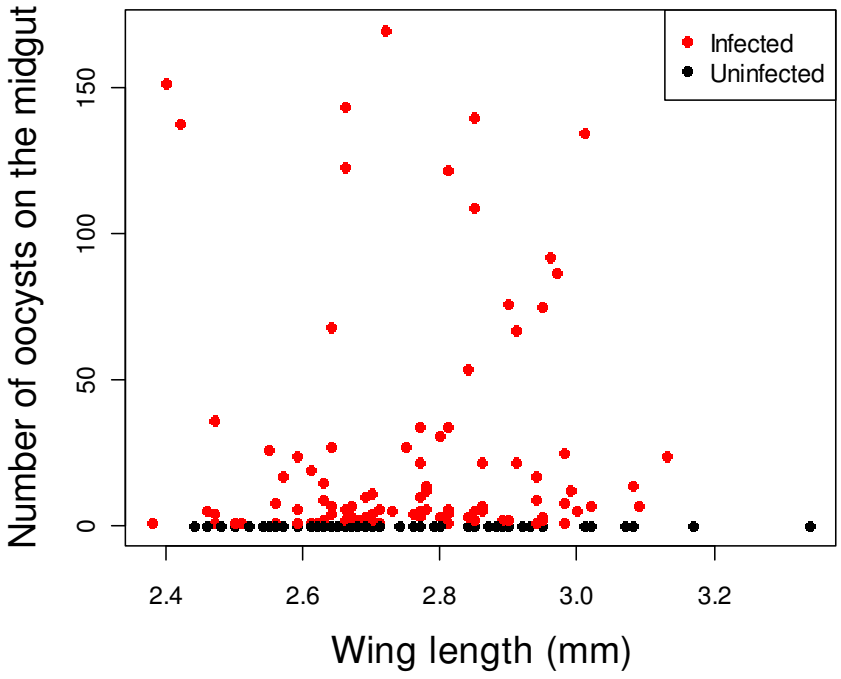
Although the sample size is small for the infected mosquitoes (n=26) the model demonstrates a significant association between mosquito size and the probability of getting infected. Infected mosquitoes had a significantly smaller wing length than uninfected mosquitoes when using HB3 *P. falciparum* in *An. gambiae s.s.* mosquitoes.

3.2.1.3The effect of mosquito size on intensity of infection

As the intensity of infection is always very low with HB3 infections, (infected range: 1-14 oocysts, median = 1 oocyst), only 3D7 data has been considered for this section.

Figure 14 shows the data from 161 *An. gambiae s.s.* mosquitoes that were fed *P. falciparum* 3D7 clone, and dissected 10 days p.i.

Figure 14. Intensity of infection and mosquito wing length. All 161 *An. gambiae* s.s. mosquitoes from eight different experimental feeds with P. falciparum 3D7 are shown, including infected (red) and uninfected (black) mosquitoes.



Two mixed effects generalised linear models, using intensity of infection as the outcome variable and wing length as the explanatory variable, whilst also including experiment as a random effects variable, provides no evidence for an association between wing length and infection intensity (Table 12). Two mixed effects linear models were used; one using infected and uninfected mosquito data, the other using only data from mosquitoes with at least one oocyst.

Table 12. Results of two mixed effects models to assess whether there is any association between wing length and oocyst burden (experiment was entered as a random effects variable) with 3D7 *P. falciparum* in *An. gambiae s.s.* mosquitoes.

Data used	p value
all data	0.5331
Only infected data	0.4739

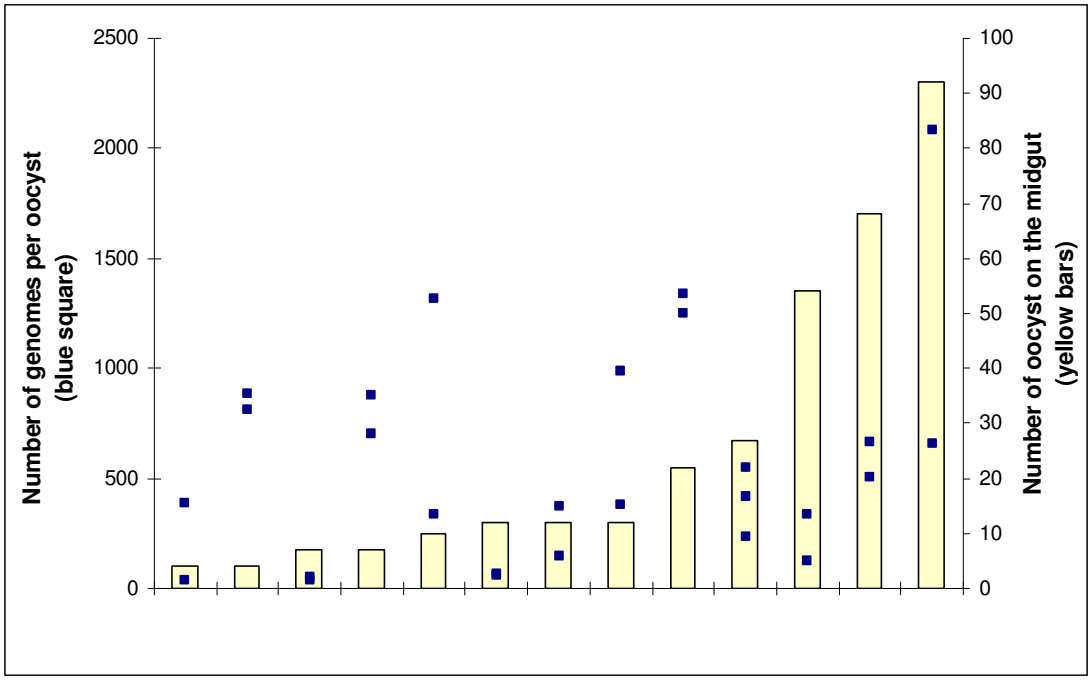
The data from 3D7 *P. falciparum* infection in *An. gambiae s.s.* mosquitoes provide no evidence that the intensity of infection is influenced by the size of the mosquito.

3.2.1.4 Variation in genomes per oocyst between mosquitoes

To assess the level of variation in genomes per oocyst between mosquitoes, an analysis of variance was carried out on 3D7 single clone infection data where at least two single oocyst samples had come from the same mosquito. A total of 28 single oocysts were dissected from mosquitoes where at least one other oocyst had also been dissected. The 28 oocysts came from a total of 13 mosquitoes. All the single oocysts were analysed by qPCR to determine the genome numbers.

Figure 15 displays the data of genomes per oocyst against the mosquitoes they were dissected from, and also shows the infection intensity of each mosquito.

Figure 15. The variation in genomes per oocyst (blue squares), for mosquitoes where at least two single oocyst samples were available. Data from *P. falciparum* 3D7 single clone infections in *An. gambiae* mosquitoes only. Intensity of infection is also shown for each mosquito (yellow bars). n oocysts=28. Each square represents the number of genomes per oocyst for each single oocyst obtained from that mosquito. Data are ranked along the x-axis by intensity of infection (number of oocysts on the midgut).



An analysis of variance test on genomes per oocyst and mosquito showed a significant p value of 0.0217 (df 12, SS 4645203, MS 387100, F value 3.07) indicating a significant difference in genomes per oocyst between mosquitoes. However there are obviously very low numbers of single oocyst samples from the same mosquito, only one mosquito had more than two samples dissected from it (mosquito 11 had 4 single oocyst samples) which makes this analysis very difficult, especially considering the wide variation in genomes per oocyst across the whole data set, but this analysis demonstrates there are some factors not measured in this study, that differ between different mosquitoes and affect the replication within the developing oocyst.

Due to the findings that genomes per oocyst did vary significantly between mosquitoes, this analysis justified using 'mosquito' as a random effects variable in all other mixed linear models considering genomes per oocyst as an outcome variable.

3.2.1.5 The effect of mosquito size on the number of genomes per 3D7 oocyst

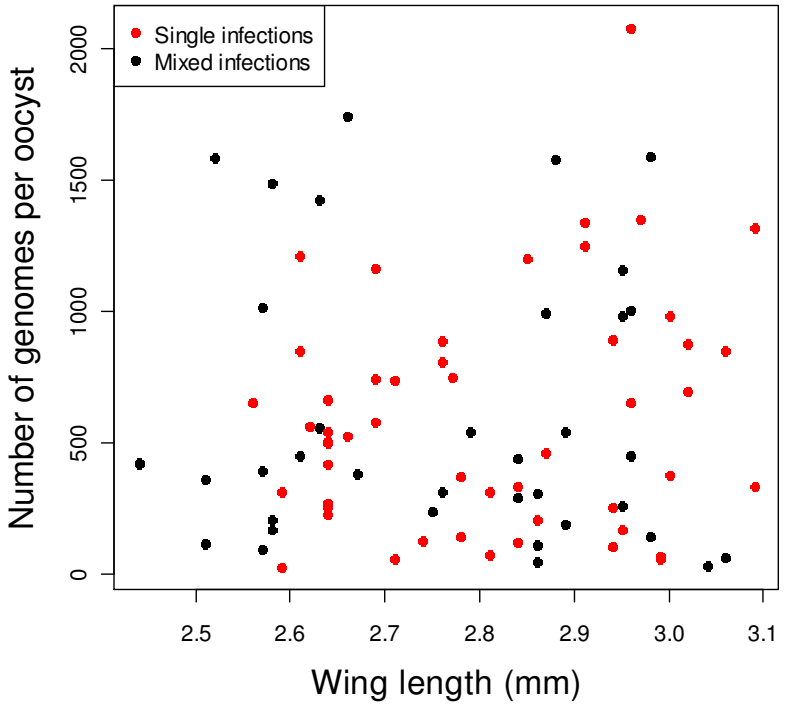
108 single oocysts were analysed using qPCR to determine the genome numbers per oocyst, 62 of those oocysts came from infections with clone 3D7 only, 46 were from infections with 3D7 and HB3, but were genotyped as 3D7 oocysts (Chapter 5). The oocysts were collected from a total of 80 mosquitoes, from 14 different experiments. A description of the data is shown in Table 13.

Table 13. Single oocyst data from single and mixed clone feeds. P values are the result of normality tests on genomes/oocyst distributions. Shapiro-Wilks values are for the distribution of the genomes/oocyst data.

***P. falciparum* clone fed**	**Number of oocysts analysed**	**Shapiro-Wilks p value**	**Median genomes per oocyst (25-75 percentile)**
3D7	62	**0.0003**	402 (201-686)
3D7 + HB3	46 3D7	**<0.001**	408 (215-977)

Data from the quantitative PCR analysis of single oocysts (Chapter 2, section 2.6) were analysed for association with mosquito size (Figure 16). Only data for 3D7 oocysts was included as the number of samples for HB3 was small, but data were pooled for 3D7 oocysts from mosquitoes fed only 3D7 and 3D7 oocysts from mosquitoes fed a mixture of 3D7 and HB3 as there was no statistically significant difference in genomes per oocyst between these two groups (Chapter 5, section 5.2.5).

Figure 16. The number of genomes per oocyst and mosquito wing length. Data is presented only for 3D7 oocysts collected from both single (red) and mixed (black) clone infections of *An. gambiae s.s.*



The data were further analysed using mixed effects linear models. The analysis was performed using number of genomes per oocyst as the dependent variable with mosquito wing length as the explanatory variable, including experiment and mosquito as random effect variables. The models were run to assess whether mosquito wing length was significantly associated with the number of genomes per oocyst. Two models were run; the first used data from oocysts from both single and mixed clone infections, the second model used only data from single clone infections. The p values from the two models are shown in Table 14.

Table 14. Results of mixed effects models for genomes per oocyst variation with wing length in *An. gambiae s.s.* mosquitoes fed either 3D7 alone or 3D7 and HB3.

Data used	p value for wing length
Single and mixed clone data	0.8529
Only single clone data	0.9572

This analysis suggests none of the variation in genomes per oocyst observed in the data can be explained by mosquito size.

3.2.1.6 The effect of mosquito size on the number of genomes per HB3 oocyst

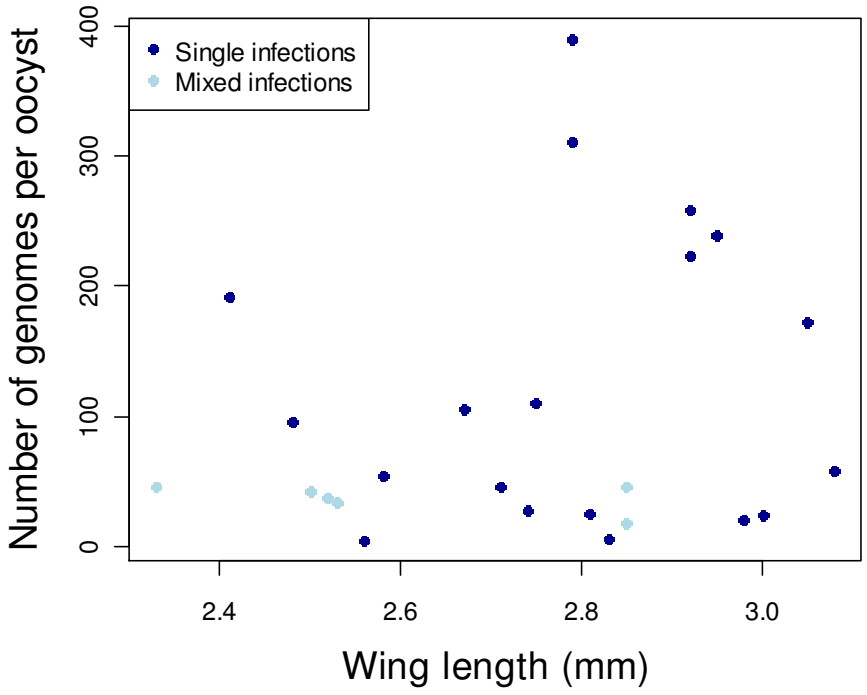
Genome numbers per oocyst were available from 31 single oocysts (23 from single clone infections and 8 from mixed clone infections) collected from a total of 31 mosquitoes, from 18 different experiments (Figure 17). A description of the data is shown in Table 15.

Table 15. Single oocyst data from single and mixed clone feeds

***P. falciparum* clone fed**	**Number of oocysts analysed**	**Shapiro-Wilks p value**	**Median genomes per oocyst (25-75 percentile)**
HB3	23	**0.0337**	140 (46-228)
3D7 + HB3	8 HB3	0.1211	40 (34-43)

There is no obvious correlation between mosquito wing length and number of genomes per oocyst (Figure 17).

Figure 17. The number of genomes per oocyst and mosquito wing length. Data is presented only for HB3 oocysts collected from both single (blue) and mixed (light blue) clone infections.



The data were further analysed using mixed effects linear model, using all the available HB3 oocyst data, as there were too few to split the analysis. The analysis was performed

using number of genomes per oocyst as the dependent variable with mosquito wing length as the explanatory variable, including experiment as a random effect variable. The model produced a non-significant p value of 0.344, suggesting no association between genomes per oocyst and wing length in *An. gambiae s.s.* mosquitoes when infecting with HB3 *P. falciparum* clone.

3.2.2 Mosquito species

A set of experiments was carried out as a pilot project to see if infections could be achieved with 3D7 *P. falciparum* in a newly established laboratory colony of *An. arabiensis* mosquitoes. The aims of these experiments were to establish if infection could be achieved, and to investigate the prevalence, intensity, and levels of genomes per oocyst produced by 3D7 in a different species of mosquito.

3.2.2.1 The effect of mosquito species on prevalence of infection

A small number of experiments were performed to compare the prevalence of *P. falciparum* clone 3D7 in *An. arabiensis* and *An. gambiae s.s.* simultaneously fed the same gametocyte-containing infectious feed (Table 16). The prevalence data from the 4 experiments is displayed in Figure 18.

Table 16. Prevalence of infection (Prev.) in *An. arabiensis* and *An. gambiae* mosquitoes fed *P. falciparum* clone 3D7 gametocytes. Fisher's Exact tests were performed to analyse the difference in infected and uninfected mosquitoes between the two species for each experiment (Expt), n=number of mosquitoes dissected. n.d.=not done.

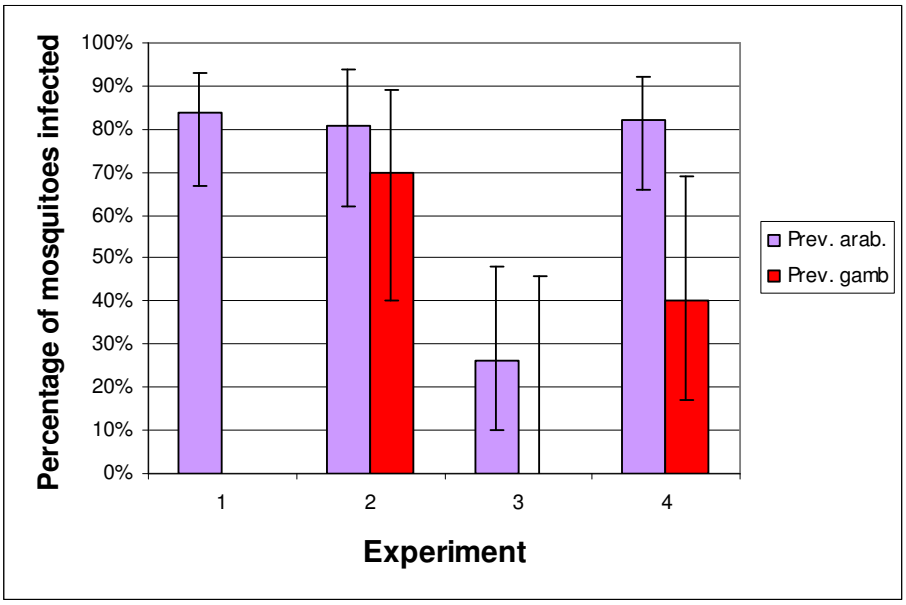
Expt	*An. arabiensis*		*An. gambiae s.s.*		Fisher's Exact P	Power
	Prev. (95% C.I.)	n	Prev. (95% C.I.)	n		
1	84% (67-93)	31	n.d.	n.d.	n.d.	n.d.
2	81% (62-94)	27	70% (40-89)	10	0.655	0.10
3	26% (10-48)	23	0% (0-39)	6	0.295	0.006
4	82%(66-92)	38	40% (17-69)	10	**0.00062**	0.68
Total		119		26		

The data show that infection of the *An. arabiensis* colony was possible with *P. falciparum* clone 3D7, and high prevalence of infection was obtained.

Fisher's Exact tests were performed on the results from the three experiments where both *An. arabiensis* and *An. gambiae s.s.* were fed with same 3D7 culture. No significant differences in prevalence were seen between mosquito species in experiments 2 and 3

(p=0.655 and p=0.295 respectively). In the final experiment the infection prevalence in *An. arabiensis* was significantly higher than that in *A. gambiae s.s.* (p= 0.00062).

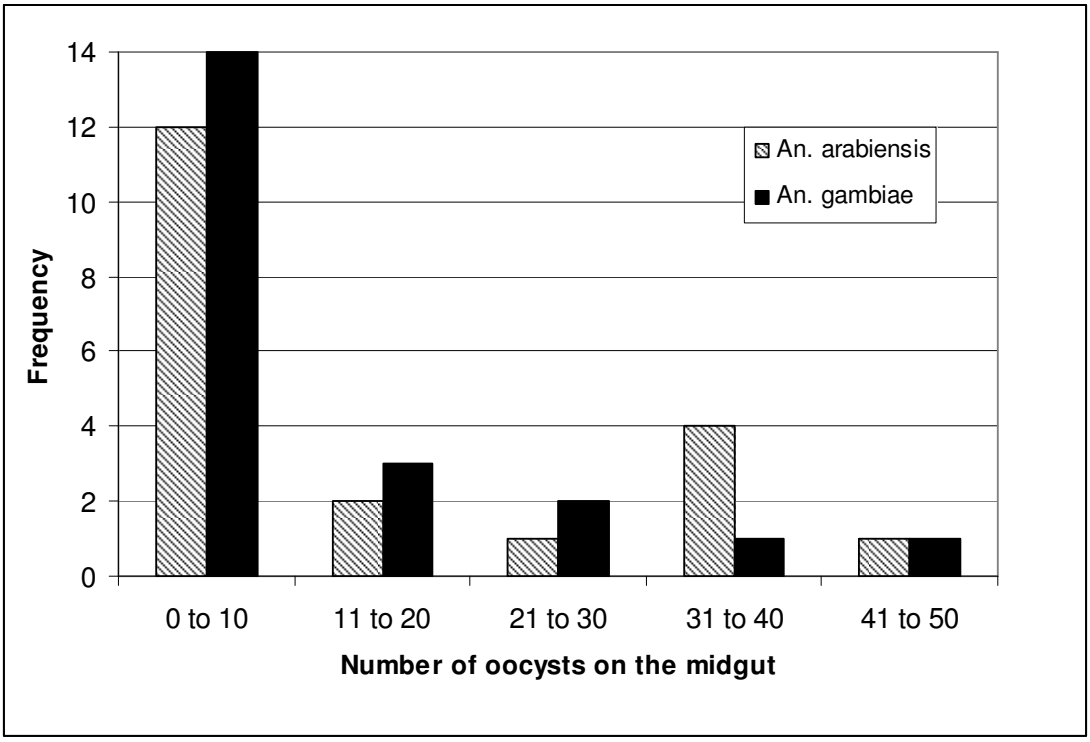
Figure 18. Prevalence of infection (% of mosquitoes infected) with 3D7 *P. falciparum* gametocytes in *An. arabiensis* and *An. gambiae s.s.* mosquitoes. Binomial 95% confidence intervals for the prevalences were calculated using Wilson score interval (Wilson, 1927)



3.2.2.2The effect of mosquito species on intensity of infection

A preliminary investigation was performed to analyse any differences in intensity of *P. falciparum* infection between the two mosquito species. Oocyst infections were found in both species in experiments 2 and 4 (Table 16) and the number of oocysts seen in these mosquitoes are summarised with an example of experiment 2 in Figure 19. As is usual for oocyst distributions there is strong non-normality, and data fit more closely to negative binomial distributions (Medley et al., 1993).

Figure 19. Histogram comparing the intensity of infection of 3D7 *P. falciparum* in *An. arabiensis* (striped) and *An. gambiae s.s.* (black) mosquitoes using only data from experiment two.



The intensity of infection in each experiment in the two mosquito species was compared by fitting the oocyst numbers to negative binomial distributions and comparing the mean and k values of the fitted negative binomials using a chi-squared test, in the GENMOD procedure of SAS (Bell and Ranford-Cartwright, 2004). Only data from experiments 2 and 4 could be analysed due to the lack of any infected *An. gambiae s.s.* mosquitoes in experiments one and three. The criteria for goodness of fit were all above 0.5 for both analyses, and the p value for the Chi^2 was non-significant for both experiment 2 and 4 (p=0.5132, p=0.318 respectively). These results suggest that there is no evidence for a difference in intensity of infection of 3D7 *P. falciparum* in the two mosquito species, although further experiments are needed to confirm this.

3.2.2.3 *Mosquito size differences in* An. gambiae s.s. *and* An. arabiensis

Mosquito wing lengths were analysed to assess whether differences in infection could be explained by differences in the size of mosquitoes (Table 17).

The overall mean wing length was slightly higher in the *An. arabiensis* group (2.925 mm) compared to the *An. gambiae s.s.* (2.868 mm), but this was non-significant (p=0.1752) when compared with a t test (Shapiro Wilks confirmed normality in both data sets p=0.203 (*An. arabiensis*), p=0.051 (*An. gambiae*)).

Table 17. Mean wing lengths in *An. arabiensis* and *An. gambiae* mosquitoes fed *P. falciparum* clone 3D7 gametocytes.

	An. arabiensis			*An. gambiae s.s.*		
Expt	**Mean wing size (mm) (range)**	**Sem**	**n**	**Mean wing size (mm) (range)**	**sem**	**N**
2	2.86 (2.73-3.19)	0.108	27	2.95 (2.72-3.06)	0.131	10
3	2.9 (2.67-3.18)	0.142	23	2.665 (2.63-3.05)	0.16	6
4	2.935 (2.6-3.22)	0.141	38	3 (2.6-3.13)	0.163	10
Totals			88			26

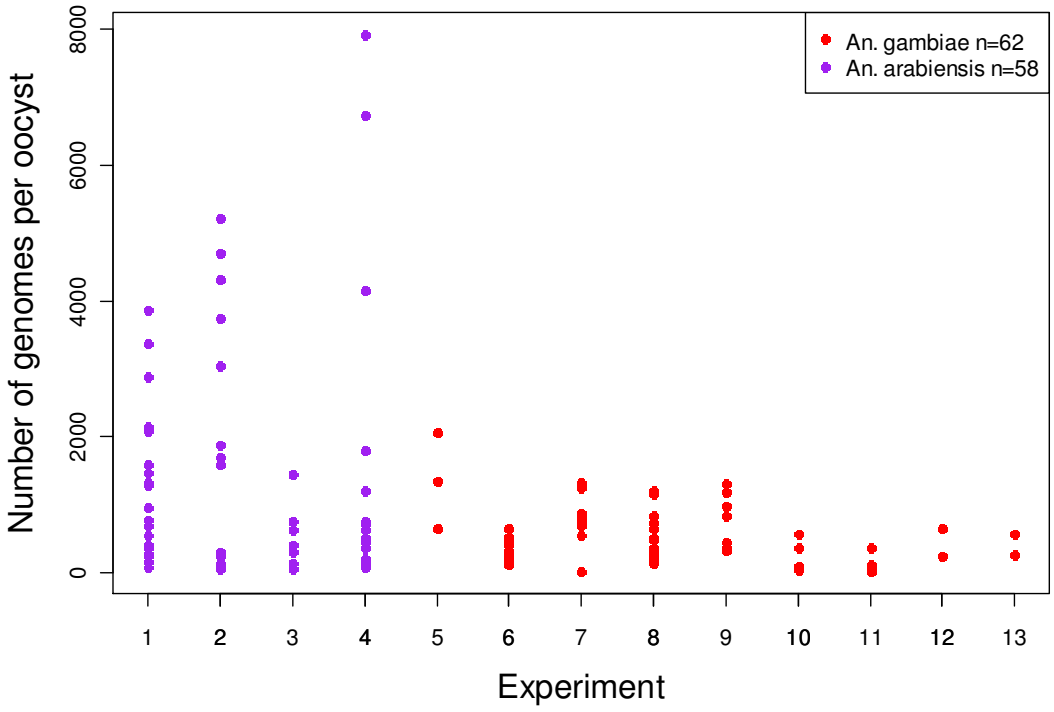
A mixed effects linear model using wing length as the dependent variable and species as the explanatory variable whilst also including experiment as a random variable, showed no association between wing length and species (p=0.1134).

These results suggest there is no significant difference in mosquito wing lengths observed between the two species of mosquito used in these experiments, and therefore the difference in prevalence is not merely a result of different mosquito size.

3.2.2.4 The effect of species on the number of genomes per oocyst

The genome numbers in a total of 58 single oocyst samples, dissected from the midguts of *An. arabiensis* mosquitoes that had been fed 3D7 *P. falciparum* gametocytes, were compared with 62 single oocyst samples from *An. gambiae s.s.* mosquitoes fed 3D7 *P. falciparum* gametocytes. The samples were collected from thirteen different experiments with *An. gambiae s.s.* and 4 different experiments with *An. arabiensis*. The raw data are shown in Figure 20, and summarised in Table 18.

Figure 20. The number of genomes per oocyst and experiment when samples were collected, displayed coloured by species of mosquito used. Data are presented only for 3D7 oocysts from single clone infections in both species.



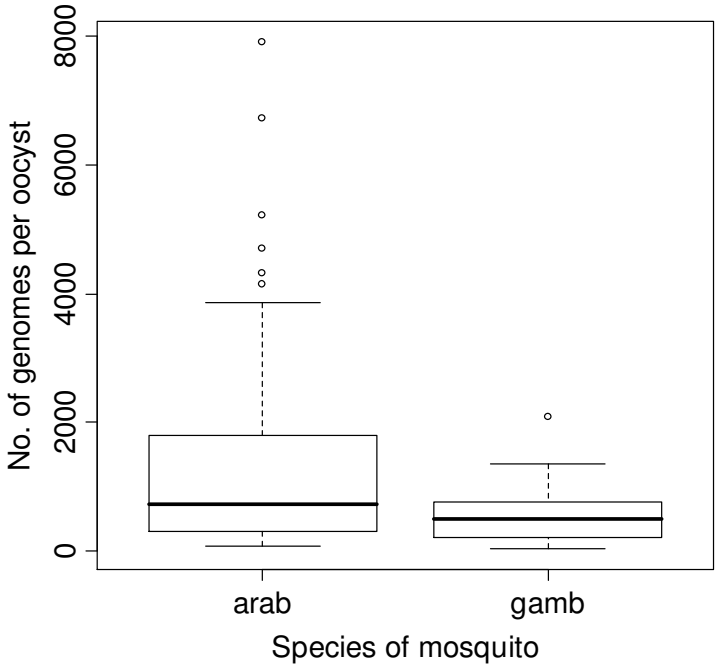
The variation in the number of genomes per oocyst is very large, especially for *An. arabiensis* (range 76-7917 genomes per oocyst for *An. arabiensis*, 27-2080 for *An. gambiae*). To assess whether the variance seen between the species is larger than that seen within different experiments using the same species, a non-parametric analysis was performed (due to some data not fitting into normal distribution). A Wilcoxon rank sum test produced a highly significant p value (0.0035, W=2355) for the difference in genome numbers per oocyst between species suggesting that oocysts developing in *An. arabiensis* mosquitoes are more likely to have a higher replication rate than those in *An. gambiae s.s.* mosquitoes.

Table 18. Median number of genomes per oocyst in *An. arabiensis* and *An. gambiae* mosquitoes fed *P. falciparum* clone 3D7 gametocytes. Data from 13 different experiments are displayed (Expt). Normality of genomes per oocyst distribution was tested by Shapiro-Wilks test.

Expt	*An. gambiae s.s.*			*An. arabiensis*		
	No. oocysts analysed	**Shapiro Wilks p value**	**Median number of genomes per oocyst (range)**	**No. oocysts analysed**	**Shapiro Wilks p value**	**Median number of genomes per oocyst (range)**
1				19	**0.028**	975 (86-3858)
2				13	0.060	1708 (78-5213)
3				9	0.076	418 (76-1457)
4				17	**<0.001**	524 (93-7917)
5	3	0.9698	1348 (655-2080)			
6	12	0.622	325 (123-666)			
7	10	0.3142	843 (27-1340)			
8	13	0.4209	529 (144-1211)			
9	7	0.3261	853 (335-1318)			
10	5	0.1361	108 (58-581)			
11	8	**0.0003**	58 (36-384)			
12	2	N/A				
13	2	N/A				
Total	**62**			**58**		

The boxplot in Figure 21 shows the data shown in Figure 20 pooled for each mosquito species.

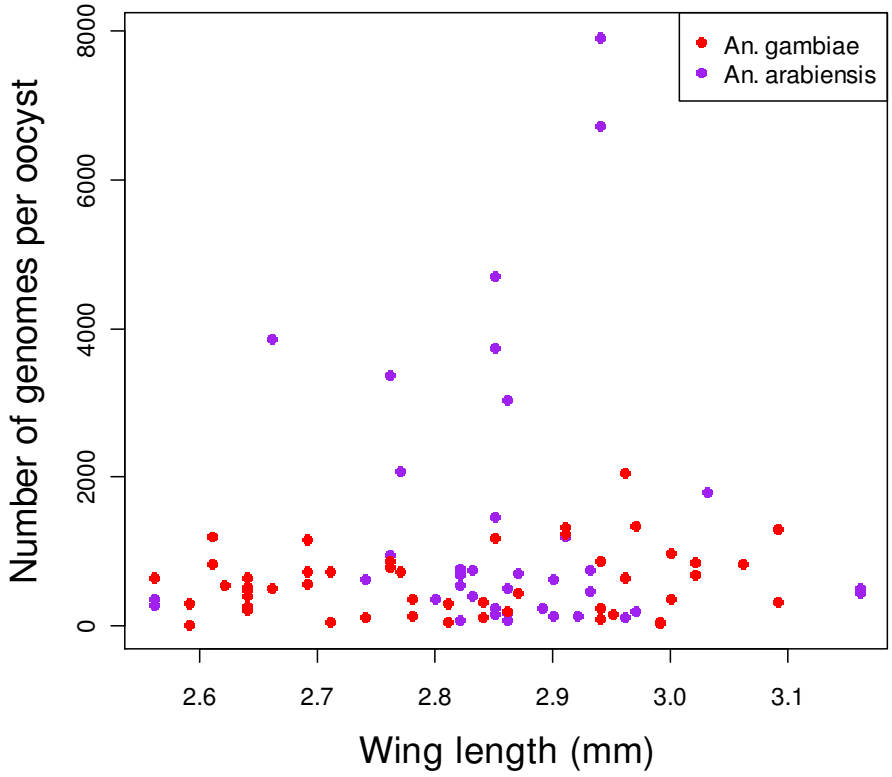
Figure 21. Genomes per oocyst from 3D7 infected *An. arabiensis* and *An. gambiae s.s.* mosquitoes. Heavy bars indicate medians, boxes show the lower and upper quartile and whiskers show the maximum and minimum. Circles are those data points considered outliers (outside 1.5 x interquartile range).



The observation of a difference in genomes per oocyst was not clearly explained by a difference in mosquito size, as there was no clear association observed between genomes per oocyst and wing length (Figure 22 and Figure 23) in this data set.

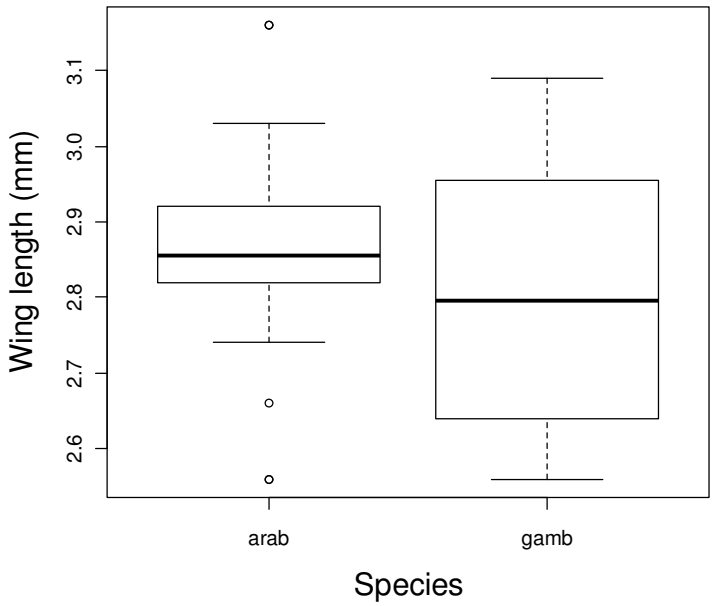
A Mann-Whitney U test (Shapiro-Wilks confirmed significant difference from normal distributions) between the two species' wing lengths found a no significant difference (p=0.176). A boxplot of this data is shown in Figure 23. Further analysis by mixed linear model (using genomes per oocyst as the dependent variable and wing length as the explanatory variable, whilst also including experiment and mosquito as random effect variables) confirmed this observation, with no statistical association between genomes per oocyst and wing length (p=0.365).

Figure 22. The number of genomes per oocyst and mosquito wing length. Data is presented only for 3D7 oocysts collected from both *An. gambiae* (red) and *An. arabiensis* (purple) mosquitoes.



This analysis of wing length confirms that any difference observed in prevalence and genomes/oocyst between the two species cannot purely be explained by a difference in wing length (as a marker of different bloodmeal sizes between the two species).

Figure 23. Wing lengths of *An. arabiensis* and *An. gambiae s.s.* mosquitoes. Heavy bars indicate medians, boxes show the lower and upper quartile and whiskers show the maximum and minimum. Circles are those data points considered outliers (outside 1.5 x interquartile range).



3.3 Discussion

3.3.1 Mosquito size

3.3.1.1 Infections with gametocytes of P. falciparum *3D7 clone*

These data produce a mixed picture on the association between the wing length of the *An. gambiae s.s.* mosquitoes and infection with *P. falciparum* 3D7 parasites. Of the eight experiments compared in section 3.2.1.1 only one (expt 4) showed a significant difference between the mean wing lengths of infected and uninfected mosquitoes, with infected mosquitoes appearing to be smaller than uninfected mosquitoes.

Overall the infected mosquitoes in experiment 4 were significantly smaller than infected mosquitoes in all other experiments apart from seven and eight. There was a large difference between the means of the infected and uninfected groups in experiment 4, and a small standard deviation in the infected mosquitoes group. This allowed a good power for the comparison analysis, even though the samples sizes were small. The other 7 experiments did not have sufficient power to reveal a statistically significant difference between infected and uninfected mosquitoes.

The data on wing length and infection probability presented here indicates an interesting area for further study, rather than providing clear conclusions about the importance of mosquito size for infection. Different experiments had different infection prevalence rates. Infection prevalence was not directly correlated with gametocyte density as shown for experiments five, six and seven where the gametocyte density in the feed was 1.2% in every case, but the overall prevalence of infection differed dramatically from 17 to 81% (gametocyte density is considered further in Chapter 4). Further experiments would therefore need to concentrate on a single experimental feed with a larger number of mosquitoes, split into size classes before the infectious feed. This would allow direct study of the hypothesis that smaller mosquitoes (possibly <2.5mm in wing length) are more likely to get infected than larger ones.

The majority of the results found here are consistent with previous studies from field-caught mosquitoes, where no correlation was found between wing length and infection with *Plasmodium* oocysts (Hogg and Hurd, 1997; Lyimo and Koella, 1992).

3.3.1.2 Infections with gametocytes of P. falciparum *HB3 clone*

Of the 7 experiments compared in section 3.2.1.2 only one showed a significant difference between the mean wing lengths of infected and uninfected mosquitoes. However, when the data from all experiments were pooled in mixed linear model with wing length as an explanatory variable, and experiment as a random effects variable, a significant association was observed between wing length and the probability of getting infected. Smaller mosquitoes had a small but significantly higher chance of infection than larger mosquitoes.

This may be explained by chance alone, as the sample sizes were very small, resulting in low power, due to the low prevalence of infection when using HB3 in *An. gambiae s.s.* mosquitoes. However, it may also indicate a true association. Further studies are required, with larger sample sizes of smaller mosquitoes.

Bigger mosquitoes may mount a better immune response to the parasite invasion (Boete et al., 2002; Suwanchaichinda and Paskewitz, 1998), which may explain why the infected mosquitoes were generally smaller than the uninfected ones. Other studies have shown small mosquitoes from laboratory-reared colonies may be more susceptible than larger mosquitoes to certain viruses (Grimstad and Haramis, 1984; Patrican and Defoliart, 1985). However, small mosquitoes may not survive long enough to serve as vectors in the field (Kitthawee et al., 1990; Nasci, 1986a; Nasci, 1986b).

3.3.1.3 Intensity of infection and correlation to mosquito size

The data presented here show no significant association between wing length and intensity of infection using 3D7 *P. falciparum* infections in *An. gambiae s.s.* mosquitoes. A mixed effects linear model showed no significant effect of wing length on the number of oocysts on the midgut. This is in contrast to the findings of Lyimo and Koella (Lyimo and Koella, 1992) that higher intensity infections were found in larger mosquitoes, but supported by the findings of Ichimori *et al,* where they used a different parasite/vector combination (Ichimori, 1989). Wild-caught mosquitoes were generally found with a low prevalence and low intensity of infection e.g. 17.5% prevalence with most infected mosquitoes having only one or two oocysts (Hogg and Hurd, 1997; Lyimo and Koella, 1992).

3.3.1.4 Number of genomes per oocyst and correlation to mosquito size

The final dependent variable that was considered, in relation to mosquito size, was the number of genomes per oocyst. Single oocyst samples collected from different experimental feeds were analysed using quantitative PCR and the results assessed for the association with the wing size of the mosquito from which they came. A mixed effects linear model found no correlation between wing length and the number of genomes per oocyst using 3D7 *P. falciparum* infections in *An. gambiae s.s.* mosquitoes. The analysis was not performed for HB3 as very few mosquitoes had more than one oocyst on their midgut.

3.3.2 Mosquito species

A set of pilot experiments considered whether the development of the *P. falciparum* parasite differed in different mosquito species. The two species used (*An. gambiae s.s.* and *An. arabiensis*) are sibling species within the *An. gambiae* complex, and are both important human malaria vectors in Africa. The *An. arabiensis* mosquito colony had only just been established at Glasgow when these experiments were conducted, so numbers were limited. These experiments should therefore be viewed as a pilot study which act as an important proof of principle that the infections in *An. arabiensis* can be carried out in the laboratory environment, as most of the published studies with this species have been with field-caught mosquitoes (Govere *et al*., 2003).

3.3.2.1 Prevalence of infection in different mosquito species

Of the three experiments comparing the prevalence of infection between the two species, two were non-significant. However, the sample sizes especially in the *An. gambiae* group were small, due to a shortage of mosquitoes being available at the time of the experiments, and therefore the power to test for associations was limited. The difference in prevalence of infection observed in experiment 4 could not be explained by a difference in wing lengths between the two species as a t-test showed no significant difference in wing lengths between the two groups of mosquitoes used. The results suggest that *An. arabiensis* is at least as susceptible, and possibly more susceptible to *P. falciparum* infection compared to *An. gambiae s.s.*

3.3.2.2 Intensity of infection in different mosquito species

Limited data meant that only two experiments could be used to analyse the difference in intensity of infection with 3D7 *P. falciparum* infections in *An. gambiae* and *An. arabiensis* mosquitoes. No significant differences were observed in the intensity of infection between the two mosquito species used, but larger studies would be needed to confirm this finding.

3.3.2.3 Number of genomes per oocyst in different mosquito species

The quantitative PCR data shows that there is a highly significant difference in genomes per oocyst between the two mosquito species, even with a large variance in genomes per oocyst in *An. arabiensis*. This finding was not explained by mosquito size differences between the two species. Oocysts from *An. arabiensis* mosquitoes had significantly higher numbers of genomes than those taken from *An. gambiae* mosquitoes.

Explanations for this finding could include higher levels of nutrition available in the midgut of the *An. arabiensis,* due to them taking a larger bloodmeal (Vaughan et al., 1994b). This would mean that although the number of oocysts per midgut might be similar between the two species the actual availability of nutrition for development of sporozoites within a single oocyst may be higher in *An. arabiensis* compared to *An. gambiae.* Some studies have suggested that the parasite does affect energy allocation in its host (Mack *et al*., 1979a; Rivero and Ferguson, 2003), and this could be variable between the two species.

Another factor that should be considered is the amount of time the *An. arabiensis* mosquitoes have been kept under laboratory conditions. The colony was only established a few months before these experiments took place, and the source of the mosquito eggs were field-sites in Tanzania. The lab-reared *An. gambiae* mosquitoes, had been in the laboratory for a much longer period. It may be possible that the *An. gambiae* mosquitoes are less efficient in providing nutrients for the parasite development which manifests as lower replication in the oocyst.

To consider the effect of species on the development of sporozoites in the oocyst, further experiments analysing single oocysts from a range of mosquito species should be carried out. Single oocysts from the same experimental feed could be collected and analysed, where the same gametocytes are fed to *An. gambiae* and *An. arabiensis* mosquitoes, as it

was observed the genomes per oocyst do vary from experiment to experiment. Samples of oocysts from field-caught *Anopheline* mosquitoes could also be an interesting area for further research.

4 Chapter 4. Parasite environmental factors influencing mosquito infection

4.1 Introduction

Environmental factors that the parasite has experienced both prior to uptake by the mosquito (either *in vitro* or *in vivo*) and then in the mosquito midgut, are likely to have an effect on the parasite infectivity and development. Three such 'parasite environmental factors' have been studied in this chapter; gametocytaemia, length of time the parasite has spent in culture, and the intensity of infection on the mosquito midgut.

Previous research has shown a positive correlation between gametocytaemia in infected patients and the infection prevalence and intensity in mosquitoes (Drakeley et al., 1999; Jeffery and Eyles, 1955). However, there was no association between the density of cultured *P. falciparum* gametocytes in the blood feed and infection in *An. gambiae* mosquitoes, no measurable parameter of *P. falciparum* gametocyte population structure reliably and consistently predicted a gametocyte culture's infectiousness to *An. gambiae* (Noden et al., 1994).

The length of time spent in asexual continuous culture is known to have a negative effect on gametocyte numbers (Ponnudurai *et al*., 1982). Gametocyte numbers usually decrease with increasing time in culture after 4-6 months (Ponnudurai *et al*., 1982). In the shorter term (2-3 months) gametocyte numbers are reasonably stable, but mosquito infectivity falls. Evidence suggests that parasites in the culture build up mutations or deletions for example in chromosome 9 (Bourke et al., 1996; Day et al., 1993) which commonly results in lowered capacity for both cytoadherence and gametocytogenesis.

Mosquitoes provide essential nutrients to the oocyst through the haemolymph (as discussed in Chapter 1) suggesting that if the midgut is heavily infected a competition for nutrients from the haemolymph may limit the parasite replication stage in the developing oocyst. Only a few studies have looked at the idea of competition for nutrients in the midgut (measured by oocyst burdens) correlating with sporozoite numbers. Vaughan (Vaughan et al., 1992) found that oocyst burdens in experimentally infected *An. gambiae* midguts were positively correlated with salivary gland sporozoite counts. This suggests there is no saturation effect from too many oocysts or too many sporozoites, and that nutrients for

further parasite population growth within mosquitoes are essentially unlimited, or at least they are up to the highest oocyst burden reported in this paper (which was 56) (Vaughan et al., 1992). Adult mosquitoes obtain non-sugar nutrition from bloodmeals, and additional bloodmeals during the extrinsic development period increase the number of sporozoites, but not the number of oocysts, possibly by synchronising oocyst development by a nutritional stimulus (Bell, unpublished and (Ponnudurai *et al*., 1989b)). This does suggest that parasite replication is to some extent limited by nutrition, at least in laboratory infected mosquitoes. As mentioned in Chapter 1, previous studies (including unpublished work) have shown no association between sporozoites per oocyst and oocyst density on the midgut (Vaughan et al., 1992).

This chapter considers three main parasite environmental factors that might influence the parasite infectivity and multiplication in the mosquito; gametocytaemia (gametocyte density of the bloodmeal), length of time spent in continuous culture by the parasite, and intensity of infection on the midgut.

4.2 Results

4.2.1 Gametocytaemia

The hypothesis of this study was that gametocytaemia would be positively correlated to infection prevalence and intensity of infection in mosquitoes; as the number of gametocytes in an infectious feed increases so too would the number of mosquitoes that get infected (as each mosquito has a higher chance of ingesting more gametocytes) and so too would the number of oocysts forming on the mosquito midgut.

4.2.1.1 The effect of bloodmeal gametocytaemia on prevalence of infection

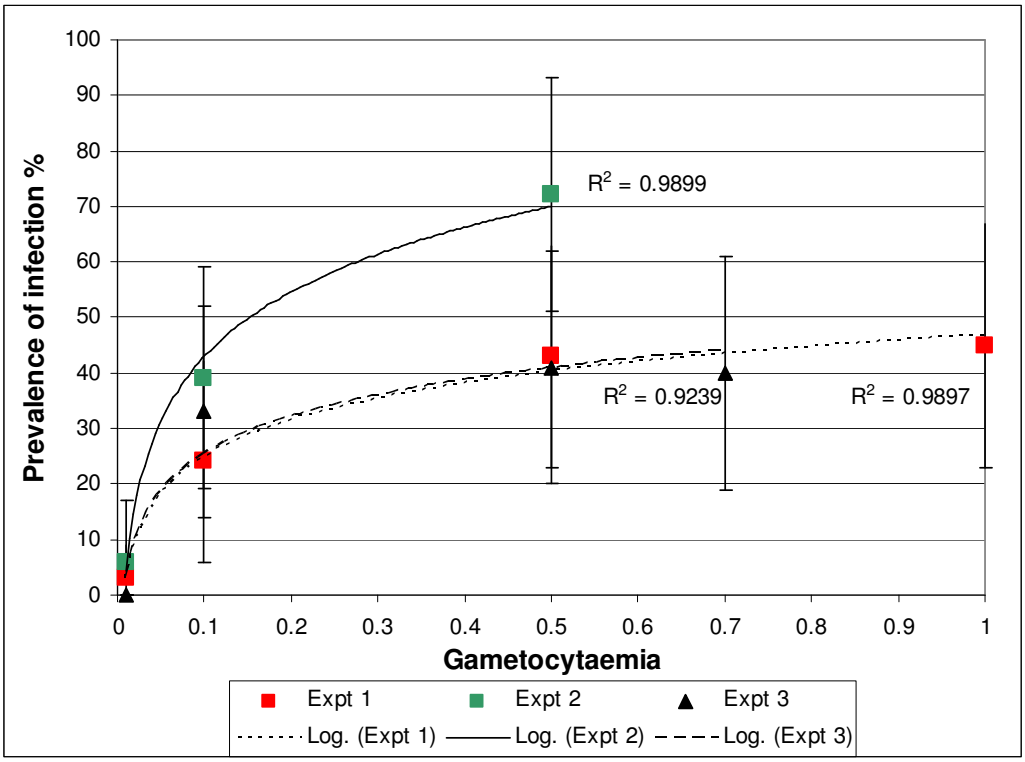
A small pilot study of three experiments was initially run specifically to determine the best gametocytaemia to use for subsequent feeds. Data from these three different infectious feeds with 3D7 *P. falciparum* are discussed first. Gametocyte cultures were grown to maturity and then diluted on the feed day to create a range of gametocytaemias to feed to the mosquitoes. All three experiments used the same 3D7 asexual culture for gametocyte production set up over a period of one month. Data of the prevalence of infection and the number of mosquitoes dissected is shown in Table 19.

Table 19. Gametocytaemia (Gams) and prevalence (Prev) of 3D7 *P. falciparum* infection in *An. gambiae s.s.* mosquitoes. Data are shown for 3 experimental feeds (Expt), and total number of mosquitoes dissected (n). Infection was defined as the presence of at least one oocyst on the midgut, 10 days post-infectious feed. Binomial 95% confidence intervals for the prevalences were calculated using Wilson score interval (Wilson, 1927).

Gams %	Expt 1		Expt 2		Expt 3	
	Prev % (CI)	n	Prev % (CI)	n	Prev % (CI)	N
0.01	3 (0-9)	30	6 (0-17)	17	0	4
0.1	24 (6-42)	21	39 (19-59)	23	33 (14-52)	24
0.5	43 (23-63)	23	72 (51-93)	18	41 (20-62)	22
0.7					40 (19-61)	20
1	45 (23-67)	20				

The gametocytaemia in each of the infectious bloodmeals used in the three experimental feeds varied from 0.01% to 1%, but not all gametocytaemia categories were fed for every experiment due to a lack of enough culture material in some experiments (Table 19).

Figure 24. Percentage gametocytaemia in the infectious bloodmeal and prevalence of infection of parasite clone 3D7. Binomial 95% confidence intervals for the prevalences were calculated using Wilson score interval (Wilson, 1927). Logarithmic regression lines are shown, with their r^2 values



The results suggest a logarithmic relationship between the prevalence of infection and the gametocytaemia of the bloodmeal using *P. falciparum* clone 3D7 in *An. gambiae s.s.* mosquitoes (Figure 25).

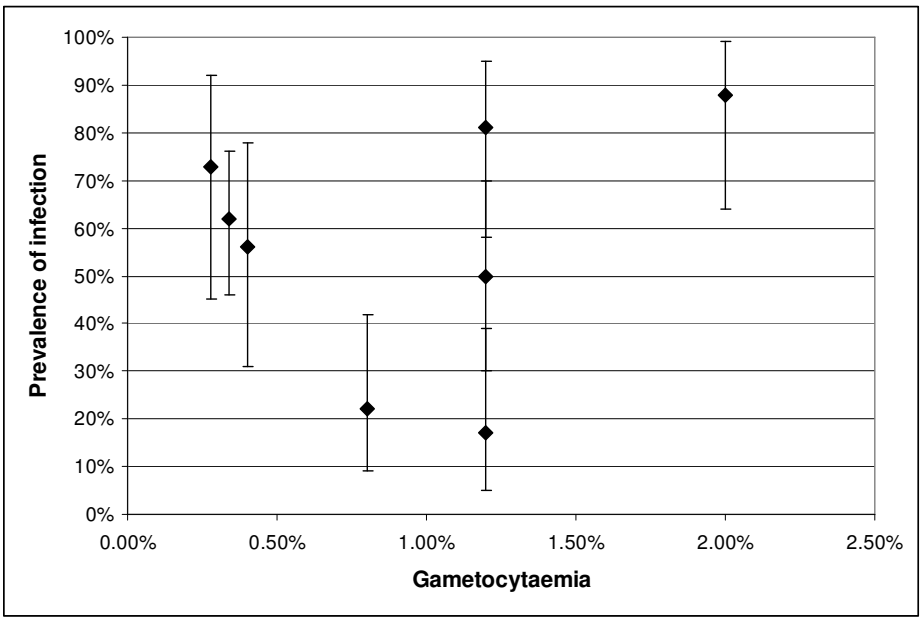
A further eight successful experimental feeds (not designed specifically to study the effect of gametocytaemia) with 3D7 *P. falciparum* gametocytes fed to *An. gambiae s.s.* mosquitoes were carried out, where each experimental feed was from a separate gametocyte culture. The variation in gametocytaemia from experiment to experiment reflects a variation in the gametocytes produced by the cultures, following a standard preparation that involves dilutions of the culture several times. The gametocytaemia in the infectious bloodmeal for each experimental feed was compared to the prevalence of infection observed in each experiment (Table 20).

Table 20. Gametocytaemia (Gams) and prevalence (Prev) of 3D7 *P. falciparum* infection in *An. gambiae s.s.* mosquitoes. Data are shown for 8 experimental feeds (Expt), and total number of mosquitoes dissected (n). Infection was defined as the presence of at least one oocyst on the midgut, 10 days post-infectious feed.

Expt	Gams	Prev (95% CI)	n
1	0.28%	73% (45-92)	15
2	0.34%	62% (46-76)	42
3	0.40%	56% (31-78)	18
4	0.80%	22% (9-42)	27
5	1.20%	50% (30-70)	26
6	1.20%	81% (58-95)	21
7	1.20%	17% (5-39)	23
8	2%	88% (64-99)	17
Total			189

The gametocytaemia in each of the infectious bloodmeals used in the eight experimental feeds varied from 0.28% to 2% (Table 20) but there was no obvious relationship with prevalence of infection (Figure 25). There were insufficient data points to examine the relationship further through regression analysis.

Figure 25. Percentage gametocytaemia in the infectious bloodmeal and prevalence of infection of parasite clone 3D7. Binomial 95% confidence intervals for the prevalences were calculated using Wilson score interval (Wilson, 1927). Each point represents a different batch of gametocytes.

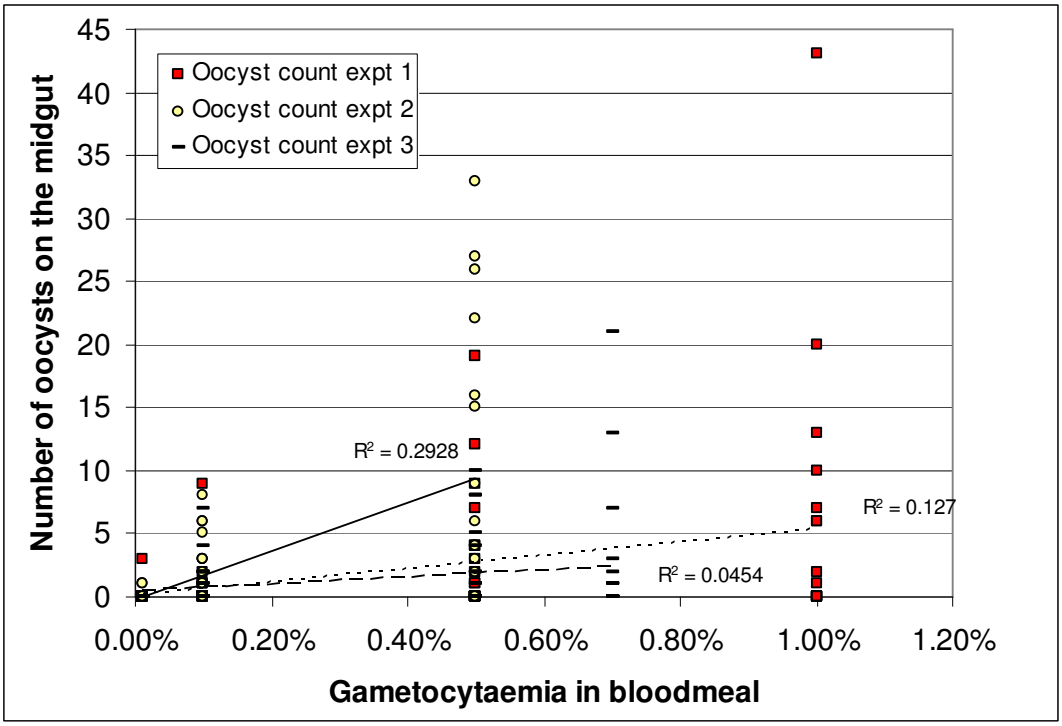


The overall conclusion from these experiments is that there is a low threshold of about 0.1% above which infection is common, however across different experiments there is a large variability in infection prevalence which is not clearly predicted by the gametocytaemia in the bloodmeal.

4.2.1.2The effect of bloodmeal gametocytaemia on infection intensity

Data from the three initial pilot experiments discussed at the beginning of section 4.2.1 were also analysed for the association between gametocytaemia and infection intensity. Data from a total of 222 mosquitoes (dissected from three different experiments) that had been fed 3D7 gametocytes is presented first. Observed infection intensity ranged from 0 to 43 oocysts on the midgut, 10 days after the infectious blood feed. Data from the three experiments are displayed in Figure 26, along with linear regression lines and their r^2 values.

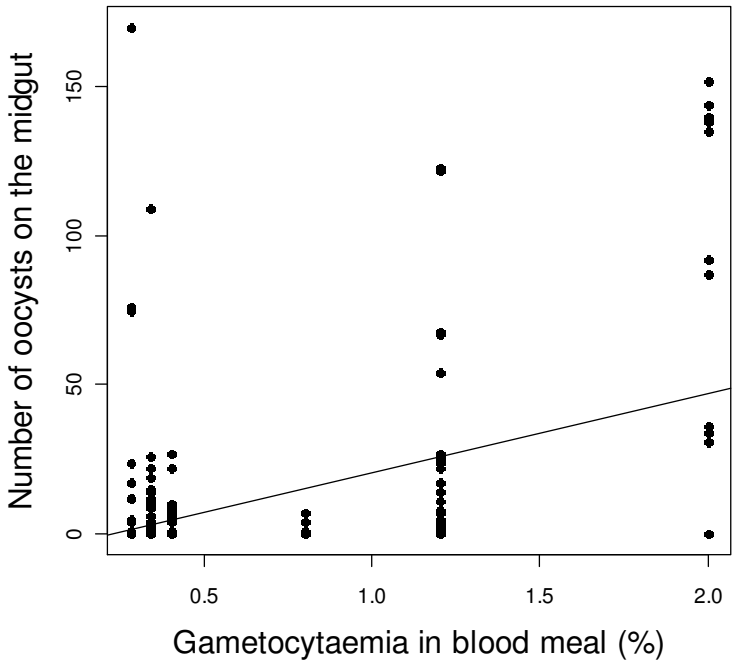
Figure 26. The effect of bloodmeal gametocytaemia on infection intensity using data from *An. gambiae s.s.* mosquitoes fed *P. falciparum* clone 3D7. The linear regression lines are shown, with their r^2 values. Both infected and uninfected mosquitoes are included.



Following a log transformation of the oocyst burden data, linear regression was used to analyse the data. There was a positive correlation for experiments one (p=0.023) and two (p=0.004), but no significant correlation for experiment three (p=0.257), between the gametocytaemia fed to mosquitoes and the intensity of infection (log oocyst burden) on the mosquito midgut ten days later. However, the linear regression models do not show a good fit to the data, as shown by the low r^2 values, due to such large variation in the infection intensity. This suggests that factors other than the gametocytaemia must be influencing the intensity of infection.

Further analysis of the gametocytaemia and its affects on infection intensity was carried out on data from eight different experiments, not designed specifically to test this hypothesis (Figure 27). A total of 92 mosquitoes (dissected from eight different experiments) that had been fed 3D7 gametocytes, and were found to have at least one oocyst on their midgut ten days post infection, were used to investigate the effect of gametocytaemia on infection intensity.

Figure 27. The effect of bloodmeal gametocytaemia on infection intensity using data from infected *An. gambiae s.s.* mosquitoes fed *P. falciparum* clone 3D7. The line shown represents the mixed linear model (intercept=-6.13, slope=26.6). Each point represents an individual mosquito from one of eight different experimental feeds.



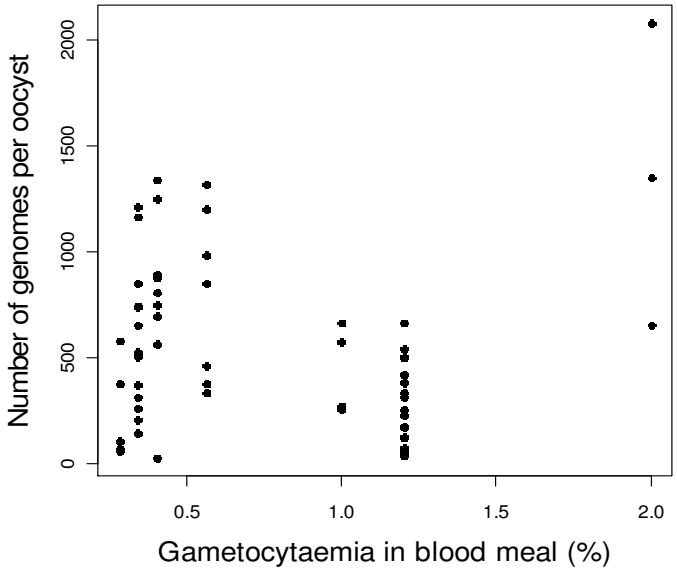
The relationship between gametocytaemia and infection intensity was investigated through a mixed linear model analysis. The model included infection intensity as the dependent variable, and gametocytaemia as the explanatory variable, whilst also including experiment as a random effect variable. The model produced a significant association between intensity of infection and gametocytaemia (p=0.0493) with a slight positive correlation (as shown by the line in Figure 27). This association is heavily influenced by the 2% gametocytaemia results and the gametocytaemia only explains a small amount of the variation found in oocyst numbers on the midgut.

4.2.1.3The effect of gametocytaemia on genomes per oocyst

The effect, if any, of gametocyte numbers in the infectious feed on the number of genomes per oocyst was investigated, although the hypothesis was that it was unlikely this would have a direct effect but would be correlated through any effect seen with infection intensity and competition for nutrients. Single oocysts collected from mosquitoes fed only 3D7 gametocytes from nine different experiments (see Appendix II for details) , genome numbers established from single oocysts showed no significant correlation with gametocytaemia (Figure 28). A mixed effects linear model using genomes per oocyst as the dependent variable and gametocytaemia as the explanatory variable, whilst also including experiment as a random effects variable gave a non-significant p value of 0.329

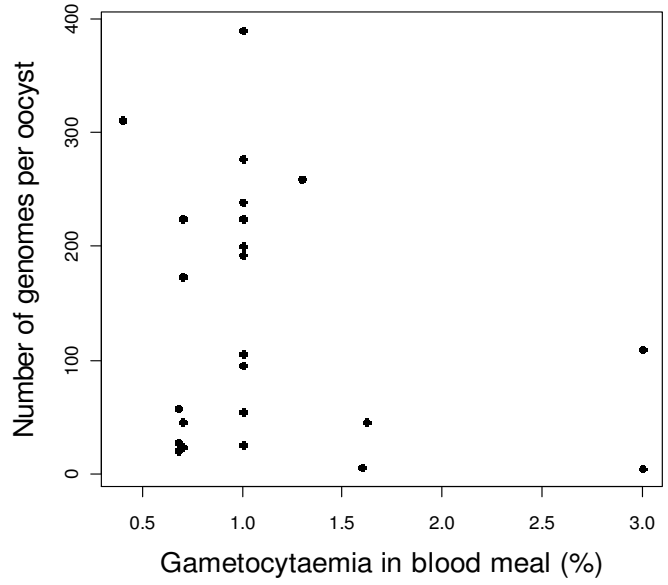
indicating no clear association between genomes per oocyst and gametocytaemia in the blood meal, when including experiment and mosquito as random variables.

Figure 28. Genomes per oocyst against the gametocytaemia in the infectious blood meal for 3D7 single clone infections in *An gambiae s.s.* mosquitoes. n=62



Similar results were found for genome numbers from single oocysts of parasite clone HB3 (Figure 29). A mixed effects linear model gave a non-significant p value of 0.204 for association between number of genomes per oocyst and gametocytaemia in the blood meal for HB3 single clone infections, when including experiment and mosquito as random effect variables.

Figure 29. Genomes per oocyst against the density of gametocytes in the HB3 single clone infectious blood meal with *An. gambiae s.s.* mosquitoes, n=23



In conclusion the data analysis showed very little of the variation in prevalence, infection intensity or genomes per oocyst was explained by the gametocytaemia in the blood meal, although a low threshold of about 0.1% is usually needed to ensure infections with *P. falciparum* 3D7 clone in *An. gambiae s.s.* mosquitoes.

4.2.2 Length of time in continuous culture (age)

The hypothesis of this study was that the length of time the parasite has spent in continuous culture would be negatively associated with infection prevalence, intensity of infection, and number of genomes per oocyst. As the parasite is kept for longer in continuous culture the probability of mutations occurring increases, and these mutations could have a negative effect on the ability of the parasite to infect mosquitoes.

4.2.2.1 The effect of age of culture on prevalence of infection

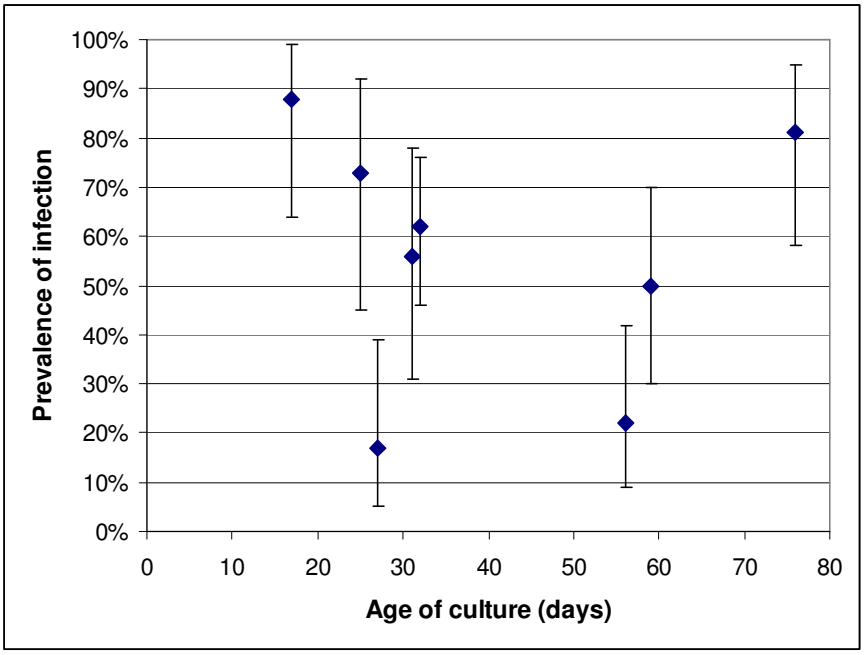
The length of time (in days) that the parasite line had been in continuous culture from thawing to feed was recorded for all eight experiments with 3D7 *P. falciparum* in *An. gambiae s.s.* mosquitoes (data shown in Table 21). The age of the culture ranged from 17 to 76 days, and prevalence of infection ranged from 17 to 88%.

Table 21. Age of the culture (age in days from thawing to feeding) and prevalence (Prev) of 3D7 *P. falciparum* infection in *An. gambiae s.s.* mosquitoes. Data are shown for 8 experimental feeds (Expt), gametocytaemia (Gams) and the total number of mosquitoes dissected (n). Infection was defined as the presence of at least one oocyst on the midgut, 10 days post-infectious feed

Expt	Age (days)	Gams	n	Prev
1	25	0.28%	15	73% (45-92)
2	32	0.34%	42	62% (46-76)
3	31	0.40%	18	56% (31-78)
4	56	0.80%	27	22% (9-42)
5	59	1.20%	26	50% (30-70)
6	76	1.20%	21	81% (58-95)
7	27	1.20%	23	17% (5-39)
8	17	2%	17	88% (64-99)
Totals			189	

No clear association between the age of the culture and the prevalence of infection was observed (Figure 30), and further analysis was not possible with the limited sample size.

Figure 30. Age of culture and prevalence of infection of 3D7. Binomial 95% confidence intervals for the prevalences were calculated using Wilson score interval (Wilson, 1927). Each point represents the prevalence of one infectious feed.

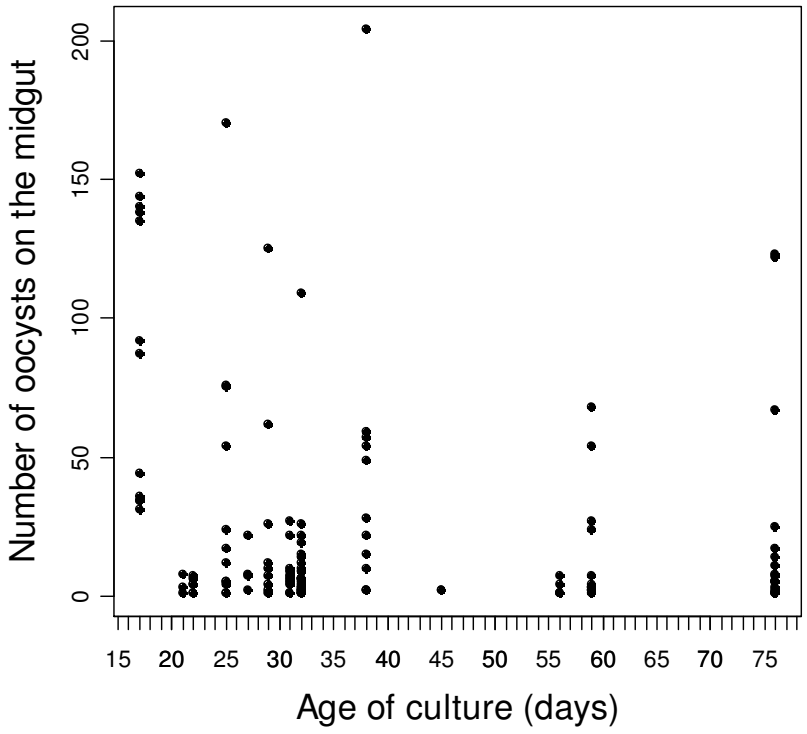


4.2.2.2The effect of age of culture on infection intensity

The relationship between age of culture and infection intensity was investigated through generalised linear modelling analysis. Scatterplots suggested no clear relationship between oocyst intensity and age of culture (Figure 31).

A mixed effects linear model analysis was performed, with oocyst intensity as the dependent variable and age of culture as the explanatory variable, including experiment as a random effects variable. The results of the mixed effects linear model confirm that there was no significant association between intensity of infection and age of culture (p=0.2204).

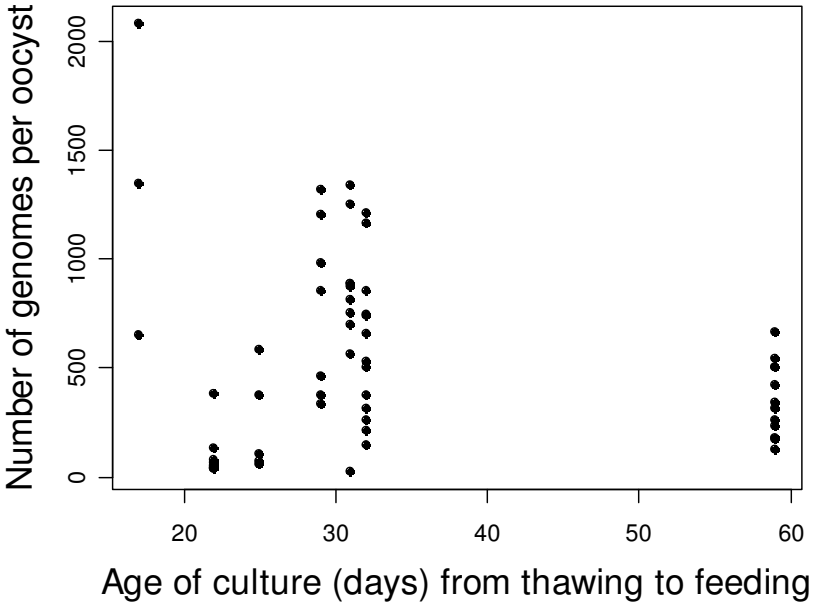
Figure 31. Infection intensity against age of culture. Data from infected *An. gambiae s.s.* mosquitoes fed *P. falciparum* 3D7 clone. Each point represents a single mosquito.



4.2.2.3The effect of age of culture on genomes per oocyst

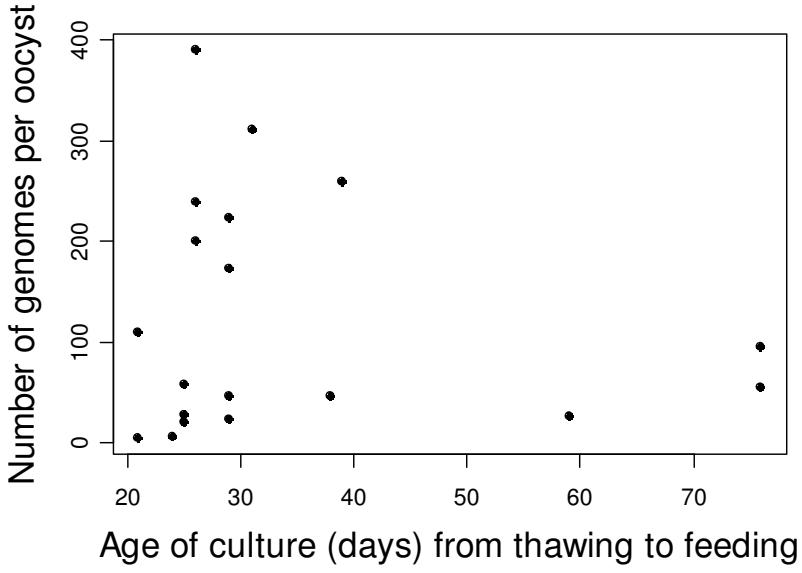
The effect of the age of the culture on the multiplication of parasites in the oocysts was investigated. There does not appear to be a significant effect of culture age below ~40 days in continuous culture. One experimental feed was performed with ~60 day old cultures, and the data suggest lower replication in these oocysts (Figure 32).

Figure 32. The effect of age of culture (days) on the number of genomes per oocyst for 3D7 single clones infections in *An. gambiae s.s.* mosquitoes, n=58



A mixed effect linear model analysis was performed to investigate the effect of culture age on genomes per oocyst using number of genomes per oocyst as the dependent variable and age of culture are the explanatory variable, whilst also including experiment and mosquito as random effect variables. There was no significant effect of culture age on subsequent parasite multiplication capacity, with a non-significant p value (p=0.3446). Similar results were obtained for oocysts of parasite clone HB3 (Figure 33), with a non-significant association (p= 0.579), although there were very few data points for this analysis.

Figure 33. The effect of age of culture fed (Days) on the number of genomes per oocyst for HB3 single clone infections in *An. gambiae s.s.* mosquitoes, n=23



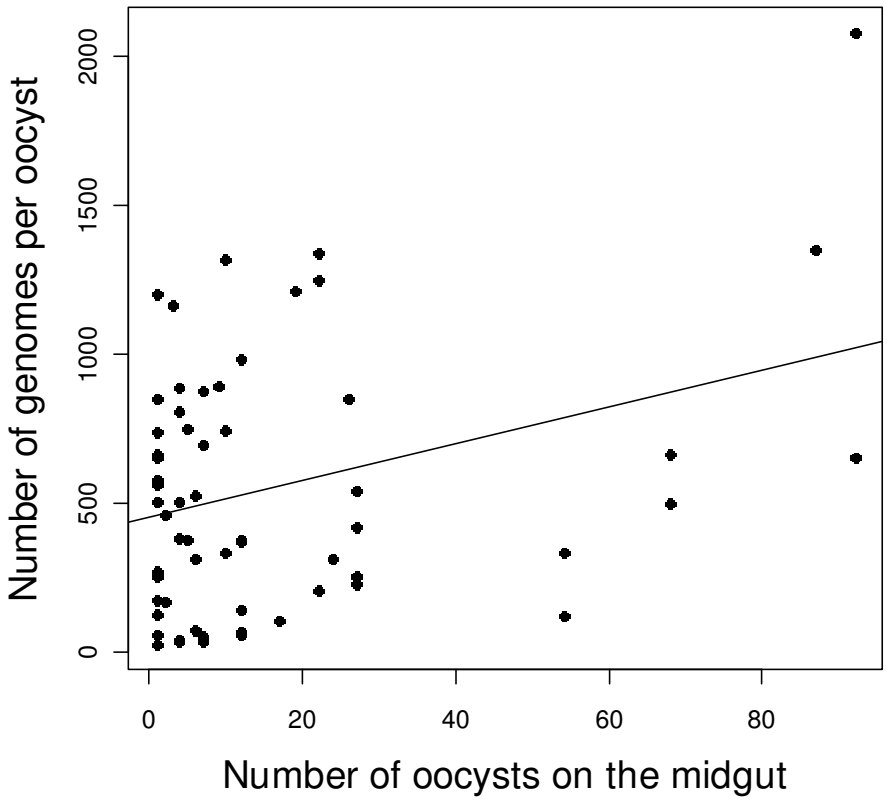
In conclusion the age of culture (up to 60 days) does not appear to influence parasite replication in the oocyst stage.

4.2.3 The effect of infection intensity on the number of genomes per oocyst in single clone infections

Increased competition for nutrients would be expected as the intensity of oocyst infection increases and this could manifest as reduced replication rates within each oocyst. To consider if this "crowding" on the midgut of the mosquito has an effect on the number of genomes per oocyst, single oocysts dissected from *An. gambiae s.s.* mosquitoes fed *P. falciparum* clone 3D7 were analysed. Data from a total of 62 single oocyst samples, collected from 9 different experiments where *An. gambiae s.s.* mosquitoes had been fed 3D7 gametocyte-infected blood, are discussed in this section. As the majority (22/23) of the HB3 samples had originated from a mosquito midgut with only a single oocyst present, analysis on the affect of crowding could not be performed on the HB3 data.

The infection intensity with 3D7 ranged from 1 to 92 oocysts per midgut and the number of genomes per oocyst ranged from 27 to 2080 (Figure 34). The data were analysed using a mixed effect linear model to assess whether the infection intensity predicted the variance in the observed number of genomes per oocyst. Using both experiment and mosquito as random effects variables, the number of oocysts on the midgut was significantly associated with the number of genomes per oocyst ($p=0.0212$, $t=2.366$, intercept=453). The association was positive (coefficient of 6.19) which means that as the number of oocysts on the midgut increased, so did the number of genomes per oocyst.

Figure 34. Number of genomes per oocyst against infection intensity, using 3D7 oocysts from single clone feeds in *An. gambiae s.s.* mosquitoes. Line shows the result of a mixed linear model (intercept 453, slope 6.185).



Overall, the data as a whole suggest that infection intensity explains some of the variance found in the number of genomes per oocyst, in a positive association.

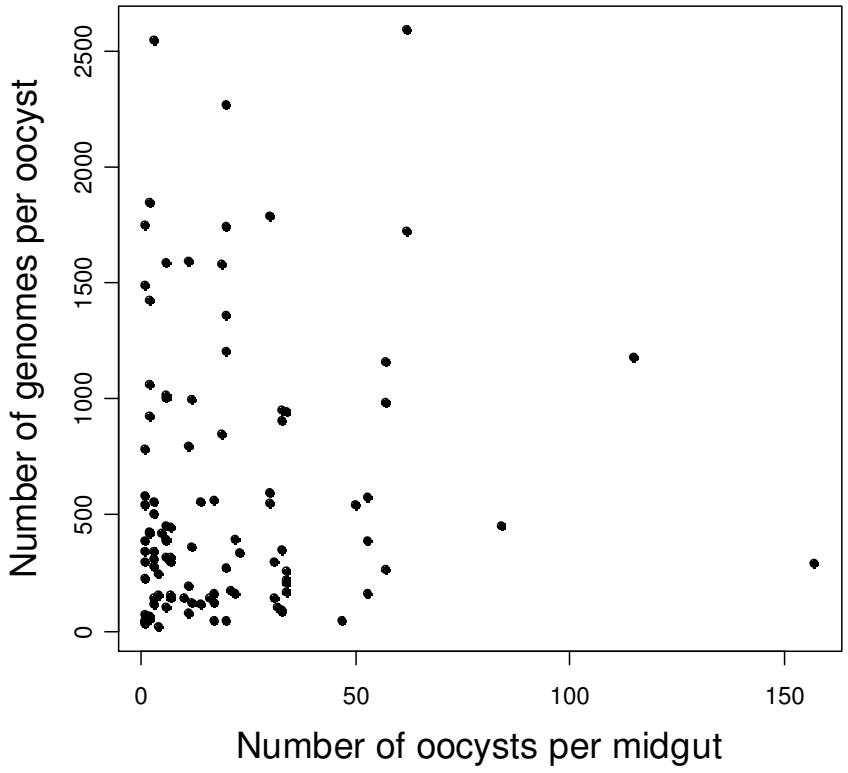
4.2.4 *The effect of infection intensity on the number of genomes per oocyst in mixed genotype infections*

The previous section considered the effect of crowding within a single clone infection on parasite multiplication within the oocyst. Competition between parasites of different genotypes may exert a different competitive pressure to that between parasites of identical genotype. Although this has not been investigated for parasite replication within oocysts, competition between genetically distinct parasites has been shown to occur in rodent models (Taylor, 1997). The hypothesis for this study was that oocysts from higher infection intensity midguts would replicate differently if other genotypes were present on the same midgut.

This was investigated by analysing oocysts from mixed clone feeds. A data set of 104 single oocyst samples collected from *An. gambiae s.s.* mosquitoes that had been fed a mixture of 3D7 and HB3 gametocytes (in a 1:1 ratio of total gametocytaemia counted), were analysed.

Looking at the overall dataset, there was no clear association between the genomes per oocyst (as determined by quantitative PCR) against infection intensity (number of oocysts per midgut) (Figure 35). The relationship between oocyst intensity and genomes per oocyst is very similar to that seen for 3D7 oocyst alone (Figure 34), although the weak positive correlation is less obvious.

Figure 35. Effect of infection intensity on number of genomes per oocyst in mosquitoes fed a mixture of 3D7 and HB3 gametocytes in *An. gambiae s.s.* mosquitoes. Each point represents a single oocyst.



Application of a mixed effects linear model to assess whether any of the observed variance in genomes per oocyst was predicted by the infection intensity (whilst including experiment and mosquito as random variables) produced a non-significant p value of 0.604, indicating that there was no significant effect of infection intensity on the genomes per oocyst in a mixed clone environment.

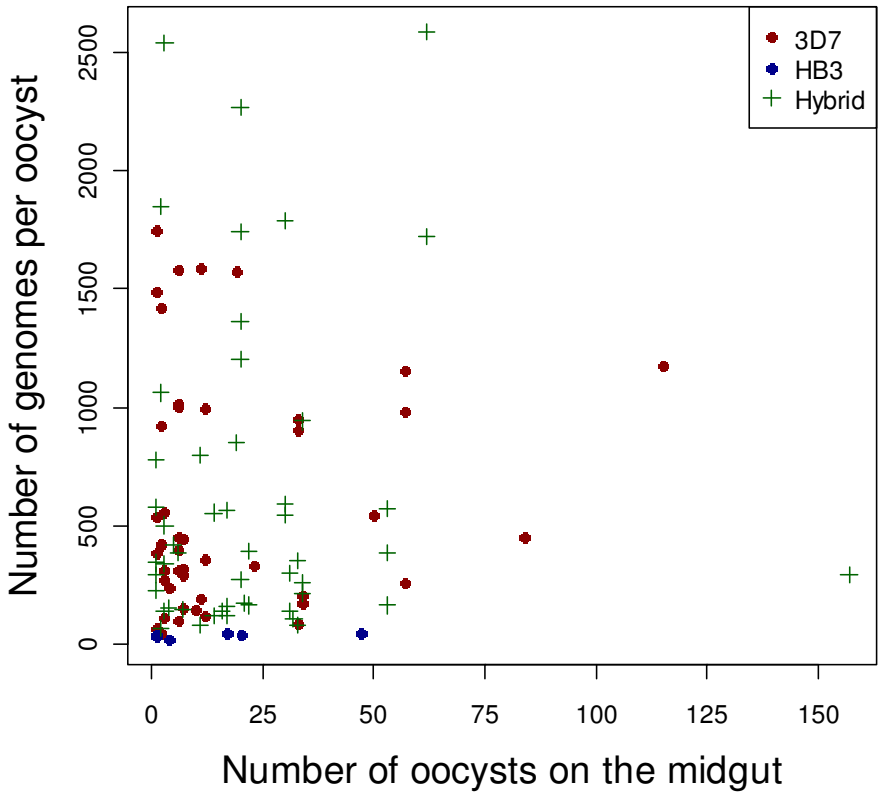
To further investigate the effect of infection intensity, the 104 oocysts were classified by their genotype (3D7, HB3, or hybrid) as described in section 2.5. The hybrid oocysts are those formed from cross-fertilisation events between 3D7 and HB3 gametes in the midgut, the other oocysts are parental-type formed from self-fertilisation events. The 104 oocysts shown in Figure 36 were categorised as 46 3D7 parental, 8 HB3 parental, and 50 hybrid oocysts (Table 22).

Table 22. Single oocyst data from mixed clone infections. Oocyst genotype determined by *msp1/2* PCR analysis

P. falciparum clone/s fed	Number of oocysts analysed	Median genomes per oocyst (25-75 percentile)
3D7 + HB3	46 3D7	408 (215-977)
	8 HB3	40 (34-43)
	50 Hybrid	367 (162-792)

There was also no obvious correlation between oocyst intensity and genomes per oocyst when the oocysts were separated into genotype (Figure 36).

Figure 36. Single oocyst data from mixed clone feeds with *An. gambiae s.s.* mosquitoes, oocyst genotypes were defined by *msp1* and *msp2* PCR analysis and are distinguishes by colour and shape, as shown in the legend.



This was investigated further using mixed effects linear models with explanatory variables of genotype and infection intensity, and mosquito and experiment as random effects variables. The results (Table 23) showed no clear evidence for a correlation of infection intensity with genomes per oocyst for 3D7 oocysts or hybrid oocysts in mosquitoes fed a mixture of 3D7 and HB3 gametocytes.

The model could not be applied for HB3 genotype oocysts because there were only 8 HB3 single oocysts found from mixed clone infections. The genomes per oocyst were all very low (18-46), with infection intensity ranging from 1 to 47 oocysts per midgut.

Table 23. Results of mixed effects linear model analyses for each oocyst genotype from mixed clone *P. falciparum* infections in *An. gambiae s.s.* mosquitoes

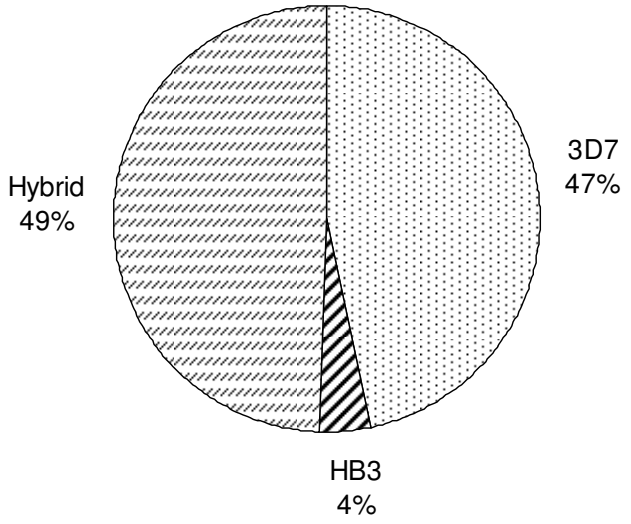
Oocyst genotype	n in sample	p value for mixed model	t value
3D7	46	0.396	0.858
HB3	8	Too small sample size for model	
Hybrid	50	0.3811	-1.079

Overall it can be concluded that the presence of other oocysts on the midgut had very little effect on parasite multiplication within the oocyst in a mixed clone environment or in a single clone environment.

4.2.5 Difficulty of isolating HB3 self-fertilisation oocysts from mixed clone feeds

Only about 4% (see Figure 37) of all the single oocysts dissected from *An. gambiae s.s.* mosquitoes, that were fed both 3D7 and HB3 gametocytes, were genotyped as HB3 parentals (products of HB3 self-fertilisation events). Previous studies have shown that mating is random in the mixed clone environment, although the gametocyte sex ratios of 3D7 and HB3 differ (Ranford-Cartwright *et al*., 1993). However the number of HB3 self-fertilisation events is low, possibly because of a lack of fitness in the HB3 female gametocyte (Ranford-Cartwright, 1995). In this study it did prove extremely difficult to obtain oocysts formed from HB3 self-fertilisation events in the midgut of mosquitoes that had been fed gametocytes of both clones.

Figure 37. Proportion of three genotypes of single oocysts collected from mixed clone feeds (typed by PCR analysis of *msp1* and *msp2*) Total number of single oocysts dissected = 214.



4.3 Discussion

4.3.1 Gametocytaemia

As the number of gametocytes fed to mosquitoes increases, the number of successful fertilisation events, and the number of ookinetes formed, should also increase, resulting in an increase in both prevalence and intensity of infection. However, previous results from *in vitro* grown cultures of *P. falciparum* have shown no association between gametocytaemia and infection in *An. gambiae s.s.* mosquitoes (Noden et al., 1994; Ponnudurai et al., 1989a). In natural infections there was a significant positive correlation between gametocytaemia and subsequent mosquito infection prevalence and intensity (Boudin et al., 1989; Drakeley et al., 1999; Jeffery and Eyles, 1955).

Within an experimental feed, dilution of gametocytes did decrease infection prevalence with a threshold-type relationship, with a plateau at about 0.5% gametocytaemia which gave more than 40% prevalence. Three specifically designed experiments, where the same culture was diluted on the feed day to give a range of gametocyte densities from 0.01% to 1%, provided some evidence for a logarithmic association between gametocytaemia and prevalence of infection, such that a threshold gametocytaemia is needed to ensure an infection (~0.1%) but the positive correlation plateaus, so that beyond a certain gametocytaemia (~0.5%) no greater prevalence is achieved by increasing the gametocytaemia in the bloodmeal. However, even within a single stock asexual culture of the same clone, fed at the same gametocytaemia, there is still a large variability observed in prevalence of infection between different experiments, which demonstrates the complex set of factors that must be influencing infection establishment in the mosquitoes.

The data from these three experiments also showed a slight positive correlation between gametocyte and infection intensity such that as the gametocytaemia in the feed increases, the number of oocysts on the midgut increases. However, there was only a weak correlation (r^2 of 0.04, 0.29 and 0.13 for the three experiments) suggesting that only some of the variation in infection intensity is explained by gametocytaemia in the bloodmeal. More experiments on this question would need to be conducted for a fuller picture on this association. *P. falciparum* isolates which were taken from infected patients and grown to gametocyte maturation before being fed to mosquitoes had different potential for gametocyte generation and prevalence of infection levels (Ponnudurai *et al.*, 1982).

Typically gametocyte densities in highly endemic areas range from 5-150 per µl and mosquitoes ingest about 1µl of blood when feeding (Rosenberg, 2008). For experiments carried out for this study; 0.1% gametocytaemia equals roughly 5000 gametocytes per µl.

When a larger data set, gathered from eight different experiments that were not designed to answer this question specifically, was used to study the association between gametocytaemia and mosquito infections, no clear relationships were seen between gametocytaemia and either prevalence of infection or infection intensity. There is clearly variability between different experiments as this has been found previously with experimental infections of *P. falciparum* (Lensen, 1996). He reported that although there appears to be an optimum gametocytaemia for subsequent oocyst production, this optimum is unpredictable and differs considerably between experiments. Unfortunately, to date there is no technique available for assessing the viability and functionality of *P. falciparum* gametocytes, so gametocytaemias are based on total counts which may include non-viable or functionally immature gametocytes.

No statistically significant associations were observed between the gametocytaemia of the infectious feed and the genomes per oocyst count with either 3D7 or HB3 *P. falciparum* clones in *An.gambiae s.s.* mosquitoes. As a small association between gametocytaemia and infection intensity was observed in the pilot experiments, a negative association between gametocytaemia and genomes per oocyst might have been expected, as competition for nutrients may become important and lead to lower parasite replication at high oocyst intensity. However no associations were observed, although this may be due to the small number of oocysts isolated from very heavily infected midguts.

4.3.2 Length of time in continuous culture (age)

Previous evidence has suggested that the length of time spent in asexual continuous culture has a negative effect on gametocyte production and infectivity (Lensen, 1996; Ponnudurai et al., 1982) and that this has been correlated to gene deletions such as those on chromosome 9 (Day et al., 1993; Kemp et al., 1992). The data collected in this thesis on age of culture and prevalence of infection, as with gametocytaemia shows no obvious relationship. The data presented here also show no significant association between the age of the culture used in the feed and the infection intensity found on the midgut. In addition, no statistically significant associations were observed between the age of the culture fed

and the genomes per oocyst count with either 3D7 or HB3 *P. falciparum* clones in *An. gambiae s.s.* mosquitoes.

One possible explanation for this is that the cultures used here had always spent less than 3 months in continuous culture, whereas previous work included parasites that had spent up to 5 months in culture (Ponnudurai *et al*., 1982). A significant effect therefore may be seen only after 3 months of continuous culture.

To study this relationship further, experiments using the same continuous culture fed to mosquitoes at equally spaced time points over a period of six months would produce a good data set for addressing the question of the effect of age of culture on infection prevalence and intensity.

4.3.3 Effect of infection intensity on the number of genomes per oocyst in single clone feeds

As the infection intensity increases, the competition for nutrients may become important for the developing oocysts on the mosquito midgut. It was therefore hypothesised that there might be a negative association between infection intensity and genomes per oocyst. Analysis of the single oocyst samples collected from infectious feeds with *P. falciparum* 3D7 clone alone showed a positive correlation ($p=0.02$) between genomes per oocyst and infection intensity. Therefore, surprisingly, the number of genomes per oocyst was positively correlated with intensity of infection in this data set. This may be explained by mosquito susceptibility such that those mosquitoes that can sustain high levels of infection intensity can also provide the most fertile environment for the developing oocysts.

Very high infection intensities are rarely seen in field-caught mosquitoes (Billingsley et al., 1994; Lyimo and Koella, 1992; Taylor, 1999). Naturally infected mosquitoes are most frequently found to be infected with low oocyst burdens; for example in a study of *An. gambiae s.s.* in Tanzania, out of the infected mosquitoes collected over a period of 4 months 91% were found to have fewer than 5 oocysts (Hogg and Hurd, 1997), and a different study found a highly over-dispersed distribution of oocysts with the most heavily infected gut containing 43 oocysts, but 95.2% of the guts holding 10 or fewer oocysts (Taylor, 1999). Therefore any difference in sporozoite production as intensity increases (> 50 oocysts per midgut) is unlikely to have a major effect on natural transmission.

4.3.4 Effect of infection intensity on the number of genomes per oocyst in mixed clone feeds

To date there have been no studies on the question of competition within the mosquito-stage of the parasite lifecycle. The data presented here, from mixed clone infections, shows no significant correlation between genomes per oocyst and infection intensity either looking at the data as a whole or dividing it by oocyst genotype. Parasite multiplication within an oocyst does not appear to be affected by the presence of parasites of different genotypes.

4.3.5 Difficulty of obtaining oocysts formed from HB3 self-fertilisation events

It has been clearly demonstrated that crossing of gametes in the mosquito occurs readily and randomly both in the field and the laboratory (Anderson et al., 2000b; Babiker et al., 1994; Ranford-Cartwright et al., 1993; Walliker et al., 1987). The reason so few HB3 parental-type oocysts are found in a mixed clone environment may be explained by the fitness of the female HB3 gametocyte, as was discussed by Ranford-Cartwright in 1995 (Ranford-Cartwright, 1995). From the observed inheritance pattern of mitochondrial genome markers in hybrid oocysts from mixed clone infections, a strong bias for mating between female 3D7 and male HB3 gametes has been reported previously (Creasey et al., 1993; Vaidya et al., 1993b). This could be explained by differences in the relative fitness of the gametes and/or zygotes produced from the two clones. Unfortunately it is not possible to shift this imbalance by just feeding more HB3 gametocytes (pers. comm. Ranford-Cartwright), so the small sample size of HB3 oocysts from mixed clone infections is just an accepted difficulty in this type of study.

5 Chapter 5. Parasite genetic effects on the parasite multiplication in the mosquito

5.1 Introduction

The journey from gametocyte to oocyst is a difficult one with high losses, however the parasite can sustain such losses because of extensive replication in the oocyst. A single oocyst, formed from the fusion of two gametocytes is thought to produce hundreds or even thousands or sporozoites. However, as discussed in Chapter 1, the variability in the number of sporozoites produced by individual oocysts, and the parasite genetic contribution to this variability have not been previously described in detail.

As discussed in Chapter 1 previous published studies have tried to establish sporozoite counts in single oocysts by counting sporozoites, released from a single isolated oocyst, under the microscope. Others have counted sporozoites in the salivary gland (plus the haemolymph in some cases) and then divided that number by the mean of the oocysts counted in the midgut. One clear limitation of the previous findings is that there was very limited sample sizes of single oocyst sporozoite counts, in one case only a single oocyst was counted.

The previous counts are very variable with a ten-fold difference (1000-10,000) in reported counts for *P. falciparum* oocysts (Pringle, 1965; Ross R, 1910). Lower counts are estimated for sporozoites in the salivary glands, suggesting losses of 80-90% in the transition from oocyst to salivary glands (Barreau et al., 1995; Myung et al., 2004; Natarajan et al., 2001).

Though quantitative PCR has been available since the early 1990s, it has not been utilised much to date to address parasite numbers in mosquitoes. Within the body of published evidence on sporozoite counts, the individual genotype of the parasites present has not been considered. Estimates from experimental infections with different laboratory clones of *P. falciparum* in *Anopheles gambiae s.s.* mosquitoes have not been carried out previously.

Differences in genome numbers per oocyst could be the result of genetic differences between parasites, as well as the environmental effects discussed previously. Variation in

the nuclear genes of different parasite clones could manifest as differences in genome numbers produced. By studying the sporozoite production in heterozygotes formed by cross-fertilisation between two different clones, it may then be possible to determine the possible causes of growth rate differences. The loci of the gene or genes that control the growth phenotype could be nuclear (in which case they may be dominant or recessive) or extranuclear. If the genes affecting growth rate are located on either of the two extrachromosomal elements, they would be inherited uniparentally through the female gamete, and in this case you would only see parental phenotypes.

From laboratory studies, mating between gametes from different clones occurs randomly, with no preferential self- (between gametes from the same clone) or cross-fertilisation (between gametes of different clones) in mosquitoes fed a 1:1 mixture of gametocytes of two parasite clones (Ranford-Cartwright *et al*., 1993).

There is limited data on cross-mating from field studies and although it has been clearly demonstrated that crossing of gametes from different clones in the mosquito occurs, the rate appears to differ depending on the study area (Anthony et al., 2000; Babiker et al., 1995; Mzilahowa et al., 2007; Paul et al., 1995; Razakandrainibe et al., 2005; Walliker, 1994). Natural populations of *P. falciparum* have been shown to exhibit extensive genetic diversity. Isolates of *P. falciparum* taken from patients in various parts of the world including The Gambia (Carter and McGregor, 1973) and Thailand (Rosario, 1981; Thaithong et al., 1984) have been shown to contain several different genotypes of parasites of the same species. These different clones may have been derived either from separate infective bites or from a single bite from a mosquito carrying genotypically distinct sporozoites. For cross-fertilisation to happen, the mosquito has to pick up a male and a female gametocyte of different genotype, so this is more likely to happen in areas of high endemicity. The abundance of genetically diverse populations in the field therefore makes any findings from the study of inheritance of phenotypic traits such as sporozoite formation, of relevance.

In summary, parasites in natural infections are genetically diverse, and cross- and self-fertilisation events can, and do, occur in naturally infected mosquitoes. It is therefore important to understand how sporozoite production varies between different parasite genotypes, and how this phenotype is inherited. Sporozoite production could also be affected by the presence of other genotypes within a single mosquito (in different oocysts). Variation in sporozoite production in hybrid vs. selfed oocysts could have a dramatic effect

on transmission dynamics and the spread of genetic mutations such as those that convey drug resistance. For example, crossing events between parasite resistant to single (but different) drugs could result in recombinant parasites resistant to both drugs. If sporozoite production was higher in hybrid oocysts this would increase the proportion of multiply resistant genotypes in the next generation.

Each *P. falciparum* parasite contains not only its nuclear genome but two additional extranuclear genomes – a 35kb, circular, double-stranded DNA molecule, located within the apicoplast (Gardner et al., 1991a; Wilson and Williamson, 1997) and an element consisting of multiple linked copies of a 6kb DNA element, located within the mitochondrion of the parasite (Vaidya et al., 1989; Wilson and Williamson, 1997). The apicoplast genome primarily encodes components of the transcription and translation machinery of the organelle (Chaubey et al., 2005) such as rRNA, tRNAs and subunits of prokaryote-like RNA polymerase (Gardner et al., 1991b; Gardner et al., 1991a). The 6kb element contains some of the enzymes of the mitochondrial electron chain such as cytochrome b, and subunits I and II of cytochrome c oxidase but does not contain all of the genes required for mitochondrial function, most of which are encoded in the nuclear genome (Vaidya et al., 1989; Vaidya et al., 1993b).

Most sexual eukaryotes show uniparental inheritance of organelle genomes, although protists, algae, and fungi can show a diverse range of inheritance patterns with sex-specific loci paying significant roles (Xu, 2005). The mitochondrial genome of nearly all animal species is inherited exclusively from the maternal parent (with two exceptions – the mussel, and a male human patient who carried mitochondrial DNA from both his parents), but unicellular eukaryotes have a slightly more diverse pattern of mitochondiral inheritance (Xu, 2005). Plastid genomes (such as plant chloroplasts) are usually inherited through the maternal line, although biparental transmission has been observed (Xu, 2005).

The inheritance of the extrachromosomal elements in *Plasmodium* has been well described as being exclusively through the female gamete (Creasey et al., 1993). Both the 6kb and the 35kb elements were found to be uniparentally inherited in the progeny of experimental crosses, with all progeny from the HB3 x Dd2 cross containing the extrachromosomal elements from the Dd2 parent (Vaidya et al., 1993b). In the 3D7 x HB3 cross, two different reports showed uniparental inheritance of the 6kb element (Creasey et al., 1993; Vaidya et al., 1993a) but inheritance of both parental types was seen in different progeny clones. Inheritance of the 35kb element was not reported for the 3D7 x HB3 cross.

If cross-fertilisation events happen equally between female HB3/male 3D7 gametes and female 3D7/male HB3 gametes, then the mitochondrial type in hybrid oocysts (from cross-fertilisation events) would be approximately evenly divided between the two parental types. However there was significant bias in mitochondrial inheritance in 3D7 x HB3 hybrid oocysts, with 58/59 of oocysts having the mitochondrial type from 3D7 (Creasey et al., 1993), and therefore being the products of fertilisation between 3D7 female gametes and HB3 male gametes. This could be partly explained by a difference in sex ratio of the two parental clones, with clone 3D7 producing significantly more female gametocytes than clone HB3 (Ranford-Cartwright et al., 1993). Modelling work also suggested a fitness defect in the female gametes of clone HB3 that could explain the results observed assuming mating between gametes was random (Ranford-Cartwright, 1995). Since the inheritance of the apicoplast genome is also through the female gamete, a similar bias is expected in the 3D7 x HB3 cross, although this has only been formally demonstrated in hybrid oocysts of the HB3 x Dd2 cross (Vaidya et al., 1993b).

Very little evidence is available to describe the natural variability in *Plasmodium* extrachromosomal elements in the field, but the rate of mutation in the *P. falciparum cytb* gene appears to be low (Sharma et al., 2001). There also seems to be extensive conservation between *Plasmodium* species in the 35kb element including; *P. falciparum, P. vivax, P. malariae,* and *P. berghei* (Tan et al., 1997).

The extrachromosomal elements are very highly conserved in laboratory strains of *Plasmodium falciparum.* From sequence analysis and restriction digestion, only a single nucleotide difference in *cytochrome b* gene was found between HB3 and Dd2 or 3D7 clones (Creasey et al., 1993; Vaidya et al., 1993b). For the 35kb element single strand chain polymorphism (SSCP) analysis was used to identify a single nucleotide difference that distinguished HB3 from Dd2 (Vaidya et al., 1993b). Generally a high level of conservation was seen for both the 6 and 35kb molecules suggesting intolerance for sequence alterations (Vaidya et al., 1993b).

In this chapter, the variability in the number of genomes present in mature single oocysts is described. Oocysts were collected from mosquitoes fed 3D7 parasites alone, HB3 parasites alone, or a mixture of 3D7 and HB3 genotypes, and the genomes per oocyst determined by quantitative polymerase chain reaction (qPCR). The number of genomes per 3D7 oocyst was compared to the number found in HB3 oocysts, to evaluate the effect of genotype on parasite multiplication. Oocysts collected from mixed feeds allowed the effect of the

presence of other parasite genotypes on parasite multiplication to be evaluated. Analysis of hybrid oocysts, formed by cross-fertilisation events between 3D7 and HB3 gametocytes, allowed the inheritance of the parasite multiplication phenotype to be investigated.

5.2 Results

A brief summary of the data collected (including infection prevalence and intensity descriptions) is presented below.

5.2.1 Prevalence and intensity of infection

Data presented in this thesis are from 32 successful infections, carried out with *P. falciparum* parasite clones 3D7 and HB3 in *An. gambiae s.s.* mosquitoes. The overall mean prevalence of infection, median intensity of infection and the number of mosquitoes dissected are presented in Table 24. As previously reported (Ranford-Cartwright et al., 1993), infection prevalence was found to be significantly different between the two clones; a t test between 3D7 and HB3 prevalence of infection gave a highly significant p value of 0.0051.

Table 24. Total number of experiments carried out with *P. falciparum* clones in *An. gambiae s.s.* mosquitoes, with the mean prevalence of infection, and the number of mosquitoes dissected also shown.

Clone fed	No. of experiments performed	Mean prevalence (range)	Median intensity (range)	No. of mosquitoes dissected
3D7	8	56% (17-88%)	1 (0-170)	161
HB3	7	21% (10-33%)	0 (0-14)	124
3D7 & HB3	17	63% (17-96%)	2 (0-225)	341

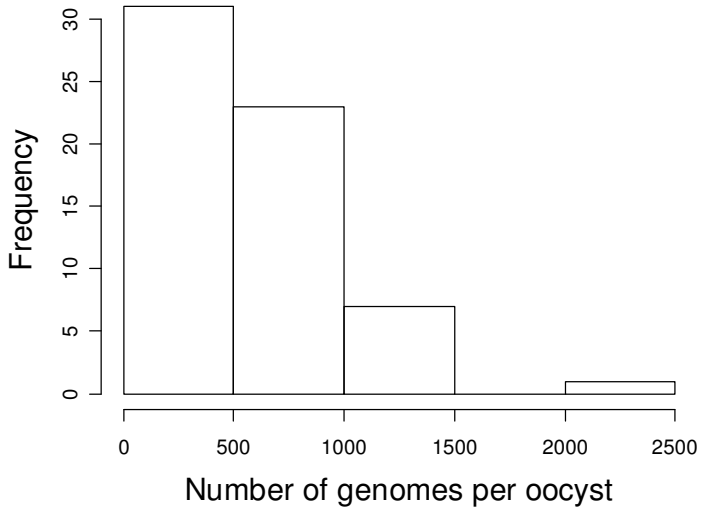
5.2.2 Parasite multiplication in 3D7 single clone infections

Single oocysts were dissected from the midguts of *An. gambiae s.s.* mosquitoes which had been given a bloodmeal containing 3D7 gametocytes 10 days earlier. Quantitative PCR was used to determine the number of genomes per oocyst (Chapter 2, section 2.6). 62 single oocysts collected from 47 different mosquitoes infected in 9 experiments were evaluated (Table 25). 16 of the oocysts were from single midguts, and multiple oocysts were removed from 13 mosquito midguts to investigate the variation in genomes per oocyst within a single mosquito.

Table 25. Number of single oocysts collected from the 9 different experiments, the number of mosquitoes from which the oocysts came, and the number of oocysts from single oocyst midguts.

Experiment	No. single oocysts collected	No. mosquitoes from which oocysts were removed	No. oocysts from midguts with only one oocyst
1	2	2	2
2	2	2	2
3	8	6	2
4	5	4	1
5	7	5	1
6	13	12	5
7	10	7	2
8	12	7	1
9	3	2	0
Total	**62**	**47**	**16**

Figure 38. The distribution of genomes per oocyst in 3D7 single clone infections. The data represent 62 single oocysts.

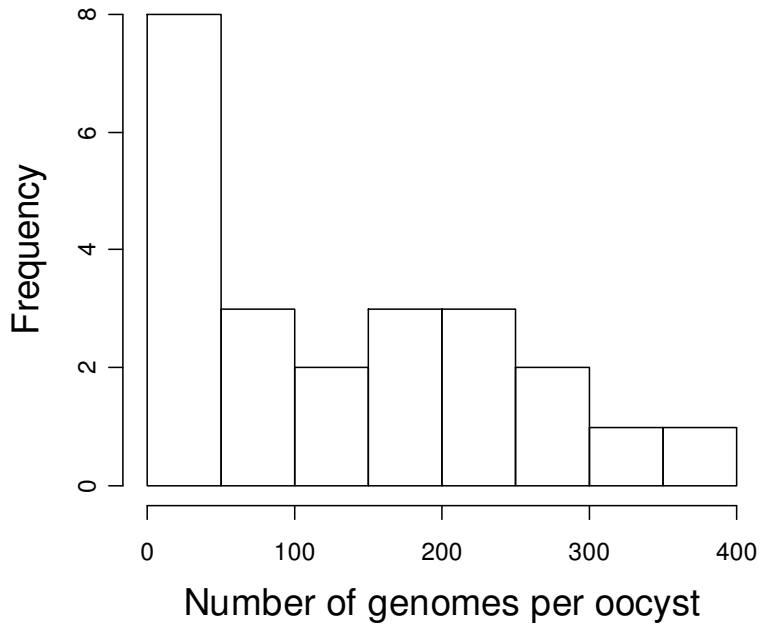


The number of genomes per oocyst ranged from 27 to 2080, with a median value of 484 and a mean value of 540 (Figure 38). The distribution of genomes per oocyst was non-normal (Shapiro-Wilks p=0.0003), showing a skew to the left, indicating that more oocysts were found with lower numbers of genomes per oocyst.

5.2.3 Parasite multiplication in HB3 single clone infections

Full qPCR data was obtained for 23 single oocysts dissected from *An. gambiae s.s.* mosquitoes offered only HB3 gametocytes in the blood meal. Figure 39 displays the distribution of the genomes per oocyst data from HB3 single clone infections.

Figure 39. Histogram of the number of genomes per oocyst for HB3 single clone infections in *An. gambiae s.s.* mosquitoes



The number of genomes per oocyst ranged from 5 to 390 with a median of 106, a mean of 135, and a non-normal distribution (Shapiro-Wilks, p=0.0337). All except one of the single oocyst samples came from a midgut with only one oocyst (one sample came from a midgut with four oocysts present).

5.2.4 Comparison of parasite replication within 3D7 and HB3 oocysts from single clone infections in An. gambiae s.s. mosquitoes

Full qPCR data was available for 85 oocysts from 16 different single clone feed experiments (four of which had data from both clones). Table 26 displays the number of samples collected from each parasite clone, the result of the Shapiro-Wilks test on the distribution of genomes per oocyst data for normality, and the median number of genomes per oocyst for each clone. The number of HB3 samples is fairly low due to the low

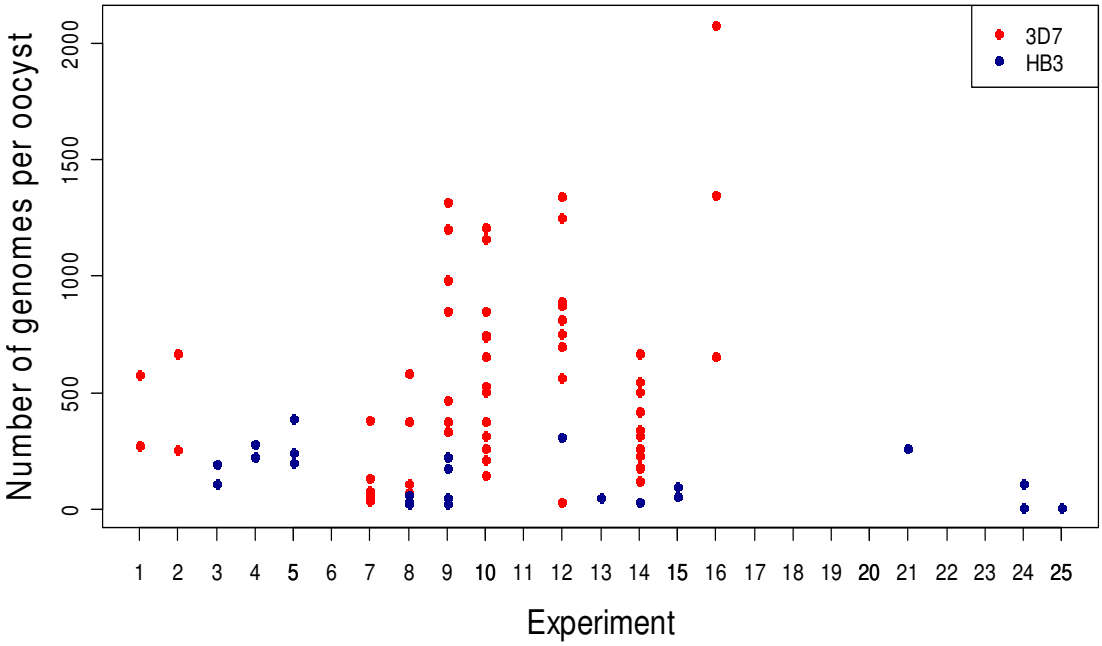
prevalence and infection intensity observed with HB3 infections, making it difficult to collect large numbers of oocyst samples.

Table 26. Single oocyst data from single clone feeds

P. falciparum clone fed	Number of oocysts analysed	Shapiro-Wilks p value	Median genomes per oocyst (25-75 percentile)
3D7	62	**0.0003**	402 (201-686)
HB3	23	**0.0337**	140 (46-228)

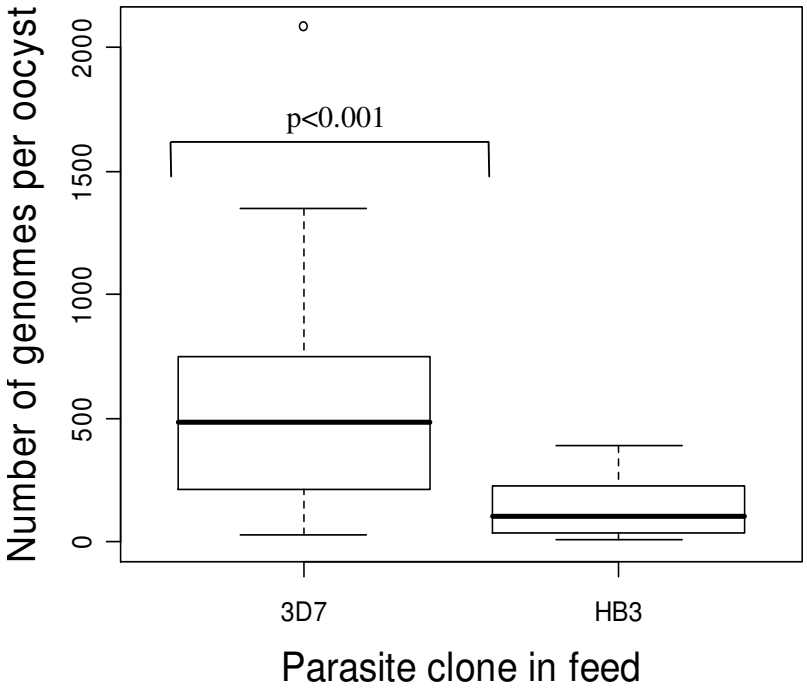
Genomes per oocyst values were non-normally distributed for both parasite clones. A scatterplot of the genomes per oocyst by experiment (Figure 40) shows the large variation found in the data, and also indicates that the variation was observed in all experiments.

Figure 40. Plot of single oocyst data from 16 different experiments (experiment numbers refer to all experiments carried out in chronological order, where no dots are shown, no oocysts from single clone infections were collected). Each circle represents a single oocyst isolated from a mosquito offered either 3D7 (red) or HB3 (blue) gametocytes.



A boxplot of the pooled data from all the single clone experiments clearly shows the difference in genome numbers between the clones (Figure 41), confirmed by a Mann-Whitney U test on the median number of genomes per oocyst ($p<0.001$).

Figure 41. Genomes per oocyst against parasite clone. The heavy bar represents the median, boxes represent the lower and upper quartiles and the whiskers show the maximum and minimum. Circles are those data points considered outliers. All oocysts were dissected 10 days post infection from An. gambiae s.s. mosquitoes fed only a single clone. n=62 for 3D7, n=23 for HB3. P value is from a Mann-Whitney U test.



A mixed effects linear model was also used to assess the association between genomes per oocyst and clone, with experiment and mosquito included as random effect variables. There was a significant difference in genomes per oocyst observed between clones ($p<0.001$, $t=-4.81$) with model parameters including an intercept of 572.3 and a coefficient for HB3 of -448, confirming that HB3 has significantly lower number of genomes per oocyst than 3D7 when developing in *An. gambiae s.s.* mosquitoes.

5.2.5 Parasite replication within parental-type oocysts from single and mixed clone infections

The hypothesis being tested in this study was that significant differences between different genotypes in single clone infections would remain in a mixed clone environment.

Mosquitoes were fed a mixture of 3D7 and HB3 gametocytes, and 104 single oocysts were isolated, of which 46 were found to be the result of self-fertilisation events between 3D7

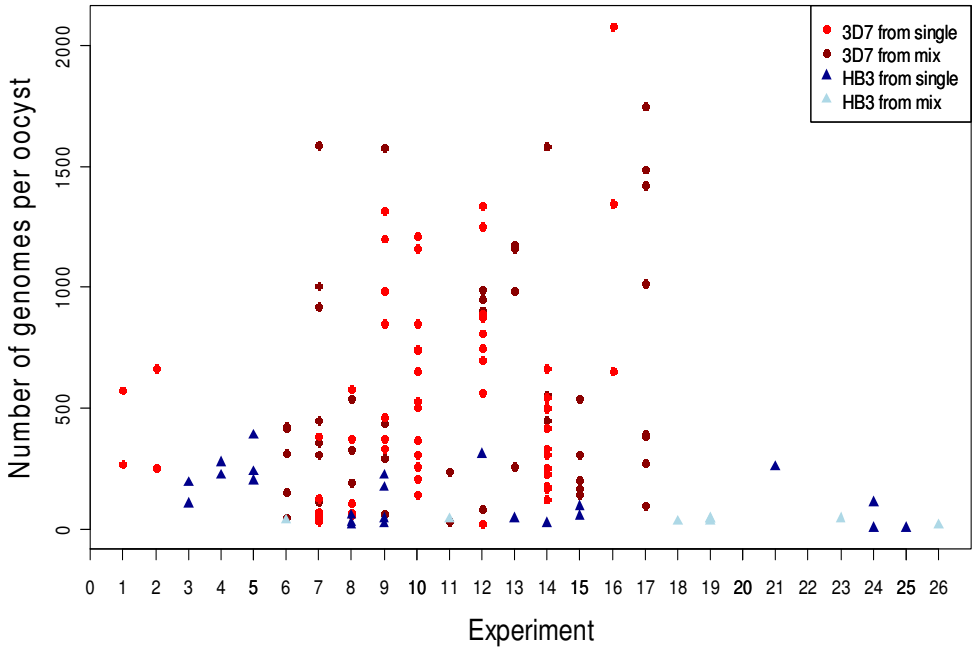
gametocytes, 8 were from HB3 self-fertilisation events, and 50 were found to be hybrid oocysts from cross-fertilisation events between 3D7 and HB3. Since all three types of oocyst are found in the same mosquito, it was possible to compare the genome numbers in 3D7 and HB3 parental oocysts taken from the same mosquito environment (Table 27). As the data distributions were significantly different from normal, non-parametric tests were used for all statistical analyses.

Table 27. Single oocyst data from mixed clone feeds

P. falciparum **clone fed**	**Number of oocysts analysed**	**Shapiro-Wilks p value**	**Median genomes per oocyst (25-75 percentile)**
3D7 + HB3	46 3D7	**<0.001**	408 (215-977)
	8 HB3	0.1211	40 (34-43)
	50 Hybrid	**<0.001**	367 (162-792)

A scatterplot of the genomes per oocyst by experiment, shows the large variation observed in genomes numbers per oocyst (Figure 42). HB3 oocysts consistently had a lower number of genomes than 3D7 oocysts (Table 27).

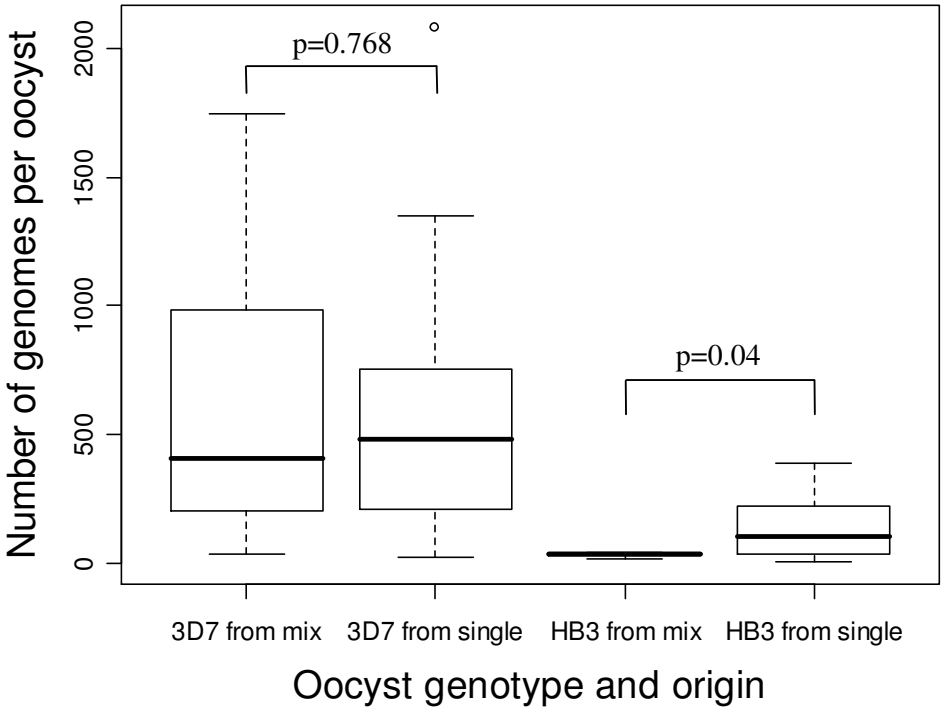
Figure 42. Plot of single oocyst data from 24 different experiments (experiment numbers refer to all experiments carried out in chronological order, where no dots are shown, no 3D7 or HB3 oocysts were collected). Each circle represents a single oocyst, with genotypes defined by *msp1* and *msp2* PCR analysis.



3D7 oocysts isolated from mosquitoes fed 3D7 gametocytes alone were then compared with 3D7 oocysts from mosquitoes fed a mixture of 3D7 and HB3 gametocytes, to assess if the presence of other genotypes affected oocyst growth and development (Figure 43). No significant differences in genomes per oocyst were observed between 3D7 oocysts from single or mixed clone feeds (Mann-Whitney U test, p=0.768). Comparison of oocysts formed from HB3 self-fertilisation events from single clone and mixed clone infections was significant (Mann-Whitney U test, p=0.0397) despite a low power (<10%) to detect a difference due to the very small sample size of HB3 oocysts from mixed clone infections.

These results suggest there is a true genetic influence over the number of genomes per oocyst, with large differences in parasite replication between the two parasite clones examined. For 3D7, parasite replication does not appear to be influenced by the presence of competing distinct genotypes. For HB3, parasite replication is significantly lower in oocysts from mosquitoes with oocysts of different genotype.

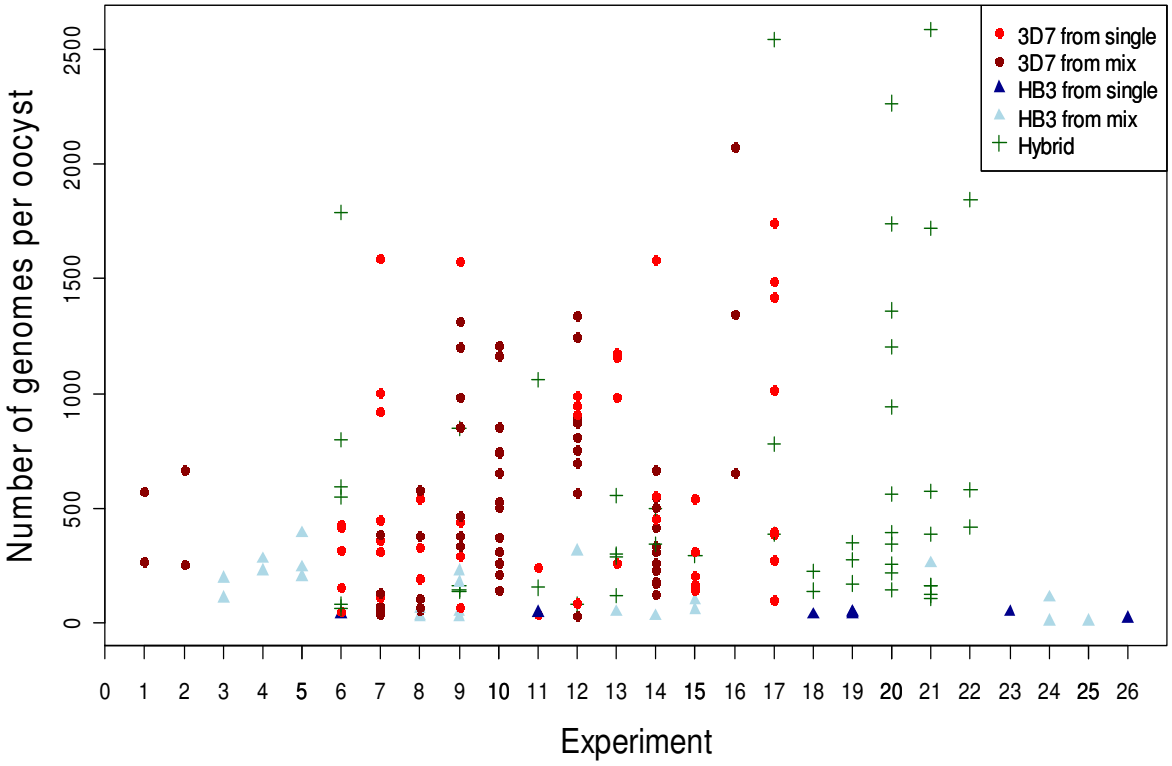
Figure 43. Genomes per oocyst against genotype of oocyst, to compare oocysts from single and mixed clone environments. Oocysts from single clone infections are labelled 'from single' and those dissected from mosquitoes fed both clones are labelled 'from mix'. p values are from Mann-Whitney U tests.



5.2.6 Parasite replication within oocysts from mixed clone infections

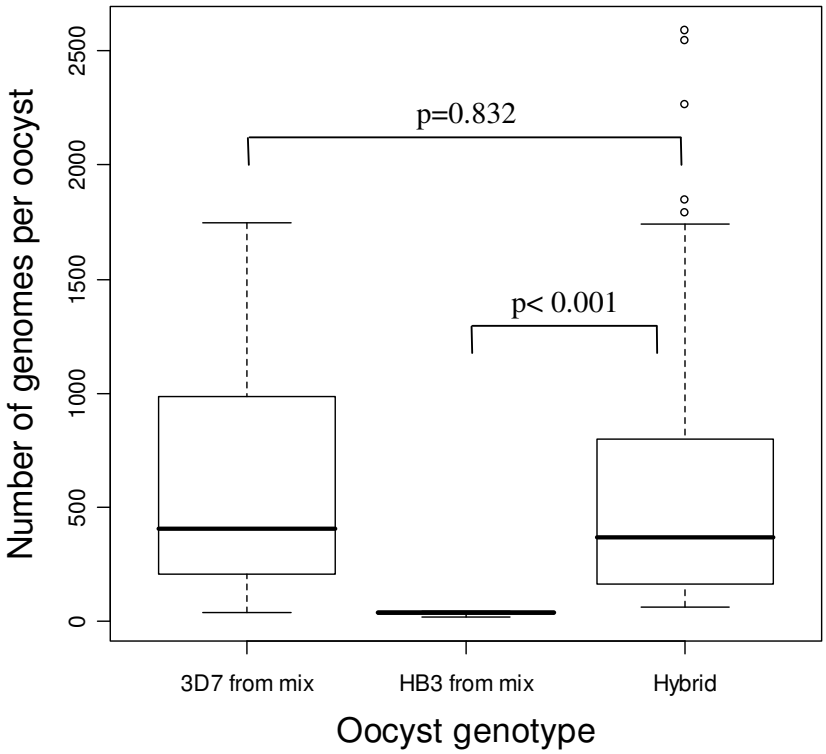
Analysis of genome numbers in oocysts formed from cross-fertilisation events (hybrid oocysts) allowed the dominance of the phenotypic trait to be established. Figure 44 shows a scatterplot of genomes per oocyst divided by experiment and genotype as defined by *msp1* and *msp2* PCR analysis. This graph displays all the single oocyst qPCR data collected from both single and mixed clone infections.

Figure 44. All single oocyst qPCR data divided by experimental feeds. Each coloured point represents a single oocyst, the genotype and origin of each oocyst is displayed by colour and shape and defined in the legend



The genome numbers found in hybrid oocysts (Table 27) were significantly higher than those seen in HB3 parental oocysts taken from mosquitoes fed a mixture of the two clones (Mann-Whitney U test, $p<0.001$). No statistically significant difference (Mann-Whitney U test, $p=0.8316$) in genome numbers per oocyst was found between 3D7 parental oocysts and hybrid oocysts (Figure 45).

Figure 45. Genomes per oocyst against genotype of oocyst, only data from oocysts from mosquitoes offered both clones are shown. Oocyst genotypes were determined by PCR analysis of two polymorphic genes, msp1 and msp2. Data shown are medians (heavy lines), lower and upper quartiles (boxes) and maximum and minimums (whiskers). Circles are those data points considered outliers. p values are from Mann-Whitney U tests.



5.2.7 Mitochondrial gene inheritance in hybrid oocysts

Each oocyst inherits the mitochondrial and apicoplast genomes from the macrogamete only (Creasey et al., 1993). The macrogamete in the cross-fertilisation event resulting in a hybrid oocyst could be either HB3 or 3D7. This was established by typing with a polymorphic mitochondrial gene marker, *cytochrome b* (Creasey et al., 1993). DNA from 50 hybrid oocysts dissected from mosquitoes offered both 3D7 and HB3 gametocytes was analysed further for the *cytb* marker as previously described (Creasey et al., 1993). Of the 50 oocysts collected, 48 were successfully amplified for the nested 439bp fragment of the *cytb* gene which contains a single base pair difference between the two clones (3D7 and HB3). RFLP typing of the gene fragment allowed the two alleles to be distinguished. All 48 oocyst samples had the 3D7 allele of *cytb*. This analysis therefore suggests that the 48 hybrid oocysts successfully typed were all formed from the mating of female 3D7 and male HB3 gametes, an observation in agreement with previous analyses of hybrid oocysts from crosses of these two parasites (Creasey et al., 1993).

Because there were no oocysts resulting from fertilisation of HB3 macrogametes, it was not possible to investigate further the contribution of variation in the extrachromosomal elements to the parasite replication phenotype.

Discussion

5.2.8 Parasite multiplication within oocysts from single clone infections

Analysis of parasite numbers within single oocyst samples suggests that, even within the same mosquito, fed the same gametocytes, there is still variation in genome numbers per oocyst, which can not be fully explained by any of the environmental factors considered in this study.

DNA replication within the developing oocyst begins following oocyst establishment on the midgut and continues until around day ten post-infection, at which point the oocyst begins to differentiate as sporozoite budding occurs. Mitotic replication begins immediately after oocyst formation and continues throughout the period of sporogony. Sporoblast formation begins around 5 days after establishment of the oocyst and continues until around day ten (Howells and Davies, 1971b). The developing sporozoites then bud off from the sporoblasts, synchronously throughout a single oocyst.

Different oocysts dissected from the same mosquito midgut were removed at the same time point, most probably after DNA replication was complete. However, there would be variation in the time at which they crossed the midgut epithelium and established as oocysts. Mature ookinetes take ~24hrs to form after the infectious feed (Chege et al., 1996; Meis and Ponnudurai, 1987), and invasion of the midgut can continue until the blood meal is fully digested and the remnants ejected from the mosquito ~48hrs after the blood meal (Briegel, 1980). It is feasible that higher numbers of parasites may be generated in 'early' oocysts (i.e. those from ookinetes that migrated first through the midgut epithelium) and that growth in 'late' oocysts may be restricted by the availability of nutrients. However this may be challenging to address experimentally as one would need to 'flush out' the bloodmeal after a set time period to remove 'late ookinetes', perhaps with some kind of mosquito enema which may be very difficult to administer with repeatable consistency.

Explanatory variables which were not considered in this study, but which may affect sporozoite production, include oocyst size, and position on the midgut. Oocysts on the midgut do vary in size (Huff, 1940; Ponnudurai et al., 1989a; Sinden and Strong, 1978), and larger oocysts may contain more sporozoites. Oocyst size is very difficult to measure

accurately, because under a coverslip there is uncontrollable variability in compression levels and without a coverslip oocysts may be positioned on top of each other. However, preliminary data indicates a positive association between oocysts (measured without a coverslip) and genome number (A.S. Bell and L.C. Ranford-Cartwright, unpublished observations). The position of each individual oocyst on the midgut was not recorded for this study, but has been previously suggested as an influencing factor on oocyst development, possibly due to the availability of nutrients in the midgut. Clumping was observed with *P. falciparum* oocysts on *An. gambiae* midgut walls by Sinden and Strong (Sinden and Strong, 1978) which was shown by Shute (Shute, 1949) to be caused by the effect of gravity on the position of the bloodmeal in resting mosquitoes following feeding. Within the clumps reported, the outer oocysts were larger than those in the centre which suggests a competition for nutrients (Sinden and Strong, 1978), however no clumps were ever observed in the experiments presented in this thesis.

5.2.9 Comparison of parasite numbers in parental-type oocysts from single and mixed clone infections

A highly significant difference in genome numbers per oocyst was observed between clones 3D7 and HB3 in *An. gambiae s.s.* mosquitoes. Although the variation in genomes per oocyst counts was found to be large, particularly in 3D7 oocysts, there were still significantly fewer genomes per oocyst in HB3 oocysts compared to 3D7 oocysts. These observations suggest that there was a significant genetic effect on replication in the oocyst.

No significant difference was observed in genomes per oocyst in 3D7 oocysts dissected from single or mixed clone infections. There was a significant difference observed in HB3 oocysts from the two different environments with lower parasite numbers in oocysts from mixed clone infections. These results suggest that the production of 3D7 sporozoites is not affected by the presence of other genetically different clones in the mosquito midgut, but that HB3 may be partially affected by the presence of 3D7 in the midgut.

Even when the two clones experience the same mosquito environment, a statistically significant difference is maintained between the genomes per oocyst counts of the two clones. This demonstrates that an important genetic component must be affecting parasite replication and by implication, sporozoite production.

5.2.10 Comparison of parasite numbers in oocysts from mixed genotype infections

Mosquitoes fed a mixture of gametocytes from two different clones could harbour three types of oocysts – those from self-fertilisation events between gametes from 3D7 or HB3, or those from cross-fertilisation events which are termed hybrid oocysts. When considering the inheritance of the trait for sporozoite production in the oocyst, there were four possible outcomes:-

1. Hybrid oocysts produced equally as many genomes as either the better or worse parent;

2. Hybrid oocysts produced an intermediate number of genomes, falling between the two values of the parents;

3. Hybrid oocysts may perform worse than either parent;

4. Hybrid oocysts may have out-performed the better of the two parents (heterosis or hybrid vigour).

There was no statistically significant difference in sporozoite numbers per oocyst between oocysts formed from cross-fertilisation events and 3D7 oocysts formed from self-fertilisation events, *i.e.* the hybrid oocysts performed as well as the better of the two parents. Hybrid oocysts developed similar parasite genome numbers to 3D7 oocysts, even when they had inherited half their DNA from the HB3 parent.

The results could therefore be caused by a gene or genes either on the nuclear genome or the extranuclear elements (the apicoplast or mitchondrial genomes). The possible explanations for the effects seen are as follows:

- Phenotype due to gene/s encoded on extranuclear elements which are inherited only from the female gamete;

- Phenotype due to gene/s encoded in the nuclear genome which may be expressed in three possible ways:

    1. expressed only in the female gamete *before* fertilisation;

2. expressed only in the female gamete *after* fertilisation;

3. expressed from both parental genomes pre- or post- fertilisation but 3D7 allele acts in a *dominant* fashion;

Each of these possible explanations is discussed in further detail to consider how one might practically test the theory in each case, although the genetic experiments will not distinguish between possibilities labelled 1 and 2 above.

5.2.10.1 Gene/s encoded on the extranuclear elements

As previous evidence shows, the extranuclear elements are inherited maternally so that each hybrid oocyst is hemizygous for the genes on the extranuclear elements (Creasey et al., 1993; Okamoto et al., 2009). One possible explanation for the results presented here is that the gene/s responsible for the "high genome numbers" phenotype is/are present on the extranuclear elements that are inherited from the female 3D7 gamete. Hybrid oocysts formed from female 3D7 and male HB3 gametes would have "high" genome numbers, whereas those formed from female HB3 and male 3D7 gametes would have "low" genome numbers. The hybrid oocysts obtained from mixed feeds all had "high" genome numbers, and all were of the first hybrid mating event (female 3D7 gamete). No hybrid oocysts were found that were the product of female HB3 and male 3D7 fertilisation, so this hypothesis could not be tested further. Additional mixed genotype infectious feeds with 3D7 and HB3 could produce the occasional hybrid oocyst formed from female HB3 and male 3D7, but attempts to produce these by biasing the ratio of 3D7:HB3 in favour of HB3 gametes were unsuccessful in generating hybrids of the required type.

Another approach to disprove this hypothesis would be to examine oocyst genome numbers in progeny clones from the 3D7 x HB3 cross. Some of the progeny clones may carry the HB3-type extranuclear elements. Over 50 different recombinant progeny clones are now available from the 3D7 x HB3 cross (Ranford-Cartwright, unpublished). Each progeny clone could be analysed (using the *cytb* PCR amplification assay described in Chpt 2) to assess the origin of the extranuclear elements. Clones found to possess the HB3 allele of *cytb* could then be cultured to gametocytes and used for in infectious feeds, individual oocysts removed, and the number of genomes per oocyst could then be determined. If replication in the oocyst is entirely due to gene/s on the extranuclear genomes, then progeny clones with the HB3 mitochondrial type (but with a recombinant

nuclear genome) would resemble the HB3 parasite clone (“low” genome numbers per oocyst), and those with the 3D7 mitochondrial type would resemble the 3D7 parental clone (“high” genome numbers per oocyst).

5.2.10.2 Gene/s encoded in the nuclear genome

Another possible explanation for the results observed is that the gene/s responsible for the sporozoite replication phenotype are located on the nuclear genome. There are three possible ways the gene/s may be expressed (as listed in Section 5.2.10 above)

5.2.10.2.1 Expression of a nuclear-encoded gene from both parental genomes, 3D7 dominant effect.

If the gene/s responsible for the “high” genome replication phenotype are present on the 3D7 nuclear genome and they are expressed at any point pre- or post- fertilisation in a dominant fashion, then all hybrid oocysts would have similar numbers of sporozoites to 3D7 oocysts from single clone infections, as was observed. Genetic linkage analysis (QTL analysis) of the progeny clones from the 3D7 x HB3 cross could be used to identify the area/s of the genome where the gene/s might lie. Single oocysts collected from mosquito infections with each progeny clone would be analysed for genome numbers. If the replication phenotype is explained by a single gene, then two phenotypes would be found in the progeny clones, each identical to one of the parent clones (“High”=3D7-like; “low”=HB3 like). Genetic maps of each progeny clone are available, constructed using microsatellite and SNP markers, across the genome, that are polymorphic between the parents. The progeny clones are typed for the markers and then it can be established which regions of the chromosome are inherited from which parent for each progeny clone (Su and Wootton, 2004). All progeny clones sharing a parental phenotype will share a region of the genome with the parental clone and each other, and this region will not be shared with progeny clones (or the parent clone) with the alternative phenotype. This linkage analysis allows the area of the genome most likely to contain the gene to be identified.

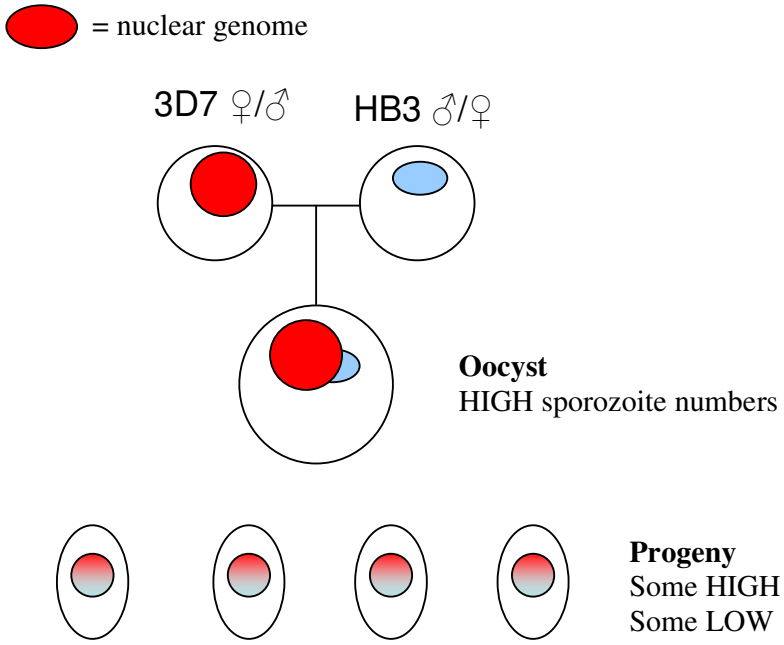
Figure 46 shows a cartoon representation of mating between 3D7 and HB3 gametes, assuming dominant nuclear gene expression (from 3D7) of a single gene responsible for high sporozoite replication.

If the replication phenotype is explained by a more than one gene, then more than two phenotypes would be found in the progeny clones – nonparental phenotypes such as

intermediate replication rates or higher replication rates than either parent. In this case (multiple genes responsible for the phenotype) the same genetic maps can be employed for QTL analysis, but a more complicated statistical analysis utilising a variety of probability-based approaches is used to obtain a LOD (logarithm of the odds) score for each marker. A LOD score is a measure of the likelihood that a marker is linked to the phenotypic trait being investigated. The LOD score compares the likelihood of obtaining the observed data if the two loci are indeed linked, to the likelihood of observing the same data purely by chance (no linkage). A LOD score of three or more is generally taken to indicate that two gene loci are close to each other (LOD score of 3 means the odds are a thousand to one in favour of genetic linkage) (Webster's Medical Dictionary, 2008).

The number of progeny clones that would need to be typed will depend on the number of genes that control the trait, and the extent of recombination in the genomes (Lander and Botstein, 1989).

Figure 46. Cartoon representation of mating between 3D7 and HB3 gametes, with dominant nuclear gene expression, assuming a single gene responsible for the high replication phenotype. Large circles represent dominant gene expression of the gene of interest.



5.2.10.2.2 Expression of a nuclear-encoded gene only in the female gamete before fertilisation

In this scenario, the gene/s responsible for the "high" genome replication phenotype are present on the 3D7 nuclear genome, but are expressed only in the female gamete (sex-specific expression). Sex-specific expression of genes in male gametocytes and gametes in

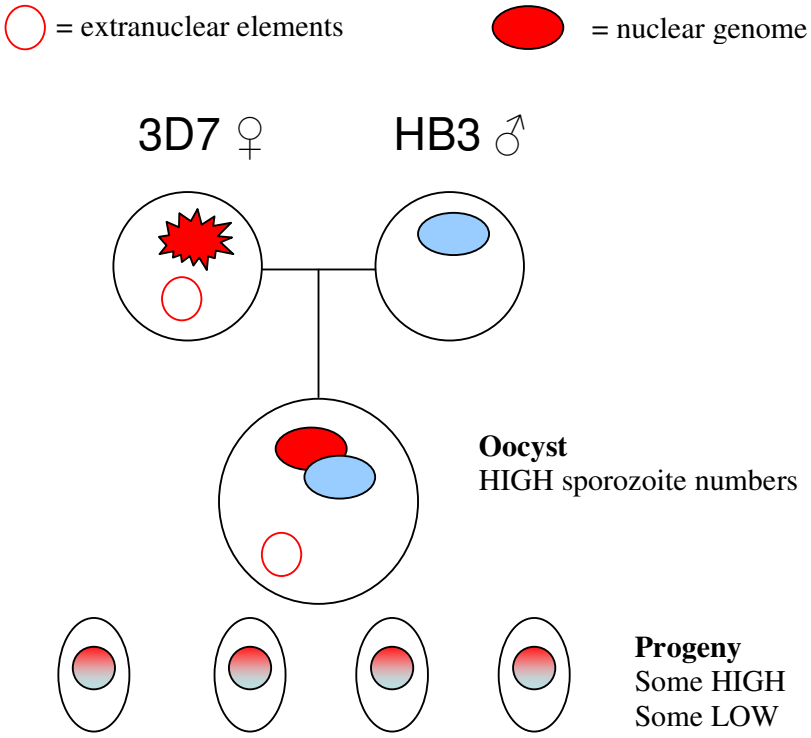
P. falciparum is known, for example PfsMR5 and alpha-tubulin II (Eksi and Williamson, 2002; Rawlings et al., 1992), and female gametocyte-specific expression has been demonstrated for two genes; Pfg377 (Koning-Ward et al., 2008) and Pfs47 (van Schaijk et al., 2006). Female-specific protein expression has also been demonstrated in *P. berghei* where 101 out of 541 proteins were female-specific in the gametocyte, and only 69 proteins were shared between the male and female proteomes (Khan et al., 2005).

Hybrid oocysts resulting from fertilisation of 3D7 female gametes would have the "high" genome number phenotype, whereas hybrid oocysts resulting from fertilisation of HB3 female gametes would have the "low" genome number phenotype. This would appear to be similar to a gene encoded on the extrachromosomal elements, as discussed in Section 5.2.10.1 above. However this mode of inheritance could be distinguished from extrachromosomal by examination of the progeny clones. In the case of a single gene conferring the trait seen, the genome numbers seen in oocysts obtained by feeding mosquitoes gametocytes of a single progeny clone would mirror the two parental phenotypes ("high" and "low"), irrespective of the extrachromosomal element type inherited. A progeny clone with the 3D7-type extrachromosomal elements is equally likely to have "high" or "low" oocyst genome numbers. If more than one locus contributes to the trait, a similar QTL analysis to that described above could be applied.

Figure 47 shows a cartoon representation of mating between 3D7 female and HB3 male gametes, assuming a single nuclear gene (from 3D7) responsible for high sporozoite replication, which is expressed only before fertilisation.

To specifically study gene expression in *P. falciparum* gametes, separation of male and female gametes would need to be carried out. To date an interesting method employing flow cytometry has been used to separate male and female transgenic gametocytes expressing green fluorescent protein in a sex-specific manner in *P. berghei* (Khan et al., 2005), and in *P. falciparum* (Eksi et al., 2008). The sex-specific expression of this gene could be confirmed by study of the already published proteomes of separated male and female gametocytes from *Plasmodium berghei* (Khan et al., 2005). The *P. falciparum* transcriptome through gametocytogenesis has also been published (Silvestrini et al., 2005; Young et al., 2005).

Figure 47. Cartoon representation of mating between 3D7 female and HB3 male gametes, with female nuclear gene expression only before fertilisation. Spiked circles represent expression of the nuclear imprinted genes of interest.



5.2.10.2.3 Expression of a nuclear-encoded gene only in the female gamete after fertilisation

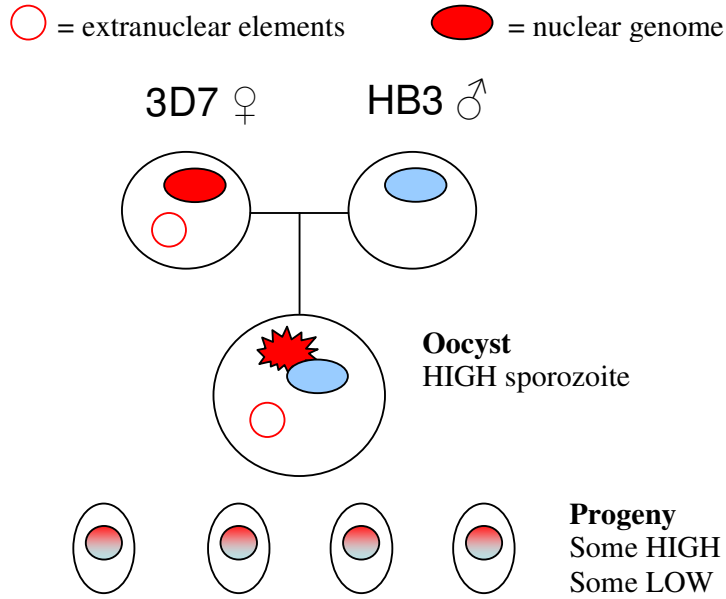
In this scenario, the gene/s responsible for the "high" genome replication phenotype are present on the 3D7 nuclear genome, but are imprinted so that they are only expressed by the female genome, after fertilisation.

Hybrid oocysts resulting from fertilisation of 3D7 female gametes would have the "high" genome number phenotype, whereas hybrid oocysts resulting from fertilisation of HB3 female gametes would have the "low" genome number phenotype.

As for the explanation above in Section 5.2.10.2.2 above, this mode of inheritance could be distinguished from extrachromosomal by examination of the progeny clones. In the case of a single gene conferring the trait seen, the genome numbers seen in oocysts obtained by feeding mosquitoes gametocytes of a single progeny clone would mirror the two parental phenotypes ("high" and "low"), irrespective of the extrachromosomal element type inherited. If more than one locus contributes to the trait, again a similar QTL analysis to that described above could be applied.

Figure 48 shows a cartoon representation of mating between 3D7 female and HB3 male gametes, assuming a single nuclear gene (from 3D7) responsible for high sporozoite replication, which is expressed only after fertilisation.

Figure 48. Cartoon representation of mating between 3D7 female and HB3 male gametes, with female nuclear gene expression only after fertilisation. Spiked circles represent expression of the nuclear imprinted genes of interest.



This explanation involves a similar phenomenon to genomic imprinting, which is a form of epigenetic gene regulation that results in expression from a single allele in a parent-of-origin-dependent manner, such as the expression of the 3D7 female nuclear gene/s *after* fertilisation in a hybrid oocyst. Examples of genomic imprinting include the mouse insulin-like growth factor II gene (DeChiara et al., 1991) and seed development in plants, which involves genes encoding Polycomb group chromatin-modifying factors (Nowack et al., 2007).

To date it is still unclear why imprinting evolved, and different patterns of imprinting occur in different classes of mammals. There are different theories to explain how the mechanism evolved including; that imprinted genes arose from sex chromosomes, or that imprinting emerged from an ancestrally-imprinted chromosome (Edwards et al., 2007). The molecular mechanisms of genomic imprinting have not been clearly described to date. Imprinted loci across the genome seem to rely on epigenetic markings of DNA methylation and/or histone modifications to distinguish parental alleles (Ideraabdullah et al., 2008).

Both DNA methylation (Hattman, 2005) and histone modification (Andrews et al., 2009) have been described in *Plasmodium*, although not in relation to genetic imprinting, and to date no genes in *P. falciparum* have been recognised as being expressed through imprinting.

If a single gene was responsible for the replication phenotype, the progeny clones from the product of female 3D7/male HB3 crosses would either be "high" or "low" phenotypes depending on whether they had inherited the gene after recombination.

5.2.11 Summary

A summary of the predicted results from each of the possible hypotheses is shown in the table below (Table 28). For simplicity only single gene effects are shown, but multiple loci may be responsible for the observed phenotype. As shown from the predicted results, it may not be possible with these experiments to distinguish between 3D7 nuclear genes expressed either before or after fertilisation.

Table 28.Possible results from future experiments with hybrid oocysts and progeny clones from the 3D7xHB3 cross. HIGH=high number of genomes/oocyst (like 3D7), LOW=low number of genomes/oocyst (like HB3).

	Hybrid oocysts		**Progeny clones**	
Hypothesised origin of effect	**3D7 ♀ + HB3♂**	**3D7♂ + HB3♀**	**Product of 3D7 ♀ + HB3♂**	**Product of 3D7♂ + HB3♀**
Extranuclear elements 3D7 gene/s	HIGH	LOW	HIGH	LOW
Nuclear ♀ 3D7 genes expressed *before* fertilisation	HIGH	LOW	some HIGH some LOW	LOW
Nuclear ♀ 3D7 genes expressed *after* fertilisation	HIGH	LOW	some HIGH some LOW	LOW
Nuclear dominant 3D7 genes	HIGH	HIGH	some HIGH some LOW	

6 Chapter 6. General discussion

6.1 Summary

The data presented here provide a much fuller and detailed picture, than anything to date, of the replication of *P. falciparum* parasites within oocysts in the natural vector *An. gambiae*. Both environmental and genetic factors have been considered as contributing to the development of sporozoites within the oocyst, but there was little evidence of environmental influences such as mosquito size or competition for nutrients in heavily infected mosquitoes. There was, however a clear genetic contribution to sporozoite production identified, although the genetic basis for this phenotype could not be determined from the data collected to date. These results contribute to understanding the complex interaction between parasite and host, and the relative contributions from genetic and environmental factors that affect parasite development in the vector. An understanding of the contribution of these various factors is crucially important for designing and evaluating intervention studies targeted at the mosquito stage, and monitoring and modelling parasite population genetics.

6.1.1 Environmental factors

The results show that for *P. falciparum* clone 3D7 there was no association between mosquito size and prevalence, infection intensity, or genomes per oocyst. There was an observed relationship between genomes per oocyst and infection intensity in the data set that included only oocysts from single clone feeds, but this association did not remain when the 3D7 oocysts from all infections were pooled for the analysis. The association seen in the single clone infection data was highly influenced by a few single oocysts from high infection intensities, which suggests that the positive association between genomes per oocyst and infection intensity may only be apparent at high levels of oocyst burden on the midgut. There was a large variability in genomes per oocyst observed between different mosquitoes, and this demonstrated the inherent variability in mosquito susceptibility in a population of mosquitoes. Variability in mosquito susceptibility to *Plasmodium* infections has been well documented even within fairly inbred laboratory lines (Vaughan et al., 1992).

The results from *P. falciparum* clone HB3 infections showed a significant association between infection prevalence and mosquito wing size, with smaller mosquitoes having a higher probability of infection than larger ones (Chapter 3, section 3.2.1.2). Although this seems counterintuitive as one might expect larger mosquitoes to take larger bloodmeals and therefore ingest more gametocytes with a consequent higher chance of infection, this finding may be explained by mosquito immune response such that larger mosquitoes mount a better immune response to infection (Boete et al., 2002; Suwanchaichinda and Paskewitz, 1998). Data from field samples suggests that there is either no association between size and infection, or that the medium sized mosquitoes are the most likely to be infective to mammalian hosts (Hogg and Hurd, 1997; Lyimo and Koella, 1992; Mwangangi et al., 2004).

The data from comparisons between the two different *Anopheline* mosquito species (Chapter 3, section 3.2.2) provide evidence for an interesting area for further work. Although there were limited sample sizes for comparison of the prevalence and intensity of infections between the two species, there was sufficient data to suggest a possible significant difference in genomes per oocyst, which was not explained by a difference in mosquito size. *An. arabiensis* showed a large variation in genomes per oocyst counts with the highest count being nearly four times as many as the highest *An. gambiae* count (7917 to 2080). This may be explained by the difference in co-evolution between *P. falciparum* and the mosquito host, due to lower level of exposure to the parasite for the *An. arabiensis* population. *An. gambiae s.s.* mosquitoes are highly anthropophilic so come into contact with *P. falciparum* far more often than their sister species *An. arabiensis* which is an opportunistic species that can be relatively zoophilic (reviewed in (Lyimo and Ferguson, 2009)). This suggests that *An. gambiae* may have evolved a better immune response to the parasite such that it limits *P. falciparum* development.

6.1.2 Parasite environmental factors

Previous evidence (as discussed in Chapter 1) suggested that environmental factors that the parasite experiences both prior to, and after, uptake into the mosquito may influence the success of infection in the vector host. Three such factors were considered in this study: gametocytaemia, length of time the parasite had been in continuous culture, and infection intensity in the midgut.

From an initial set of pilot experiments, gametocytaemia in the bloodmeal appeared to show a logarithmic association to prevalence of infection, such that increasing gametocytaemia increased the prevalence of infection only up to a threshold gametocyataemia of about 0.5% (~25,000 gametocytes/µl), although this value varied with different parasite cultures. A minimum threshold for infection of about 30 ookinetes per blood meal has been reported (Vaughan et al., 1994b). A minimum threshold level of gametocytaemia is therefore unsurprising as there are such great losses for a variety of reasons, for example poor fertilisation, poor conversion to ookinetes, and losses across the midgut epithelium.

The results show some evidence for a positive association between gametocytaemia and infection intensity (Chapter 4, section 0). As gametocytaemia increased the infection intensity also increased but there was still a large range of intensities; in fact the range increased as the gametocytaemia increased. It seems surprising that some mosquitoes still only carry a few oocysts, even at high gametocytaemias but this may be an example of the range of susceptibility in the mosquito population. Previous studies on *P. falciparum* gametocyte densities and mosquito infectivity produced conflicting conclusions, and there are many factors (including many that were not measured within this study e.g. blood factors, asexual parasitaemia, sex ratios) that may act together to influence the infectivity of a gametocyte population (reviewed in (Sinden, 1991)).

Crowding on the midgut (increasing oocyst intensity) could cause competition for nutrients, and could therefore have a negative effect on the number of genomes per oocyst. However the data suggested a slight positive association between genomes per oocyst and infection intensity such that when there were more oocysts on the midgut, more genomes per oocyst were found. This association was only observed in the 3D7 single clone infection data and not when the mixed clone data was analysed.

A positive association between intensity of infection and parasite replication may demonstrate the range in mosquito ability to sustain infection. It could be that those mosquitoes that sustain a high density of infection also support a high productivity in parasite multiplication, although if this was the case it seems surprising not to see the same association in the mixed clone data as there were higher infection intensities in that data set.

Previous work had found no association between sporozoites per oocyst and oocyst density with NF54 *P. falciparum* and *An. gambiae* mosquitoes (Vaughan et al., 1992), although sporozoites were measured as an average number per oocyst rather than measured directly. As the range in genomes per oocyst has been demonstrated to be large when measured directly, the Vaughan study may not give an accurate picture of any association between these two variables.

Factors such as the gametocytaemia in the infectious feed, the length of continuous culture time, and the infection intensity or crowding on the midgut were considered but no evidence was found for any clear relationship between these explanatory variables and genomes per oocyst counts either in single or mixed clone *P. falciparum* infections in *An. gambiae* mosquitoes within the ranges studied.

A factor that was not considered in this study is position on the midgut. It was very difficult to record the physical position of the oocyst on the midgut but there is a suggestion that this might explain some of the variation in genomes per oocyst. Previous hypotheses are that those oocysts on the periphery of 'clumps of oocysts' are larger than those in the centre, perhaps as a result of greater access to nutrients (Sinden and Strong, 1978), although clumps of oocysts were never seen in this study. There is also an effect attributed to gravity where oocysts develop at the malpighian tubule end of the midgut (Shute, 1949).

6.1.3 Parasite genetic factors

Although there was a large variation in genomes per oocyst within a single parasite clone, a clear genetic factor was observed in the variation in genomes per oocyst between clones. *P. falciparum* clone HB3 had significantly fewer genomes per oocyst than clone 3D7 when the feeds were carried out with *An. gambiae* mosquitoes. This finding will allow the identification of genetic loci contributing to the trait of parasite replication in the mosquito through linkage analysis (or quantitative trait locus analysis) of the progeny clones of a cross between 3D7 and HB3.

This observed difference in sporozoite production was maintained when the clones were in the mixed clone environment, showing that the production of sporozoites in the oocyst is not affected by the presence of other parasite genotypes in the mosquito.

It is worth noting that several of the HB3 oocysts collected from both single and mixed clone feeds could not be fully analysed by qPCR due to the threshold level (~2 genomes) of the technique and the generally very low numbers of genomes in HB3 oocysts. This suggests that there has probably been an underestimate of the difference in genomes per oocyst between 3D7 and HB3 and that parasite replication in HB3 oocysts can be very low indeed. Several of the single oocyst samples could not be counted in quadruplicate due to threshold detection level of the technique. One possible solution to overcome this would be to try and concentrate the elution volume from the DNA extraction, which could be considered for future experiments.

There was no evidence for a difference in genomes per oocyst between 3D7 and hybrid oocysts, as discussed in Chapter 5. The results could be explained by the products of a gene or genes either on the nuclear genomes or the extranuclear elements, and may also be stage-specific in expression. A full discussion of the possible genetic explanations and ways to test each hypothesis is included at the end of Chapter 5.

In general the findings presented here suggest that in endemic areas where many genetically distinct clones are circulating, the multiplication step in the mosquito may be at the level of the better of the two parents that cross-fertilised in the midgut. This finding may influence the predicted speed at which advantageous mutations may spread through parasite populations, although this will also depend on the location of the responsible gene or genes i.e. nuclear or extranuclear.

Parasite replication could be affected by genes involved directly in DNA replication, or by genes involved in transport of required nutrients into the developing oocyst. Polymorphisms in these genes could lead to faster (or slower) parasite replication.

Although this was not something considered in this study, it may be that time to maturity could have an impact on the genotype of the sporozoites that first make it to the salivary gland, and that these might be the first to be released in an infectious bite. This might provide an explanation for bias numbers of recombinant progeny found in chimps from crosses, which could be explained by the theory that hybrid oocysts are quicker to develop and therefore the first to release sporozoites to the salivary gland. There is evidence for asynchronous release of sporozoites from *P. berghei* and *P. falciparum* (Dawes et al., 2009; Meis et al., 1992).

6.2 Conclusions

The aims of this thesis were to investigate influences on sporogony. The findings are that the mosquito environment has little effect on parasite replication, but the parasite genetic background is of primary importance, and that the replication trait may be inherited in a dominant fashion or maternally through gene/s located on the extranuclear elements.

Appendix I. Media / solutions

Thawing solution I: in ddH_20 and 0.22µm sterile-filtered; stored at 4^oC.
12 % (w/v) NaCl (BDH Laboratory Supplies)

Thawing solution II: in ddH_20 and 0.22µm sterile-filtered; stored at 4^oC.
1.6 % (w/v) NaCl (BDH Laboratory Supplies).

Thawing solution III: in ddH_20 and 0.22µm sterile-filtered; stored at 4^oC.
0.9 % (w/v) NaCl (BDH Laboratory Supplies);
0.2 % (w/v) dextrose.

Freezing solution: in ddH_20 and 0.22µm sterile-filtered; stored at 4^oC.
0.65 % (w/v) NaCl (BDH Laboratory Supplies);
3 % (w/v) D-sorbitol (Koch Light Laboratories Ltd.);
28 % (v/v) glycerol (BDH Laboratory Supplies).

Incomplete RPMI 1640 medium: in ddH_20, adjusted to pH 7.2 and 0.22µm sterile filtered; used within four weeks; stored at 4^oC.
10.4 g/l GIBCO RMPI 1640 (+ L-glutamine, -$NaHCO_3$) (Invitrogen);
5.94 g/l (25 mM) 1-piperazineethane sulfonic acid (HEPES) (Sigma);
50 mg/l (0.37 mM) hypoxanthine (Signma).

Complete RPMI 1640 medium: used before turns pink / within two weeks; stored at 37^oC.
500 ml incomplete RMPI 1640 medium;
21 ml of sterile-filtered 5 % (w/v) $NaHCO_3$ (Fisher Scientific);
ddH_20; used within one week; stored at 4^oC;
50 ml (10% v/v) sterile heat-inactivated (1 hour at 56^oC) pooled human AB serum; stored at -75^oC.

Giemsa's buffer solution: in ddH_20, adjusted to pH 7.2 to 7.4; stored at room temperature.
3 g/l (21.1 mM) Na2HPO4 (BDH Laboratory Supplies);
0.6 g/l (4.4 mM) KH2PO4 (Fisher Scientific Ltd).

Giemsa's stain: made fresh daily; stain methanol fixed blood smears for 20 mins, and rinse slides in H_20.

5 % (v/v) Geimsa's stain solution, Gurr improved R66 (BDH Laboratory Supplies);

95 % (v/v) Giemsa's buffer solution.

Glucose solution for adult mosquitoes: in ddH_20; stored at 4^oC.

5 % (w/v) D-glucose (Fisher Scientific Ltd);

0.05 % (w/v) para-amino-benzoic acid (PABA) (Fisher Scientific Ltd).

Appendix II. Summary of data collected

Prevalence and intensity of infections

Data presented in this thesis are from 32 successful infections, carried out with *P. falciparum* parasite clones 3D7 and HB3 in *An. gambiae s.s.* mosquitoes. The overall mean prevalence of infection, median intensity of infection and the number of mosquitoes dissected are presented in Table 29.

Table 29. Total number of experiments carried out with *P. falciparum* clones in *An. gambiae s.s. s.s.* mosquitoes, with the mean prevalence of infection, and the number of mosquitoes dissected also shown.

Clone fed	No. of experiments performed	Mean prevalence (range)	Median intensity (range)	No. of mosquitoes dissected
3D7	8	56% (17-88%)	1 (0-170)	161
HB3	7	21% (10-33%)	0 (0-14)	124
3D7 & HB3	17	63% (17-96%)	2 (0-225)	341

Single oocyst collections

In total, 26 experiments were carried out for the purposes of single oocyst dissection, hence the experiment number runs from 1 to 26 including both single and mixed clone feeds.

Single clone feeds

For single clone samples, single oocysts were dissected from *An. gambiae s.s.* mosquito midguts 10 days after an infectious feed with *P. falciparum* gametocytes of either 3D7 or HB3 clone. The numbers of oocysts collected are shown in Table 30.

Table 30. Number of single oocysts collected from single clone feeds with *An. gambiae s.s.* mosquitoes.

Expt #	No. of oocysts collected 3D7	No. of oocysts collected HB3
1	2	
2	2	
3		2
4		2
5		3
7	8	
8	5	3
9	7	4
10	13	
12	10	1
13		1
14	12	1
15		2
16	3	
21		1
24		2
25		1
Total	**62**	**23**

Mixed clone feeds

For mixed clone samples, single oocysts were dissected from *An. gambiae s.s.* mosquito midguts 10 days after an infectious feed with a mix of *P. falciparum* gametocytes of both 3D7 and HB3 clones. The numbers of oocysts collected are shown in Table 31.

Table 31. Number of single oocysts collected from mixed clone feeds with An. *gambiae s.s.* mosquitoes.

Expt #	No. of oocysts collected	Genotype		
		3D7	HB3	Hybrid
6	12	5	1	6
7	8	8		
8	3	3		
9	8	4		4
11	5	2	1	2
12	5	4		1
13	8	4		4
14	5	3		2
15	6	5		1
17	11	8		3
18	3		1	2
19	6		3	3
20	11			11
21	8			8
22	3			3
23	1		1	
26	1		1	
Total	**104**	**46**	**8**	**50**

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