Novel approaches to sex-ratio studies in *Plasmodium falciparum*

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ABSTRACT

Malaria parasites are able to alter the number of male and female gametocytes produced, thereby changing the sex ratio of the reproductive stages. Alteration of the sex ratio has been suggested to increase the reproductive oputput under different conditions, thereby maximizing the transmission success and influencing the epidemiology of this disease.

The only existing method for quantifying sex ratios of *P. falciparum* is based on the visual identification of male and female gametocytes by light microscopy. However, this method is limited, as it only allows studying microscopically detectable gametocyte densities found in natural infections and parasites at sub-microscopic levels are not accounted for. A new and more accurate method for estimating sex ratios of *P. falciparum*, which includes large sample sizes obtained by magnetic purification, is described here. In this study light microscopy, immunofluorescent antibody detection (IFA) and quantitative Real-Time PCR (qRT-PCR) were used to estimate sex ratios in *P. falciparum*.

The qRT-PCR assay allowed measurement of mRNA expression levels for sex-specific and sexual stage proteins in a multiplex analysis. The female specific protein Pfg377 (PFL2405c) and the putative male specific protein Alpha-tubulin II (PFD1050w) were used to quantify either female or male gametocytes, with Pfs16 (PFD0310w) and Pfs25 (PF10_0303) serving to quantify the total number of gametocytes present. Results suggest that Alpha-tubulin II is not in fact a male specific protein, whereas Pfg377 was found to be only present in female gametocytes. Different patterns of gene expression during gametocytogenesis have been identified for the four key sexual stage genes in a multiplex assay. Relative expression data of Pfg377 versus Pfs16 or Pfs25 reveal that the quantification of sex ratios from small volumes of

gametocyte-positive blood is possible, and should be applicable for using finger-prick peripheral blood samples from gametocyte carriers.

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ABBREVIATIONS

ΔCt	Delta Ct
ΔΔCt	Delta Delta Ct
3D7	Gametocyte producing strain of *P. falciparum*
ACT	artemisinin-based combination therapy
AE	Amplification efficiency
AL	Artemether plus lumefantrine
ASP	Amodiaquine plus pyrimethamine sulphadoxine
ATP	Adenosine triphosphate
AQ	Amodiaquine
AS	Artesunate
BCP	1-Bromo-3-chloropropane
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine serum albumin
cDNA	copy DNA
CM	Complete media
COT	Cotrimoxazole
CQ	Chloroquine
Ct	Cycle threshold
DAPI	4',6-diamidino-2-phenylindole
dH_2O	Distilled water
DHC	Dynein heavy chain
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleoside tri-phosphate
EPO	Erythropoietin
FACS	Fluorescent activated cell sorting
FITCI	Fluorescein isothiocyanate
g	Relative centrifugal force
gDNA	genomic DNA
GSA	Gametocyte surface antigens
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICM	Incomplete media

IFA	Immuno-fluorescent-antibody-test
IgG	Immunoglobulin G
LMC	Local mate competition
MACS®	Magnetic activated cell sorting
min.	Minutes
mRNA	messenger RNA
NAG	N-acetyl glucosamine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pcv	packed cell volume
PlasmoDB	*Plasmodium falciparum* Genome Database
PSP	Pyrimethamine sulphadoxine plus probenecid
Q	Quinine
qRT-PCR	Quantitative Real-Time PCR
QT-NASBA	Real time quantitative nucleic acid sequence-based amplification
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT-PCR	Reverse transcriptase PCR
SE	Standard error
sp.	Species
SP	Pyrimethamine sulphadoxine
SR	Sex ratio
TBE	Tris-Borate-EDTA
TBI	Transmission blocking immunity
TRITC	Tetramethyl Rhodamine Iso-Thiocyanate
UV	Ultra violet

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1 INTRODUCTION

1.1 General introduction to malaria parasites

Plasmodium, a protozoan parasite, is the causative agent of the vector borne disease malaria. This parasite is widely spread in the animal kingdom and is known to occur in animals such as reptiles, birds, rodents and primates. There are four classic species affecting humans; *P. vivax*, *P. malariae*, *P. ovale* and *P. falciparum*, the most severe form of this disease. However, zoonotic species such as *P. knowlesi*, which are transmitted from monkeys to humans via mosquitoes are now being discovered (Ollomo et al., 2009), as diagnostic methods improve.

Infections with malaria parasites are non lethal when treated promptly and appropriately and the spread of this disease can be reduced with the use of insecticide treated nets (ITNs), for example. Nevertheless, malaria infections are responsible for over one million deaths per year, mostly affecting children in Africa under the age of five (Snow *et al.*, 1999). The African continent bears a disproportionate amount of the human cost, where around two-thirds of the population are considered to be at risk (Snow *et al.*, 2005). However, malaria is a public health issue throughout the tropics and accounts for at least 300-500 million infections per year (Gardner *et al.*, 2002).

Poverty, poor infrastructure, civil wars and the emergence of drug and insecticide resistance have made it difficult to fight the battle against malaria. Also, increased air travel, has allowed this parasite to enter non-endemic countries and the emerging resistance to newer drugs such as artemisinin combination therapy (ACTs) is an alarming discovery (Noedl *et al.*, 2008). Vaccines are under development and recent reports of the RTS,S/AS02A vaccine are promising, as protection during a 45 month period in children has been shown (Sacarlal *et al.*, 2009). Combined efforts and pooled resources are necessary to evaluate the best strategies to better control this parasite. Such strategies would include the distribution of ITNs and accessibility of appropriate antimalarial drugs to people living in malaria endemic regions.

Malaria parasites belong to the phylum Apicomplexa. All apicomplexans are unicellular and parasitic, while most share an apical complex structure. This structure is present in the 'sporozoites stage' and believed to be involved in the invasion process of the host cell. Many apicomplexans also share an apicoplast, believed to have originated by endosymbiosis of an alga; however the function of this structure is still unknown.

Most apicomplexan parasites alternate between reproducing asexually and sexually, thus sharing similar life cycle strategies with each other (Roberts *et al.*, 2005). The family Plasmodiidae contains the malaria species, which all share a dipteran final host, but considerable variation in the host cell preference and the timing of asexual and sexual replication among the malaria species exist.

Studying malaria parasites in the laboratory is important to gain a better knowledge of the biology of this organism and to also allow the development of improved surveillance and control measures against this disease. However, the culturing of the asexual and especially the sexual stages is time consuming, costly and technically demanding, which makes studying this parasite in the laboratory difficult. *P. falciparum* is the only human malaria parasite that can be reliably cultured in the laboratory, but the investigation of non-human malaria parasites has greatly helped us to increase our understanding of this disease. Rodent malaria, such as *P. berghei* and *P. chabaudi*, has allowed studying this parasite in the laboratory, whereas bird and lizard malaria species have allowed us to observe malaria parasites in natural ecological systems.

Significant advances in molecular techniques have led to the sequencing of genomes of several malaria species, thereby facilitating studying these parasites on a molecular level. Freely available Genomic Databases such as PlasmoDB have contributed to the advancement of malariology. However, novel molecular methods are still needed for monitoring these parasites in natural infections. No method currently exists that allows the accurate quantification of the male and female sexual stages, which are responsible for the transmission of this disease. The development of such techniques is pivotal for understanding the reproduction dynamics of this parasite.

1.2 *Plasmodium falciparum* life cycle overview

Malaria parasites are transmitted to humans during the feeding of an earlier infected female *Anopheles* mosquito, where the human serves as the intermediate host (asexual reproduction) and the mosquito as the final host (sexual reproduction), see figure 1.1.

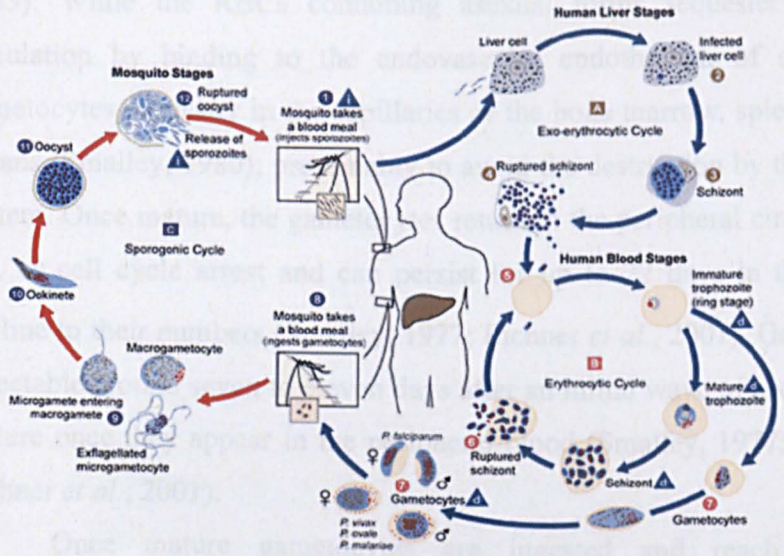


Figure 1.1: Life cycle of *P. falciparum*. The asexual blood stages are the merozoites (invasion), trophozoites (feeding) and schizonts (cell division) stages, whereas the sexual blood stages are the gametocytes (sexual reproduction). The blue arrows reveal the human stage (intermediate host) and red arrows reveal the mosquito stage (final host). Adapted from the CDC website (http://www.dpd.cdc.gov/dpdx).

During the feeding process mature sporozoites, ranging in number from 10 to 100,000, are injected from the salivary glands into the blood stream of its human host (Sherman, 1998). Once sporozoites enter the circulatory system, they circulate to the liver invading parenchyma cells where they mature into asexual intracellular hepatic schizonts (pre-erythrocytic cycle).

After an average of six days, the schizonts rupture and one schizont can release up to 30,000 merozoites into the blood stream. From here begins the asexual blood stage that causes the disease malaria (erythrocytic cycle), with each cycle taking 48 hours. Once the merozoites enter the erythrocytes, they develop into trophozoites, which subsequently undergo schizogeny, releasing each up to 32 merozoites 48 hours after invasion. Multiple rounds of this asexual reproductive phase take place, rapidly building up the population of malaria parasites within the human host, leading to the

pathology of this disease. Schizonts are committed to release either asexual or sexually determined merozoites (Bruce *et al.*, 1990), which will subsequently either undergo another round of asexual reproduction, or form the sexual stages - gametocytes.

All sexually committed parasites from one schizont will either form into male or female gametocytes (Bruce *et al.*, 1990; 1996; Smith *et al.*, 2000; Silvestrini *et al.*, 2005). While the RBCs containing asexual forms sequester from the peripheral circulation by binding to the endovascular endothelium of organs, the immature gametocytes sequester in the capillaries of the bone marrow, spleen, and possibly other organs (Smalley, 1980); presumably to avoid the destruction by the reticulo-endothelial system. Once mature, the gametocytes return to the peripheral circulation and remain at G_1, or cell cycle arrest and can persist for up to 22 days in the blood without any decline to their numbers (Smalley, 1977; Eichner *et al.*, 2001). Gametocytes are usually detectable around seven to eleven days after an initial wave of asexual parasites and are mature once they appear in the peripheral blood (Smalley, 1977; Smalley *et al.*, 1977; Eichner *et al.*, 2001).

Once mature gametocytes are ingested and reach the mosquito gut, gametogenesis (gametocyte activation) is triggered by a combination of factors. Such factors include a decline in temperature, and other mosquito factors which the gametocytes encounter inside the gut of their final host. After around ten minutes of gametocyte activation, the crescent, or 'sausage' shaped sexual stages form into a round shape, during which the gametocytes break free from the red blood cell (RBC). During this activation process the female gametocyte transforms into one macrogamete, whereas the male gametocyte undergoes three rounds of mitotic division, forming eight microgametes. The release of the microgametes, which contain mobile flagella, is referred to as exflagellation. Once a microgamete encounters a macrogamete, they fuse together, during which fertilization occurs and the zygote forms. Each zygote then undergoes meiotic division, forming into an ookinete with four haploid nuclei. This motile cell crosses the mosquito's midgut wall, enters the haemocoel and develops into an oocyst on the outer side of the stomach. The oocysts then undergo sporogony over a period of 10 to 14 days depending on ambient conditions and host species (Sherman, 1998).

Once mature, the oocyst ruptures, releasing sporozoites into the haemocoel from where they migrate to the salivary glands of the mosquito. Approximately 2000 sporozoites are produced from a single zygote. Compared to the large number of

sporozoites found in the salivary gland of an infected mosquito, only few are injected into the intermediate host during the mosquito's blood meal. Interestingly, mosquitoes infected with sporozoites have shown to have a more persistent feeding behaviour, than those not infected, thereby possibly manipulating their final hosts to increase the chances of transmission to the intermediate host (Koella *et al.*, 1996).

1.3 Gametocytogenesis

1.3.1 Overview of gametocytogenesis

Gametocyte development is referred to as the process of gametocytogenesis. This developmental step begins when a sexually committed merozoite forms into a gametocyte (Smith *et al.*, 2000), which then undergoes five developmental stages (see figure 1.2), before they are activated to form into either one (macrogamete) or approximately eight gametes (microgametes) inside the gut of a mosquito.

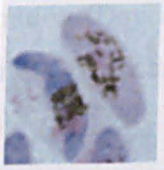
Stage	I	II	III	IV	V
					
Characters	Indistinguishable from trophozoite, granules appear darker	Parasites elongates within the erythrocyte, becomes D shape	Parasites are D shaped, distorted erythrocyte	Elongated and thin parasite	Crescent shaped parasites
Timing	0-2	1-4	2-8	6-9	7-23
Cell cycle	G1	G1	G0	G0	G0
Location	Sequestered	Sequestered	Sequestered	Sequestered	Peripheral circulation

Figure 1.2: Identification of the five gametocyte stages. *P. falciparum* gametocyte characters, development in days of natural infections and the localization during gametocytogenesis are shown. Pictures were taken on day 1, 3, 5, 7 and 11 from cultures and were prepared on thin film slides stained with Giemsa. The picture of stage V parasites reveals the purple stained female gametocyte on the left and the pink stained male gametocyte on the right. Adapted from Sinden *et al.*, 1983 and Talman *et al.*, 2004.

The five developmental stages (I-V) of *P. falciparum* were first described from induced infections in *Aotus* monkeys (Hawking *et al.*, 1971). The most obvious characteristic of mature gametocytes is their distinctive elongated, rounded shape. From stage II onwards gametocytes develop a rigid, crescent shape; compared to the amoeboid shape of the asexual stages. The subpellicular membrane develops from late stage I onwards and is located underneath the parasitophorous vacuole membrane (Sinden, 1982). Electron micrographs revealed that gaps within this membrane were found to be filled with microtubular networks (Sinden, 1983) and it is these microtubules that are likely to account for the crescent shape (Sherman, 2005). Mature gametocytes are more 'rounded' in shape in contrast to the acicular (needle shaped) shape present in the early sequestered gametocyte stages. It has therefore been hypothesized that the rounded shape facilitates the gametocytes' release into the peripheral circulation, whereas the acicular shape may aid in the sequestration (Nacher, 2004).

Laboratory studies have shown that when *P. falciparum* is cultured over longer periods, the parasites may lose the ability to undergo gametocytogenesis (Bhasin *et al.*, 1984). A 0.3 Mb region of chromosome 9 was identified to be involved in gametocytogenesis, as deletions in this region affected gametocyte formation (Day *et al.*, 1993; Alano *et al.*, 1995c), leading to the assumption that this region plays a vital role in triggering sexual development.

1.3.2 Sexual commitment of parasites

Previously it was believed that gametocytogenesis only occurs after a few rounds of asexual reproduction; however one recent study has shown that gametocyte mRNA was already present after one single round of erythrocytic schizogeny (Schneider *et al.*, 2004). It may be possible that in seasonal transmission areas, the timing of gametocyte induction has been shaped by natural selection and that certain parasite lineages may produce gametocytes earlier than others.

It is still not clear what exactly triggers the switch from asexual to sexual development. Several potential local environmental factors that cause 'stress' to the parasites have been suggested to increase gametocyte numbers *in vivo* and *in vitro*. Such factors include high parasitemia (Bruce *et al.*, 1990), chemotherapeutic drugs (Ono *et al.*, 1993), anaemia (Nacher, 2002; Trager, 1992), hormones such as insulin and progesterone (Lingnau *et al.*, 1993), immune responses from the vertebrate host (Ono *et*

al., 1986) and the beginning of the transmission season (Rosenberg *et al.*, 1990). It is likely that these parasites respond to signal pathways, as it has been suggested that cAMP-dependent protein kinase pathways are responsible for triggering gametocytogenesis, as parasites lose the ability to undergo gametocytogenesis when a substantial decrease in cAMP-dependent protein activity has been observed (Kaushal *et al.*, 1980; Read *et al.*, 1991).

Gametocyte production has been shown to be highly variable, as some isolates continuously have high gametocytes conversion rates compared to others (Alano *et al.*, 1990), while dissimilar gametocyte conversion rates were observed between clones and even among isolates (Graves *et al.*, 1984). It has been suggested that commitment to gametocytogenesis may be genetically predisposed, but can respond to environmental stimuli (Reece *et al.*, 2008). This implies that different gametocyte isolates are likely to be more successful by producing certain gametocyte densities, but have the plasticity to change this according to the changing environment of the hosts.

In the laboratory *P. falciparum in vitro* cultures have revealed that when asexual parasites multiply at high densities, gametocyte production is limited. However, as soon as asexual proliferation decreases, gametocyte production increases (Dyer *et al.*, 2003), see figure 1.3. It has been proposed that asexual multiplication represses the induction of gametocytogenesis and that production of the sexual stages is therefore highly regulated.

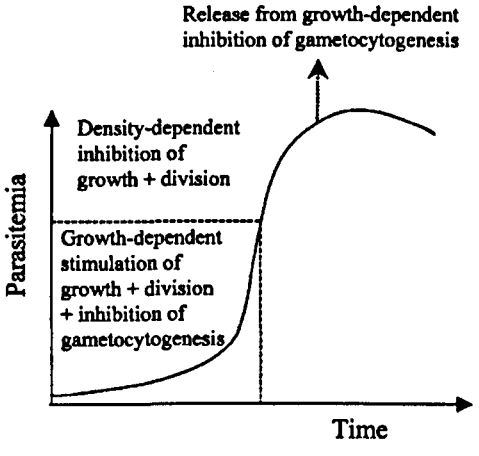


Figure 1.3: Growth curve of *P. falciparum in vitro*. Representation of asexual and sexual growth of malaria parasites over time, suggesting that sexual reproduction is dependent on asexual density (Dyer and Day, 2003).

It is not fully clear why *P. falciparum* produces such low densities of gametocytes in comparison to the large densities of asexual parasites. One theory suggests that low gametocyte numbers may be due beneficial due to immune responses targeting gametocytes specifically, as low gametocyte numbers will elicit a lower immune response and may therefore not be so easily destroyed. High asexual parasitemia may serve as a shield, to reduce the destruction of gametocytes by the hosts immune system (Taylor *et al.*, 1997a). Little is known about gametocyte mediated immunity, however one recent paper supports this theory by identifying plasma antibodies that recognize gametocyte surface antigens (GSA), which were associated with lower gametocyte densities (Saeed *et al.*, 2008). It has also been reported that there is a trade off between investment in the sexual stages and mosquito mortality, as ingestion of high gametocyte densities can affect the survival rate of the host, as it has been reported that the oocyst may destruct the abdominal organs inside the mosquito host (Klein *et al.*, 1986). From this observation it can be suggested that low gametocyte numbers would increase the chances of transmission.

Observations from natural infections have shown that in seasonal transmission areas, gametocyte densities are very low and only peak with the onset of the wet season (Ouedraogo *et al.*, 2008). This suggests that a sudden increase in gametocyte density may be advantageous, as the immune system has not been exposed to gametocytes during the dry season and gametocytes are less likely to be affected by the hosts immune system when this increased gametocyte production occurs (Taylor *et al.*, 1997a). Therefore regulating the timing and number of parasites that commit to the sexual stages must play an important role for maximizing transmission.

Whether competition increases gametocyte commitment is unclear, as human hosts co-infected with *P. malariae* and *P. falciparum* have shown an increase in gametocyte commitment of *P. falciparum in vitro* (McKenzie *et al.*, 2002), whereas other studies did not show such an increase of gametocyte production *in vivo* (Wargo *et al.*, 2007; Bousema *et al.*, 2008). Different host constraints may shape competition in different ways and innate and environmental factors may both play a role in triggering gametocytogenesis.

1.4 Differences between male and female gametocytes

1.4.1 Sex determination

It is known that sex chromosomes are not involved in the sexual differentiation process of the malaria parasites, as a haploid parasite clone can form into a haploid sexual stage (Carter *et al.*, 1988). Also, DNA extracted from asexual and sexual stages showed no difference in the number of chromosome-size DNA molecules (Van der Ploeg *et al.*, 1985), therefore sex determination is likely to occur by differential gene expression, resulting in the expression of mutually exclusive sexual traits.

Since male and female gametocytes play very different roles in the process of fertilization, it is therefore logical that they vary on an ultrastructural level. The most distinct visual difference is that female gametocytes are slightly larger than male gametocytes and more acicular shaped, which can often lead to them being confused with stage IV male and female gametocytes. On a cellular level, female gametocytes store large amounts of untranslated RNA, contain more osmiophilic bodies and carry larger amounts of ribosomes and endoplasmic reticulum (Janse *et al.*, 2004). These ribosomes are likely to be needed for later fertilization of the macrogamete and contain many nucleic acids. It is also the large amounts of ribosomes present in the female gametocyte that allows distinguishing between the sexes (see section 1.4.3 for further information).

1.4.2 Identification of male and female gametocytes

In 1880 Charles Louise Alphonse Laveran identified an exflagellating gametocyte in a blood smear taken from a febrile patient and as a result of this discovery the link between parasite and disease was established (Bruce-Chuvatt, 1981). In many settings, the 'gold standard' for the enumeration of malaria parasites still relies on the visual method of light microscopy. In contrast to the advanced techniques available to quantify asexual and sexual parasites of *P. falciparum* (Babiker *et al.*, 2008b), sexing gametocytes *in vivo* still relies on the presence of five characters determined by light microscopy (Carter *et al.*, 1988), see figure 1.4 :

1.) Males are smaller than females.
2.) The nucleus is larger in males than in females.
3.) The ends of the cells are round in males and more angular in females.
4.) The cytoplasm of males stains pink and that of the females purple.
5.) The granules of the malaria pigment are centrally located in females and more widely scattered in males.

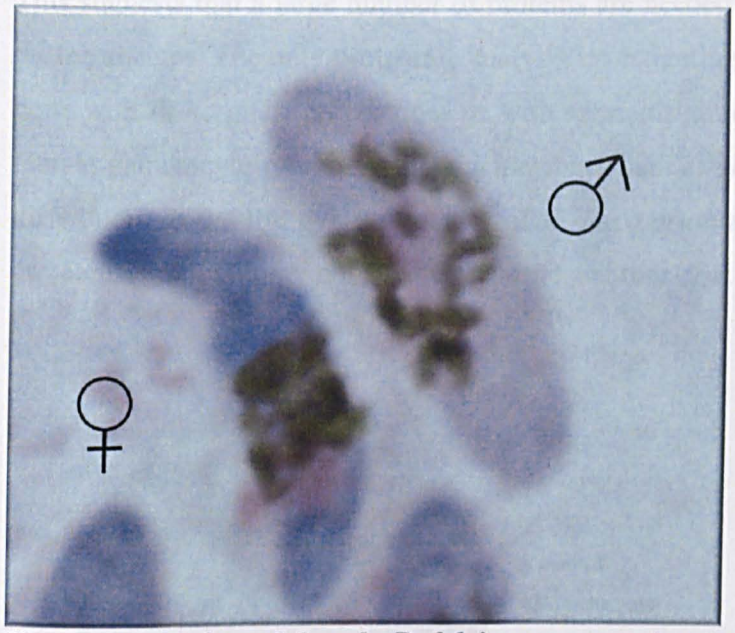


Figure 1.4: Male and female *P. falciparum* gametocytes from a day 11 culture. The male gametocyte (pink) is seen on the right and the female gametocyte (purple) can be seen on the left. Pictures were magnified x 1000 and were taken from thin film slides stained with Giemsa.

When stained correctly, male gametocytes stain pink and female gametocytes stain purple. Mature female gametocytes contain substantially more ribosomes than male gametocytes (Janse *et al.*, 2004). The purple staining of female gametocytes occurs because ribosomes are basophilic, thereby being more receptive of hydrogen ions, compared to neutral or acidic substances found in male gametocytes and thus causing the Giemsa to stain in different colours in male and female gametocytes (Shaw *et al.*, 1996). Nonetheless, sexing gametocytes by eye, is time consuming and subjective and cannot account for gametocytes below submicroscopic levels (see chapter 5 for further information).

Individual *P. falciparum* gametocytes from *in vitro* cultures however have been sexed with alternative methods at low densities, such as: electron microscopy (Sinden, 1982), *in situ* hybridization (Baker *et al.*, 1995), immunoelectron microscopy (Alano *et*

al., 1995b) and IFA (Immuno fluorescent antibody test) (Bruce *et al.*, 1990; Rawlings *et al.*, 1992; Severini *et al.*, 1999; Silvestrini *et al.*, 2000a; van Schaijk *et al.*, 2006; de Koning-Ward *et al.*, 2008).

1.4.3 Male and female gametocyte proteomics

Proteomic analysis of *P. falciparum* identified more stage specific proteins in gametocytes than in any other life cycle stage (Lasonder *et al.*, 2002), see figure 1.5. This suggests that a large number of proteins are needed for the sexual development of these parasites. The only proteomic analysis investigating sex specific proteins has been done with *P. berghei* and provides us with some insight on the differences of male and female gametocyte proteins in *P. falciparum* (Khan *et al.*, 2005). However, due to the differences in the life cycle strategies of *P. falciparum* and *P. berghei,* the *P. berghei* proteomic analysis may not accurately represent that of *P. falciparum*.

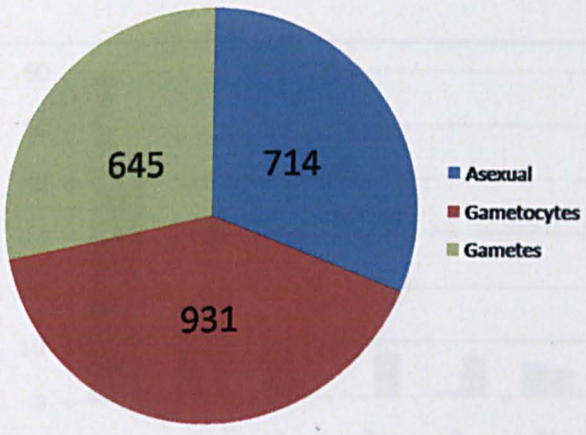


Figure 1.5: Protein distribution in *P. falciparum.* Representation of the number of proteins observed using high accuracy mass spectrometric analysis of *P. falciparum*, taken from Lasonder *et al.*, 2000.

Khan *et al.* 2005 were able to identify sex specific proteins by separating transgenic male and female gametocytes by flow cytometry; following this the gametocytes separate proteomes were analyzed using mass spectrometry. This study identified approximately 650 sex specific proteins in male gametocytes and approximately 541 proteins in female gametocytes. Of these proteins, 36% were male specific and 19% were female specific and a large number of proteins were found to be hypothetical proteins. Female gametocytes were found to be richer in ribosome and mitochondrial peptides, whereas the male gametocytes contained more proteins involved in DNA replication and those associated with axoneme and flagella and motor molecules, such as dyneins, see figure 1.6. A large number of sex specific kinases and phosphatases were also identified, which are often associated with signal transduction pathways, suggesting that the male and female gametocytes may have different modes of signalling.

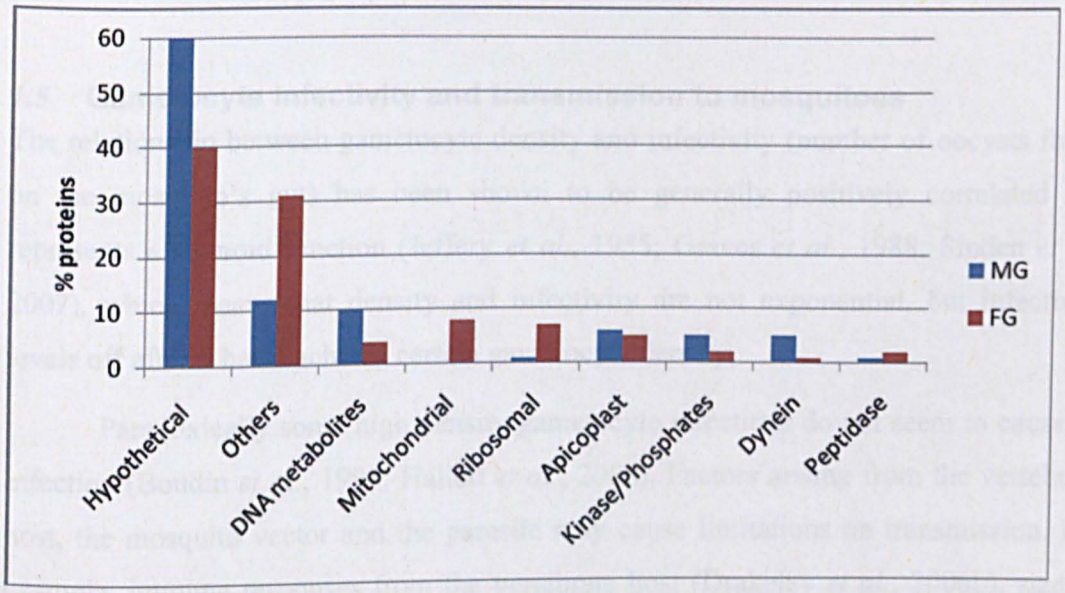


Figure 1.6: Distribution of male and female proteins in *P. berghei* gametocytes. The majority of identified proteins were found to be predicted (hypothetical); however a large number of proteins could not be grouped, taken from Khan *et al.*, 2005. MG stands for male gametocyte and FG stands for female gametocyte.

Overall the proteomic analysis coincides with the different function gametocytes serve in the preparation for the formation of gametes, as male gametocytes need to prepare for three rounds of mitosis, and the gamete production, whereas female gametocytes need to prepare for meiosis and post zygote growth in the mosquito midgut (Janse *et al.*, 2004). To this date less than a handful of sex specific proteins have been studied in *P. falciparum*. Khan's study however has discovered a large number of novel proteins that were found to have orthologs in *P. falciparum*. In *P. falciparum*, the only protein described to be strictly male specific is Alpha-tubulin II (PfB0400w), which has recently been reported to also occur in the female gametocytes and the asexual stages (Khan *et al.*, 2005; Kooij *et al.*, 2005; Fennell *et al.*, 2008) and only three female specific proteins have been studied Pfg377 (Alano *et al.*, 1995b; Severini *et al.*, 1999), Pf77 (Baker *et al.*, 1995) and Pfs47 (van Schaijk *et al.*, 2006). Proteomic studies that investigate sex specific proteins are needed to gain more insight into the sexual development and biology of this parasite.

1.5 Gametocyte infectivity and transmission to mosquitoes

The relationship between gametocyte density and infectivity (number of oocysts found on the mosquito's gut) has been shown to be generally positively correlated and represents a sigmoid function (Jeffery *et al.*, 1955; Graves *et al.*, 1988; Sinden *et al.*, 2007), which means that density and infectivity are not exponential, but infectivity levels off after it has reached a certain gametocyte density.

Paradoxically some high density gametocyte infections do not seem to cause an infection (Boudin *et al.*, 1993; Hallett *et al.*, 2006). Factors arising from the vertebrate host, the mosquito vector and the parasite may cause limitations on transmission. For example, immune responses from the vertebrate host (Drakeley *et al.*, 2006b), size of the mosquito (Lyimo *et al.*, 1992), and reproductive limitations on the number of ookinetes may affect transmission (Al-Olayan *et al.*, 2002). Combinations of complex factors are likely to influence transmission, which have made it difficult to understand the dynamics behind it.

Several field studies have observed that gametocyte density is highest in infants and decreases with age in endemic transmission areas yet, in areas where transmission intensity is low, adults may be the main source of transmission, but gametocytemia does not directly reflect infectivity (Boudin *et al.*, 1993; Drakeley *et al.*, 2006a). Host immunity to these asexual parasites explains this phenomenon, as infants have not been exposed to asexual parasites in high transmission areas, whereas adults have build up immunity due to previous exposure. However, adults may play a more important role in low transmission areas, as they are more likely to pass on gametocytes (Paul *et al.*, 2003), as behavioural aspects of adults (increased exposure at night time) are also likely to play a role. In low transmission areas submicroscopic levels of gametocytes are commonly found in asymptomatic carriers and may be responsible for maintaining transmissions occurring in these areas (Shekalaghe *et al.*, 2007).

The vast majority of gametocytes are never taken up by mosquitoes and are broken down in the human host and naturally acquired transmission blocking immunity (TBI) can reduce or even fully block transmission (Mendis *et al.*, 1987; Mulder *et al.*, 1994). The development of vaccines which induce TBI are currently being investigated and may aid in the control of malaria parasites in the future (Saul, 2007). The identification of gametocyte surface antigens (GSAs), could also serve as novel targets for transmission blocking vaccines (Saeed *et al.*, 2008). Sexual stage proteins, especially Pfs230 and Pf48/45, have been particularly well studied, as antibodies against these proteins have been shown to have transmission blocking effects and may be of interest for the future development of vaccines (Roeffen *et al.*, 1996; Healer *et al.*, 1999).

1.6 Gametogenesis

Once gametocytes have entered the mosquito gut, gametogenesis is triggered. This process is triggered by a combination of factors, such as reduction in temperature, plus the presence of a mosquito factor (including xanthurenic acid), which has shown to trigger exflagellation (Billker *et al.*, 1998), also the downstream activation of a calcium dependent protein kinase is required for many of the underlying events (Billker *et al.*, 2004). Recently it has been shown that specific inhibition of the parasite cGMP-dependent protein kinase prevents the initial rounding up step of *P. falciparum* gametogenesis. Chelation of intracellular calcium did not prevent rounding up

suggesting that a hierarchy of signalling events are needed for the induction of gametogenesis (McRobert *et al.*, 2008). Several pathways are believed to regulate RNA and protein synthesis, of which some are repressed by translational control in the gametocytes until gametogenesis is triggered, as with Pfs25 for example (Paton *et al.*, 1993). This translational repression allows a large number of proteins to be rapidly synthesized, needed for the process of fertilization.

Distinct morphological changes can also be observed soon after gametogenesis is triggered; male and female gametocytes lose their crescent shape and become completely rounded in shape, while turning into micro- and macro- gametes respectively. Male gametes have been observed to form exflagellation centres, which can be visualized as an aggregation of microgametes, likely caused by the sticky nature of the proteins found on the flagella. These centres are believed to aid in drawing the stationary female gametes towards them. Once the gametes have touched, their plasma membranes fuse, allowing fertilization and formation of a diploid zygote (Sinden, 1983), which will undergo meiosis and several mitotic steps to continue its development inside the mosquito.

1.7 Sex ratios

1.7.1 Introduction of sex ratio studies

Evolutionary theory suggests that where fitness and investment of male and female individuals are equal, species will tend towards producing equal numbers of males and females, thereby maximising the reproductive output (Fisher, 1958; Charnov, 1982). However, even slight relative differences in the fitness or cost of the two sexes should produce a dramatic shift in the sex ratio (Charnov, 1993).

Plants, mammals, reptiles, insects and disease causing agents like malaria are capable of allocating different amounts of resources into one sex of the offspring than to the other to maximize their reproductive output. Evolutionary sex ratio theories have been widely studied in the animal kingdom (Hardy, 2002) and allow us to understand selection pressures that shape sex ratios and thereby influencing the organism's reproductive success.

In nature many different forms of sex ratio manipulation exist, for example, some parasitic wasps can adjust the sex ratio of their offspring according to the size of their offspring's host, thereby maximizing investment into the offspring (Charnov,

1982). With these wasps, host size does not affect fecundity in the males, but larger females can produce more eggs, therefore these parasitic wasps benefit from laying female eggs into larger hosts. Also, some mite species are known to manipulate the sex ratio of their offspring according to population density, food availability and according to the seasonal changes, allowing them to adapt to the continuously changing environmental circumstances (Hardy, 2002). Sex allocation has also been reported to occur in higher vertebrate animals, such as in red deer, where population density affects the sex ratio of the offspring (Kruuk *et al.*, 1999), or in rhesus monkeys where the mother's status has been hypothesized to influence the sex ratio of her offspring (Simpson *et al.*, 1982).

1.7.2 Introduction to *P. falciparum* sex ratio studies

In malaria parasites, the sex ratio is calculated as the total number of male gametocytes observed, divided by the total number of male and female gametocytes observed, thereby revealing the proportion of males. In *P. falciparum,* one merozoite will give rise to either one male gametocyte, which produces approximately eight gametes, or one female gametocyte which produces a single gamete (Inselburg, 1983; Silvestrini *et al.*, 2000a). Smith *et al.,* (2000) suggested that the sex of male and female gametocytes must be equally costly in their production, as the number of gametocytes produced per sexually-committed schizont was similar for both sexes. This study used an immuno fluorescent antibody test (IFA) plaque assay and was thereby able to determine the number of male and female gametocytes emerging from one schizont. Assuming cost and fitness are equal in the male and female gametocytes, one would anticipate a female biased sex ratio, of eight female to one male gametocyte, or a sex ratio of approximately 0.1. However, sex ratios of malaria parasites can vary greatly among isolates, clones, and over the course of an infection (Graves *et al.*, 1984; Alano *et al.*, 1990; Sowunmi *et al.*, 2009).

For example, *P. falciparum* gametocyte sex ratios observed in natural infections in Nigeria have been found to range from being almost completely female biased (e.g. the sex ratio = 0.044) to being almost entirely male biased (e.g. the sex ratio = 0.69) (Sowunmi *et al.*, 2009), see figure 1.7. It is not yet fully understood why such variation of sex ratios of malaria parasites exist, but since sex ratios can influence the transmission success (Burkot *et al.*, 1984; Robert *et al.*, 1996a; Schall, 2000; Mitri *et al.*, 2009), it is important understanding the factors that shape the sex ratio dynamics of

these parasites. Also, identifying methods that may shift the sex ratio in an unfavourable way may be of interest for the development of better malaria intervention strategies, as in the form of transmission blocking drugs, for example.

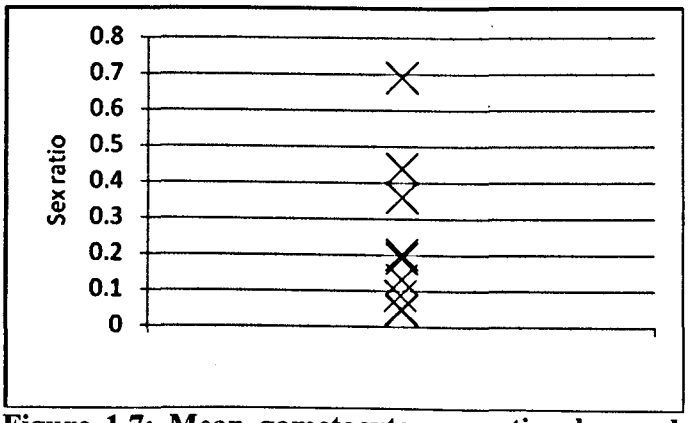


Figure 1.7: Mean gametocyte sex ratio observed from natural infections. These gametocyte sex ratios were observed at enrolment in a Nigerian study. This graph represents the variation of gametocyte sex ratios observed in natural infections; see Sowunmi *et al.*, 2009.

1.7.3 Factors influencing *Plasmodium* sex ratios

In vitro studies revealed that sex ratios can be clone specific, as some sex ratios varied among clones taken from the same isolate, whereas others were found to maintain their sex ratio even after several rounds of sub-culturing (Burkot *et al.*, 1984).

Osgood *et al.*, 2002, revealed that in *P. mexicanum* sex ratios are inheritable. This study was done by replicating natural infections using infecting naïve lizards and thereby showing that the sex ratios remained the same in both hosts, suggesting that the sex ratios are based on a genetic component. Conversely, another study has revealed that sex ratios can be manipulated with environmental stimuli. Paul *et al.* (2000) showed that erythropoietin (EPO), a hormone that triggers erythropoiesis during anaemia, has a direct effect on increasing the sex ratio in *P. vinckei* and *P. gallinaceum*. These parasites can react to environmental stimuli (anaemia) and change their sex ratio accordingly. In summary, studies have shown that the changes of sex ratios differ:

- due to the presence of other genotypes within an infection (*in vitro* and *in vivo*) (Schall, 1989; Schall, 1996; Drew et al., 2007; Reece et al., 2008)
- among different genotypes (*in vitro* and *in vivo*) (Read *et al.*, 1995; Reece *et al.*, 2008)
- depending on the parasitemia (*in vivo*) (Pickering *et al.*, 2000)

- in relation to anaemia/host immune responses (*in vitro*) (Paul *et al.*, 2000)
- with drug treatment (*in vitro*) (Schall, 1996; Sowunmi *et al.*, 2003b; Sowunmi *et al.*, 2008a, b; Sowunmi *et al.*, 2008c; Sowunmi *et al.*, 2009)
- over the course of an infection (*in vitro* and *in vivo*) (Osgood *et al.*, 2002; Paul *et al.*, 2002b; Robert *et al.*, 2003; Reece *et al.*, 2008).

1.7.4 Local mate competition theory

One sex allocation theory that has been well studied in the fig pollinating wasp has now also been applied to malaria parasites. This theory has been termed 'local mate competition' (LMC), which predicts that where siblings must compete to mate, a sex ratio (proportion of males) bias will develop towards the females to maximize the reproductive output (see figure 1.8). However, when outbreeding (cross fertilization) is likely to occur a more male biased sex ratio will be advantageous (Hamilton, 1967) to maximize the reproductive output relative to the 'competing clones'. Such sex allocation will allow maximizing the number of the F2 (second filial generation) generation.

In the case of the fig pollinating wasps, the sex ratio of the offspring will be more female biased when the female wasp lays her eggs into a fig that has not been previously fertilized, contrary a more male biased sex ratio will be observed if the female wasps lays her eggs into a previously fertilized fig (Hamilton, 1967; Herre, 1985). Read *et al.*, 1992 were the first to apply LMC theory to *P. falciparum* parasites from Papua New Guinea. Since malaria infections are either monoclonal (containing one genotype), or polyclonal (containing several genotypes), it was hypothesized that a gametocyte sex ratio would be more male biased in the presence of other genotypes, just as it has been observed with the fig pollinating wasp, see figure 1.8. Read's study predicted correctly that in areas where transmission is low, most infections were monoclonal and female biased. Selfing rates in Papua New Guinea were later confirmed by using molecular techniques and revealed that sex ratios correlate with the number of clones within an infection (Paul *et al.*, 1995). Soon after these studies, gametocyte sex ratios of *P. falciparum* were studied in Cameroon, a high transmission area, and as predicted, it was observed that the sex ratio was less female biased, correlating with the higher numbers of polyclonal infections (Robert *et al.*, 1996a).

A recent paper by Reece *et al.*, 2008 is the first study were rodent hosts where infected with different *P. chabaudi* genotypes to manipulate the parasites sex ratios. As predicted, the sex ratios observed were more male biased in the presence of other genotypes. This study was done using a novel quantitative Real-Time PCR (qRT-PCR) assay that quantifies sex specific mRNAs of *P. chabaudi,* thereby revealing the sex ratios of the parasites observed. From these results Hamilton's formula, 'F=1-2r', which was established in 1976, where 'r' equals the sex ratio and 'F' reveals the inbreeding rate, can now be used to elucidate whether a monoclonal or a polyclonal infection is present within the host and may provide us with vital information of malaria population structures (Hamilton, 1967; Reece *et al.*, 2008; Schall, 2009), see figure 1.9.

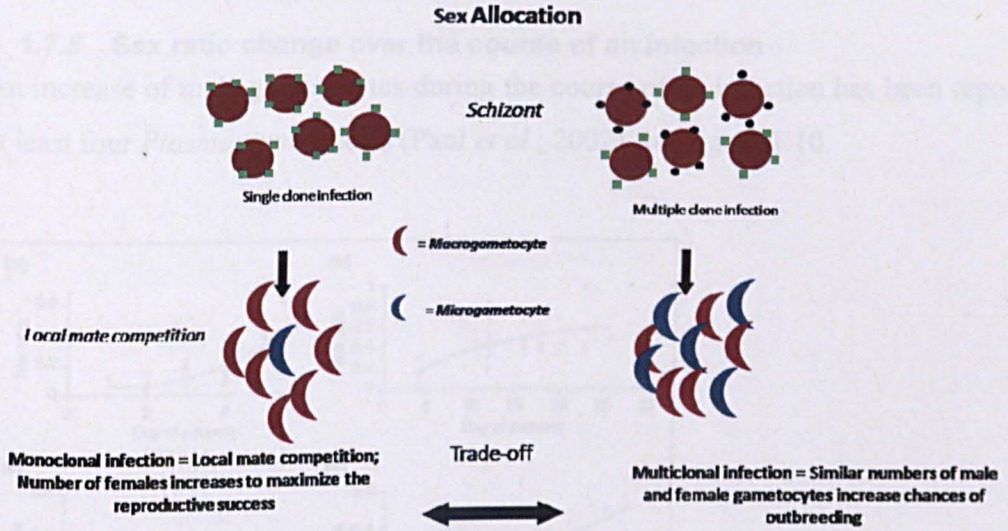


Figure 1.8: Local mate competition theory in *Plasmodium.* Schematic diagram illustrating the differential investment into offspring of monoclonal versus polyclonal infections. Local mate competition has been hypothesized to occur when related parasites are present within an infection, allowing maximizing the reproductive output for that clone in the F2 (the second filial generation), relative to the other clones.

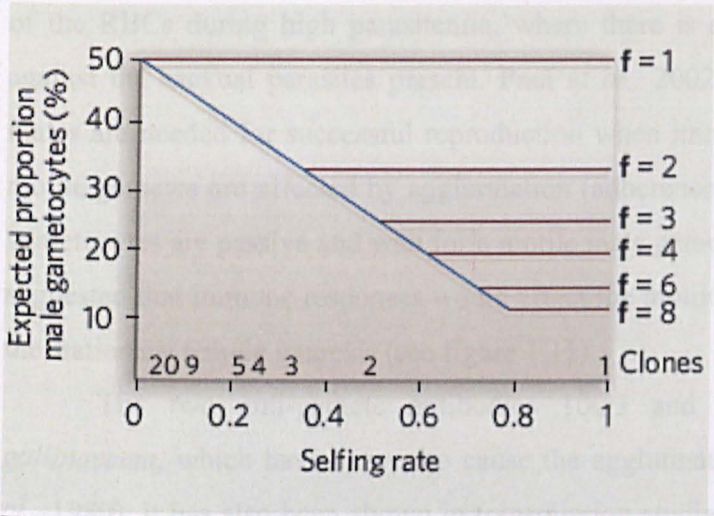


Figure 1.9: Selfing rates and sex ratios. Representing the sex ratio theory from Hamilton (1967), where 'f' equals the fecundity of male gametocytes and the expected sex ratio is shown, taken from Schall *et al.*, 2009.

1.7.5 Sex ratio change over the course of an infection

An increase of male gametocytes during the course of an infection has been reported in at least four *Plasmodium* species (Paul *et al.*, 2002), see figure 1.10.

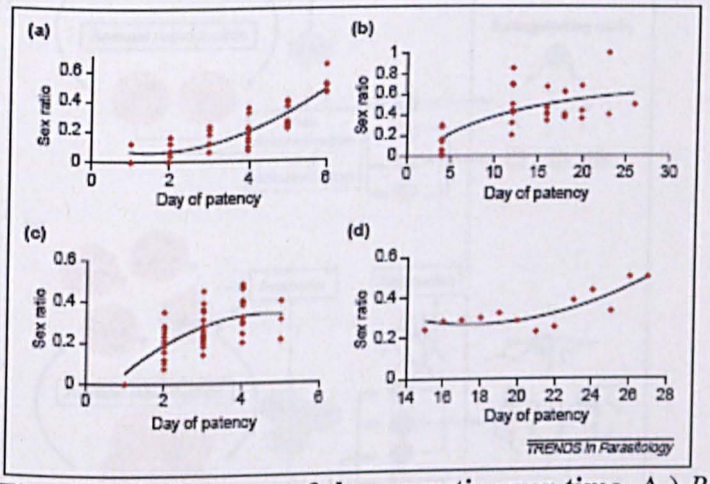


Figure 1.10: Increase of the sex ratio over time. A.) *P. vinckei*, B.) *P. berghei*, C.) *P. gallinaceum* and D.) *P. falciparum,* taken from Paul *et al.*, 2002.

It is not yet clear why the sex ratio becomes more male biased over the course of an infection. In 2000 Paul *et al.*, found that natural and artificial induction of erythropoiesis in the vertebrate host, chickens in this case, caused an increase in male gametocytes. EPO is naturally released by the body during anaemia and signals to the body that more RBCs need to be produced. In malaria infections anaemia is caused by the destruction

of the RBCs during high parasitemia, where there is also a strong immune response against the asexual parasites present. Paul *et al.*, 2002 therefore suggested that more males are needed for successful reproduction when immune responses are high, as the motile gametes are affected by agglutination (adherence) with antibodies. Since female gametocytes are passive and wait for a motile male gametocyte to encounter, it has been suggested that immune responses would affect the motile male stages more compared to the stationary female gametes (see figure 1.11).

The two anti-gamete antibodies 10G3 and 11C7 were identified in *P. gallinaceum*, which have shown to cause the agglutination of male gametes (Rener *et al.*, 1980). It has also been shown in transmission studies of *P. falciparum*, that a more male biased sex ratio was more infective (Robert *et al.*, 1996a), which supports Paul's theory that more males are needed for fertilization in during high parasitemia infections. No study has yet provided any evidence of sex specific immune reactions mediated more towards one of the sexes (Read *et al.*, 1992; Paul *et al.*, 1995; Read *et al.*, 1995).

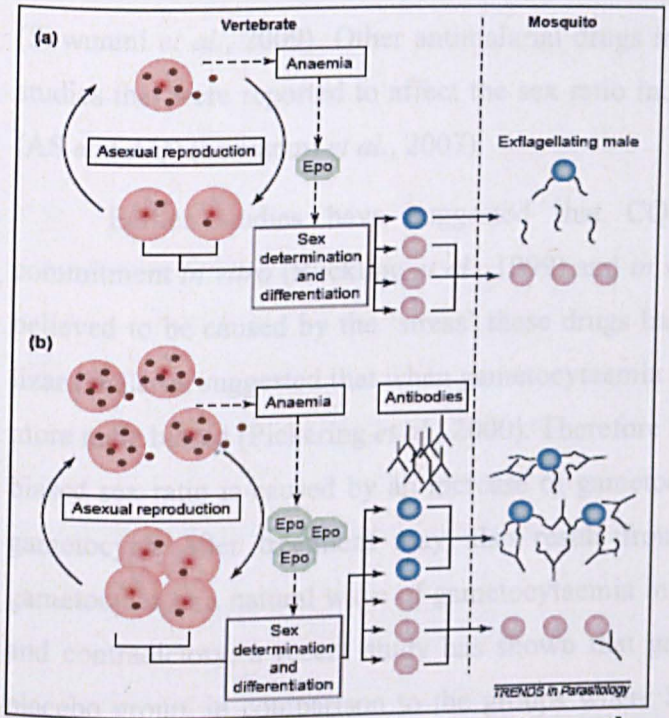


Figure 1.11: Possible effects of immunity on the sex ratio. Schematic representation explaining why the sex ratio may become more male biased over the course on an infection. A.) Representation of an infection where anaemia and immune responses are low, B.) Hypothesis suggesting that when anaemia and immune responses increase, fertilization is affected by the increased agglutination of antibodies of the male gametes, taken from Paul *et al.*, 2002.

1.7.6 Antimalarials and sex ratios

The effects of drug treatment on the gametocyte sex ratio have recently become of increased interest (Sowunmi *et al.*, 2003b, a; Talman *et al.*, 2004; Sowunmi *et al.*, 2005; Sowunmi *et al.*, 2007; Sowunmi *et al.*, 2008a, b; Sowunmi *et al.*, 2008c; Stepniewska *et al.*, 2008; Kar *et al.*, 2009; Sacarlal *et al.*, 2009; Sowunmi *et al.*, 2009). Two *in vitro* studies reported no effects of CQ in relation to the gametocyte sex ratio of rodent malaria (Reece, 2003; Talman *et al.*, 2004), however, several *P. falciparum in vivo* studies have suggested that antimalarial drugs may affect the gametocyte sex ratio.

Drugs that have been proposed to cause a shift in the sex ratio include, ACTs (Sowunmi *et al.*, 2009), amodiaquine (AQ) (Sowunmi *et al.*, 2007; Sowunmi *et al.*, 2008a, 2009), artesunate (AS) (Sowunmi *et al.*, 2009), cotrimoxazole (COT) (Sowunmi *et al.*, 2005; Sowunmi *et al.*, 2009), CQ (Sowunmi *et al.*, 2003a) and pyrimethamine sulphadoxine (SP) (Sowunmi *et al.*, 2003b; Sowunmi *et al.*, 2005; Sowunmi *et al.*, 2009). All drugs were described to cause an increase in the sex ratio, except treatment with AS and ACTs, which have been suggested to cause a more female biased sex ratio (Sowunmi *et al.*, 2009). Other antimalarial drugs investigated in *P. falciparum in vivo* studies that were reported to affect the sex ratio included: artesunate (AS) and AS-AQ (AS and AQ) (Sowunmi *et al.*, 2007).

Earlier studies have suggested that CQ treatment increases gametocyte commitment *in vitro* (Buckling *et al.*, 1999) and *in vivo* (Talman *et al.*, 2004), which is believed to be caused by the 'stress' these drugs have on the parasites. A study, using lizard malaria, suggested that when gametocytaemia increases the sex ratio may become more male biased (Pickering *et al.*, 2000). Therefore it may be possible that a more male biased sex ratio is caused by an increase of gametocytaemia. However, an increase of gametocytes after treatment may also result from the release of the sequestered gametocytes or a natural wave of gametocytaemia in an acute infection (Butcher, 1997) and contradictory, a recent study has shown that gametocyte load was highest in the placebo group, in comparison to the groups where SP or SP in combination with AS were administered (Dunyo *et al.*, 2006). It should also be noted that CQ interferes with the natural EPO production (el Hassan *et al.*, 1997), and this is may be a reason why no change of sex ratio was observed during CQ treatment (Paul *et al.*, 2002b).

Also, ACTs have been suggested to have the opposite effect compared to other antimalarial drugs, by decreasing the gametocyte prevalence (Strickland *et al.*, 1986; Dutta *et al.*, 1989; Price *et al.*, 1996; Sutherland *et al.*, 2005) and the administration of the artemisinins have been reported to suppress transmission (Drakeley *et al.*, 2004). Yet one study suggested that ACTs do not have a complete effect on transmission, due to the duration of the gametocyte carriage and the submicroscopic gametocytaemia that can still lead to transmission (Bousema *et al.*, 2006). It is possible that Sowunmi *et al.*, (2009) observed a more female biased sex ratios of *P. falciparum* in the presence of ACTs, because no drastic increase of gametocytaemia occurred, which would support Pickering *et al.*, (2000) findings, that the sex ratio is influenced by the gametocytaemia. To this date it is not fully clear what effects antimalarial drugs have on the sexual stages due to the contradictory data observed.

Sowunmi *et al.*, 2009 hypothesized that antimalarial drugs may affect the longevity of the gametocytes, thereby leading to the different half-lives of the male and female gametocytes, which lead the sex ratios to become more male biased during the course of an infection. This study also suggested that there was a negative relationship between anaemia and the sex ratio observed at enrolment, suggesting that anaemia and therefore EPO production may also shape sex ratios in human malaria. Theoretically it is possible for drugs to have differential effects towards one sex of the gametocytes. SP for example, affects DNA synthesis, which could possibly affect male gametocytes more than female gametocytes, as the males undergo three rounds of mitotic divisions before fertilization (Sherman, 1998).

So far, all studies suggesting that certain drugs may affect the sex ratio have been studied by light microscopy and at times only 70% of gametocytes were sexed (Sowunmi, Balogun *et al.* 2009), which may have led to the analysis of unreliable data. If the missed 30% of gametocytes have a sex bias (e.g. all males, or females), this would have a drastic shift in the measured sex ratio, in that the sex ratio may be observed to be more male biased than it actually is, see figure 1.12. Novel molecular techniques are needed to reliably quantify sex ratios from natural infections to elucidate the effects antimalarials drugs may have on the transmission stages.

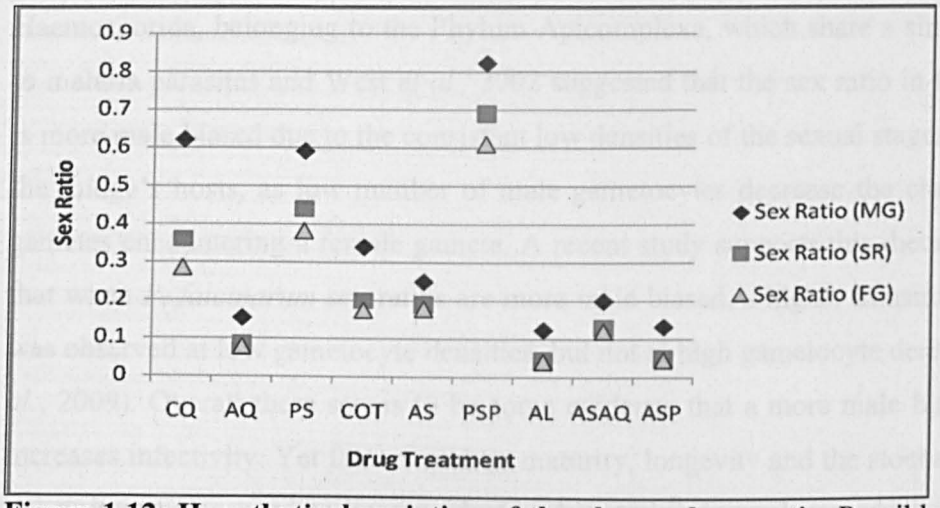


Figure 1.12: Hypothetical variation of the observed sex ratio. Possible variations of observed sex ratios. Gametocyte mean sex ratios (SR) are shown from patients before enrolment (Sowunmi *et al.,* 2009). SR is the mean sex ratio observed, the sex ratio (MG) reveals the sex ratio if all unsexed gametocytes were male and the sex ratio (FG), reveals the sex ratio if all non-identified gametocytes where accounted for as females.

1.7.7 Sex ratio and infectivity to mosquitoes

Studies using *P. falciparum* (Burkot *et al.*, 1984; Robert *et al.*, 1996b), *P. mexicanum* (Schall, 2000) and *P. gallinaceum* (Paul *et al.*, 2000) have found that infectivity of mosquitoes increases when the sex ratio is more male biased, yet another study did not find such a correlation (Noden *et al.*, 1994). The observations that an increase in infectivity occurs when the sex ratios become more male biased supports Paul's theory, as more males are needed to successfully find a female for fertilization to occur in the presence of large numbers of antibodies (Paul *et al.*, 2000).

A study using *P. chabaudi* found that mixed clone infections yielded higher numbers of oocysts than single clone infections (Taylor *et al.*, 1997b), which may have been caused by a more male biased sex ratio, frequently observed in polyclonal infections (Reece *et al.*, 2008). Therefore, mixed clone infections could be more infectious, as more males are produced to increase the fecundity. It was also shown that a lowered antibody concentrations, in comparison to the absence of antibodies, actually increased the transmission success (Ponndurai, 1987), possibly increasing the chances of the gametes forming clusters that are similar to exflagellation centres, thereby increasing the chances of encountering a female gamete.

Haemosporida, belonging to the Phylum Apicomplexa, which share a similar life cycle to malaria parasites and West *et al.*, 2002 suggested that the sex ratio in these parasites is more male biased due to the consistent low densities of the sexual stages found within the midge's hosts, as low number of male gametocytes decrease the chances of male gametes encountering a female gamete. A recent study supports this theory, as it found that when *P. falciparum* sex ratios are more male biased, a higher transmission success was observed at low gametocyte densities, but not at high gametocyte densities (Mitri *et al.*, 2009). Overall there seems to be some evidence that a more male biased sex ratio increases infectivity. Yet factors such as maturity, longevity and the stochastic nature of transmission also need to be considered when trying to evaluate which factors might play a role on the transmission of this parasite.

Collecting reliable data on transmission studies from natural infections is difficult, due to the clustering nature of gametocytes within the host (Pichon *et al.*, 2000). Clustering may also explain some of the differences observed in relation to gametocyte density and gametocyte infectivity. Finger prick blood samples may not represent the same gametocytaemia and sex ratio as it would be obtained from a mosquito feeding in nature. Clustering of gametocytes may further be an adaptation to increase the chances of the mosquito picking up more than one clone with one blood meal, thereby increasing the chances of outbreeding.

1.8 Molecular detection of gametocytes

Since the completion of the *Plasmodium falciparum* genome project in 2002 (Gardner *et al.*, 2002), further significant advances in the fields of *Plasmodium* proteomics and genomics have been made. Genomic databases have been made freely available online, allowing international researchers to access wider amounts of information more easily and thereby providing a vital tool for bioinformatics studies. In the field of malaria research, PlasmoDB (http://plasmodb.org/) is the main reference database. The latest release, PlasmoDB 5.5, contains information on six *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. yoelii*, *P. berghei*, *P. chabaudi* and *P. knowlesi*) detailing also information on the different stages of the life cycles of these parasites.

Data available from PlasmoDB include genomic sequence retrieval, gene/protein expression profiles, identification of SNPs (Single Nucleotide Polymorphisms), identification of GO (Gene Ontology) annotations, identification of orthologs/homologs,

and the identification of genes that target, or belong to certain domains (Aurrecoechea *et al.*, 2009). Understanding changes in gene expression of the malaria parasite is important, as genomic sequence data are meaningless without understanding which genes are expressed in which time frame. Recent studies have significantly improved our understanding of protein expression in malaria parasites (Florens *et al.*, 2002; Lasonder *et al.*, 2002; Hall *et al.*, 2005; Khan *et al.*, 2005). Targeting sexual stage proteins has become of interest for the development of transmission blocking drugs and vaccines, for example (Chotivanich *et al.*, 2006; Wu *et al.*, 2008).

A wide range of qRT-PCR assays that quantify mRNA levels from the sexual stages of malaria parasites have been developed in recent years (Babiker *et al.*, 2008b). It is now possible to detect and quantify gametocytes at sub-microscopic levels (Schneider *et al.*, 2007; Shekalaghe *et al.*, 2007). Accurate monitoring of gametocytaemia for example, is important for understanding the effects drug treatment has on the transmission of this parasite (Jeffery *et al.*, 1955; Babiker *et al.*, 2008a; Barnes *et al.*, 2008) and qRT-PCR assays offer an attractive alternative to microscopy.

Despite the critical role gametocyte sex ratios play in relation to transmission and the evolution of *P. falciparum*, sex ratio theories are still poorly understood and sexing gametocytes still relies on the visual identification of the five characters described by Carter *et al.*, (1988). Obtaining large sample sizes from *in vivo* studies along with the difficulty of sexing gametocytes by eye has left the relationship between gametocyte sex ratios and transmission elusive. To this date no assay currently exists that quantifies sex ratios of any human malaria parasite. Studying sex ratio dynamics in the field would provide vital information on transmission dynamics and particularly whether drug treatment affects transmission. In addition, monitoring sex ratios will reveal information on population dynamics, which may be important for monitoring the spread of drug resistance, for example.

The recent advancement of molecular techniques has allowed an increase in the sensitivity and accuracy of parasite detection. These sensitive techniques are especially important for gametocyte detection, as they occur at much lower densities than asexual parasites. Gametocytes frequently occur at submicroscopic levels, and thus molecular techniques are more effective than microscopy for detection of these elusive transmission stages (Shekalaghe *et al.*, 2007). A meta-analysis of 42 studies found that on average 50.8% of the prevalence of infections was only detected by microscopy in comparison to

PCR prevalence (Okell *et al.*, 2009). This study included asexual parasites which are far more abundant and therefore easier to detect compared to gametocytes.

Identifying patients that have sexual stage parasites at submicroscopic levels is important for malaria surveillance, as these can still lead to transmission (Schneider *et al.*, 2006) and submicroscopic gametocyte carriers exist at all levels of endemicity (Abdel-Wahab *et al.*, 2002; Shekalaghe *et al.*, 2007). Submicroscopic gametocyte levels are common after treatment and studying the effects of drugs, such as ACTs on gametocytes is important and can only be done on a large scale with molecular techniques, such as QT-NASBA (real time quantitative nucleic acid sequence-based amplification) (Bousema *et al.*, 2006; Oesterholt *et al.*, 2009).

Babiker *et al.*, 1999 showed for the first time that by using RT-PCR and targeting Pfs25 a more accurate detection of gametocytes was possible. Detection limits were 1-2 gametocytes per microlitre, which would be the equivalent to examining 1000 fields of a thick blood smear, taking around 50 minutes. In 2004, QT-NASBA was developed, which exhibits the same accuracy for gametocyte detection (Schneider *et al.*, 2004). Reece *et al.*, 2008 combined existing methods (Drew *et al.*, 2007) by quantifying the number of gametocytes from specific genotypes and also quantifying the ratio of male to female gametocytes within the infection. This was achieved by using a relative quantitative approach lacking absolute measurement of mRNA abundance using qRT-PCR of *P. chabaudi* parasites. The assay was based on targeting a common gametocyte gene (PB000198.00.0) and the male gametocyte gene (PB000791.00.0), which is a putative dynein heavy chain first described by Khan *et al.*, 2005 in *P. berghei* (see Chapter 4).

2 RATIONALE AND AIMS OF THIS STUDY

2.1 Rationale

Studying *P. falciparum* sex ratios is important, as sex ratios have been reported to influence the transmission success (Burkot *et al.*, 1984; Robert *et al.*, 1996a; Mitri *et al.*, 2009), population genetics (Reece *et al.*, 2008; Schall, 2009) and thereby further influence the epidemiology of this disease. Environmental factors, such as the host's drug treatment (Sowunmi *et al.*, 2003b; Sowunmi *et al.*, 2008a, 2009) have also been suggested to affect the gametocyte sex ratio. Identifying factors that can shift the sex ratio in an unfavourable way to obstruct transmission could be of great interest to serve as a transmission blocking strategy.

The only existing method that allows the quantification of male and female gametocytes of *P. falciparum in vivo* is standard light microscopy. Gametocytes occur at low densities in natural infections and thin film slides need to be prepared with special precautions to allow differential staining of the sexes. Sex determination is time consuming, subjective and requires a trained eye. Recent molecular studies and the advancement of molecular techniques have allowed the rapid and accurate quantification of parasites *in vivo* below microscopic levels (Babiker *et al.*, 1999; Schneider *et al.*, 2004; Babiker *et al.*, 2008a; Reece *et al.*, 2008).

Yet gametocyte sex ratios remain elusive, due to the lack of a reliable quantification assay. It is therefore necessary to establish an assay that will allow the accurate and sensitive quantification of *P. falciparum* gametocyte sex ratios from natural infections. This assay can then be used to evaluate sex ratios from patients and help to evaluate what effects anti-malarial drug (and possibly other drugs) treatments have on the gametocyte sex ratio.

2.2 Aims of this study

1. **The primary rationale of this project is to establish a sensitive qRT-PCR assay that allows quantifying male and female *P. falciparum* gametocytes from peripheral blood samples taken from natural infections.**

A) To investigate 'novel' sex specific proteins to allow gametocyte sex ratio quantification using a qRT-PCR assay.

Hypothetical sex specific dyneins, previously only described in *P. berghei* (Khan *et al.*, 2005), will be investigated to use as targets for the qRT-PCR assay. These dyneins will be investigated in cultured parasites. See chapter 4 for further information of the investigation of dyneins in *P. falciparum* gametocytes.

B) To establish gametocyte sex ratios from purified 3D7 gametocytes using light microscopy.

Sex ratios will be first established by standard light microscopy using cultured stage V gametocytes, during which a purification process will allow establishing sex ratios from high gametocyte densities. The sex ratios measured by light microscopy will allow validating the observed sex ratios from the IFA (see chapter 5) and will later allow to 'calibrate' the relative expression data established by the qRT-PCR assay (see chapter 6). See chapter 5 for further information on sexing gametocytes by light microscopy.

C) To establish gametocyte sex ratios from purified 3D7 gametocytes using IFA.

Protein expression of Pfg377 (female gametocyte) and Alpha-tubulin II (male gametocyte) will be observed in cultured parasites during gametocytogenesis using IFA. This will allow observing the expression patterns of one sexual stage and one sex specific protein from purified gametocytes. From these observations it will be determined at what stage sex ratios can be measured using IFA. This will be the first time IFA will be used for sex ratio quantification with high gametocyte densities and activated gametocytes. The sex ratios measured using

IFA will allow obtaining information of the sex specificity of Pfg377 and Alpha-tubulin II and will also allow comparing the sex ratio data obtained using light microscopy. Therefore light microscopy and IFA sex ratio data will allow to 'calibrate' the relative expression values obtained through the qRT-PCR assay (see chapter 6). See chapter 5 for the investigation of sexual stage and sex specific proteins using IFA.

D) To develop a qRT-PCR assay that quantifies relative expression values of sexual stage and sex specific proteins.

The assay will be developed to target transcription levels of several sexual stage (Pfs16, Pfs25 and Alpha-tubulin II) and one sex specific protein (Pfg377) during gametocytogenesis of cultured parasites. These transcription levels will provide relative expression data that can be related back to the sex ratios determined by light microscopy and IFA. See chapter 6 for the development of the qRT-PCR assay.

E) To use the developed qRT-PCR assay to analyze samples from natural infections.

The qRT-PCR assay will be used to measure sex ratios from peripheral blood samples from gametocyte positive patients collected in The Gambia in 2001. See chapter 6 for the application of the qRT-PCR assay from natural infections.

3 MATERIALS AND METHODS

3.1 Overview of materials and methods

Three main methods were used for the development of novel techniques for the quantification of gametocyte sex ratios. These methods include: light microscopy (using Giemsa stain), IFA and qRT-PCR, see figure 3.1. See Appendix 8.1 for the buffers, solutions and media used in the following experiments. See attached CD for IFA gametocyte images (IFA folder) and the Excel sheets for the quantification of the relative expression (Relative expression folder).

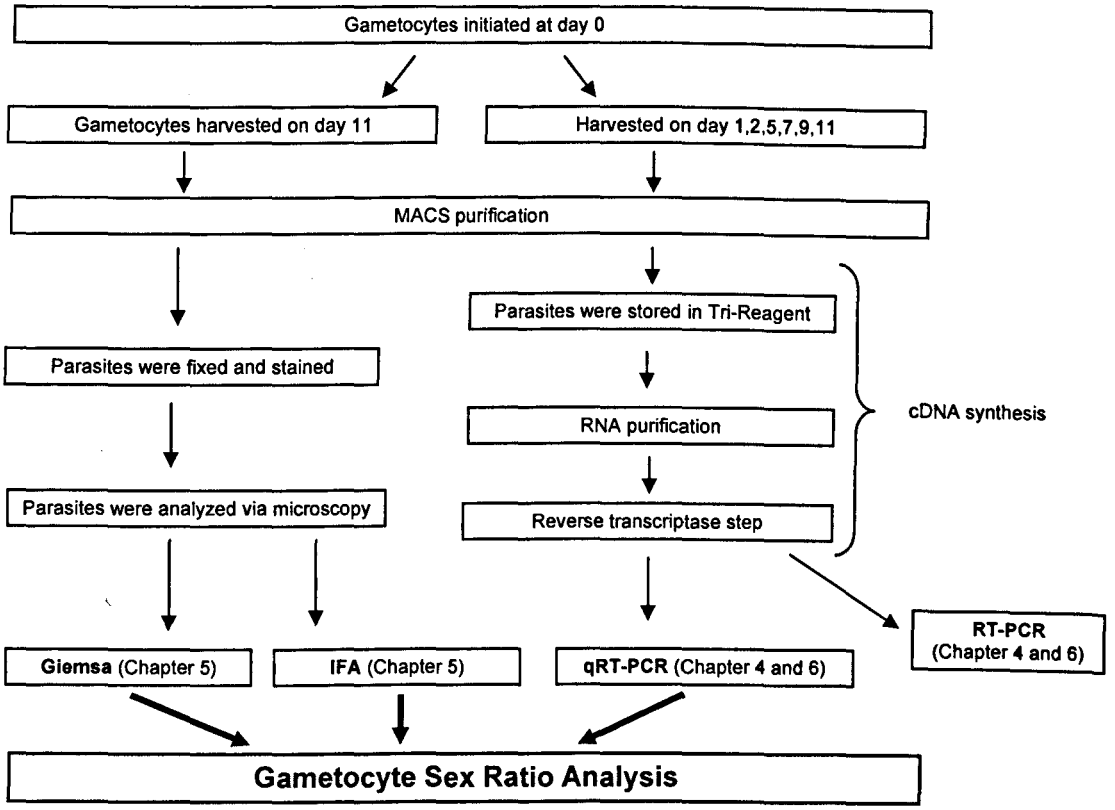


Figure 3.1 Schematic overview of the methods used for the sex ratio analysis of *P. falciparum*. Gametocytes were cultured, harvested between day 0 and day 11 and MACS® purified. Parasites were either used for microscopy (light microscopy and IFA), or for RT-PCR or qRT-PCR.

3.2 *P. falciparum* culture

3.2.1 Parasite culture

Parasites were cultured from the *P. falciparum* cell line 3D7A (MRA-151; MR4-Malaria Research and Reference Reagent Resource Centre, Manassas, VA, USA) using the standard methods with slight modifications (Trager *et al.*, 1976; Carter *et al.*, 1988; Fivelman *et al.*, 2007). Parasites were maintained in T75 cell culture flasks (Iwaki, Japan) containing AB+ erythrocytes (donated by the author), and complete media (CM). Media was warmed to 37°C for culturing and otherwise stored in the fridge for up to five days at 4°C. Cultures were incubated at 37°C and gassed for 1 minute every day (3% O_2, 4% CO_2, N_2; BOC). Parasites were kept between 0.1-15% parasitemia at a haematocrit of 2-5%. In order to enhance the invasion of the merozoites and to prevent high numbers of double infections, parasites at the trophozoite/schizont stage were placed onto a shaker overnight.

3.2.2 Preparation of erythrocytes

AB+ blood was washed at least twice with an equal volume of cold incomplete media (ICM) at 2500 x g for 5 minutes at 4°C. Each time the buffy coat, containing leucocytes and platelets, was removed and the supernatant containing plasma was discarded. The blood pellet was then resuspended in 50% ICM and stored at 4°C for no longer then 5 days.

3.2.3 Thawing cryopreserved parasites

Cryopreserved parasites were removed from the liquid nitrogen tank, transported in a small container containing liquid nitrogen, and were rapidly thawed at 37°C in a water bath. Samples were transferred into a sterile 15ml tube and an equal volume of thawing solution 1 (see appendix for details on the thawing solution) was added slowly drop by drop, and samples were centrifuged at 500 x g for 5 minutes. The supernatant was discarded and 5ml of the thawing solution 2 was added to the pellet to prevent osmotic lysis. The pellet was centrifuged as before, and the supernatant was discarded. Finally, 5ml of thawing solution 3 was added to the pellet and centrifuged as before. The pellet was washed twice in ICM and resuspended in CM at a 5% haematocrit and maintained as previously described.

3.2.4 Cryopreservation

When cultures were mainly synchronous and had a parasitemia higher than 5% rings, they were suitable for cryopreservation. Parasites were transferred to a 10ml tube, centrifuged at 500 x g for 5 minutes at 20°C, the supernatant was removed and replaced (slowly, drop by drop, mixing gently between each drop) with the pre-warmed freezing solution (see appendix for details on the freezing solution), which was then transferred into a 1.5ml screw-capped ampoule (Nunc). Ampoules were stored in liquid nitrogen at 196°Cfor long term storage.

3.2.5 Parasite monitoring

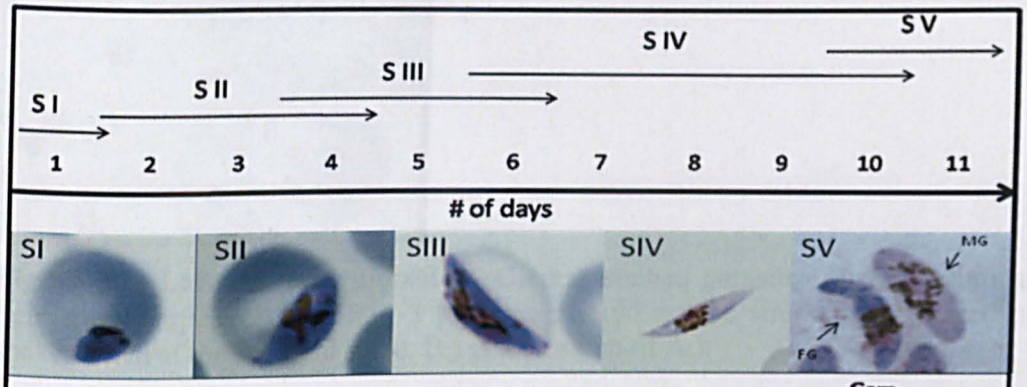
In order to maintain parasite cultures, thin blood smears were taken daily from each flask. Approximately 20µl of the culture containing a 50% haematocrit was spread on a clean glass microscope slide and air dried. Blood films were methanol-fixed before being stained with 10% Giemsa (BDH). Slides were washed off with water and air dried (this time using a normal hair drier to speed up the drying process). For the examination of parasites a drop of immersion oil (Zeiss, Germany) was placed on the slide and parasites were examined under the x 100 objective (oil immersion lens) of the light microscope containing a x 10 magnified eye piece (Leitz, Germany), allowing to magnify parasites x 1000. The percentage parasitemia was then determined using a counting graticule with 2 squares, where all RBC's inside the small square ($1/4^{th}$ in size of the large square) were counted, this including any RBC's touching two previously specified sides of the square. All the parasitized cells were then counted within the big square. Counts were repeated in different fields until over 200 RBC's were counted in total. The number of parasitized cells was then divided by all RBC's multiplied by nine and the total value was further multiplied by 100, this percentage revealed the parasitemia of a culture.

3.2.6 Synchronization of asexual parasites

Parasites were only synchronized when a mixture of ring and trophozoite stages were present. In this case, parasites were incubated for 5 minutes at 37°C with 3.5 volumes of 15% D-sorbitol solution. Parasites were then gently vortexed (breaking the mature parasites RBCs) for 1 minute and washed twice in pre-warmed ICM and maintained as described before. This allowed obtaining synchronized ring stage cultures.

3.2.7 Growing gametocytes

A culture was always started with freshly thawed 3D7A parasites, as they have higher conversion rates of asexual parasites that have been cultured over longer periods. Fresh blood was added (maximum 2 days old) to the parasites and a low parasitemia was maintained to avoid stressing parasites too early. Parasites were monitored daily; CM was exchanged and gassed at the same time every day. When parasitemia exceeded 15%, CM was changed in the morning and in the early evening. Growing gametocytes requires at least 13 days, of which 11 days are needed for the gametocytes to mature, see figure 3.2.



Stage	Day	Parasitemia	Hematocrit	# of flasks	# of flasks	Gam. Gas?	NAG?
Troph.	-3	2%	2%	2	4	No	No
Rings	-2	10-15%	1.5-3%	2	4	No	No
Troph.	-1	3%	5%	10	16	No	No
Rings	0	10-18%	3%	10	16	Yes	Yes
I/dying troph.	1	10-15%	3%	10	16	Yes	Yes
I/II/dying troph.	2	10-15%	3%	10	16	Yes	Yes
II/dying troph.	3	10-15%	3%	10	16	Yes	Yes
II/III	4	3-8%	3%	10	16	Yes	Yes
III	5	3-8%	3%	10	16	Yes	Yes
III/IV	6	3-8%	3%	10	16	Yes	No
IV	7	3-8%	3%	10	16	Yes	No
IV	8	3-8%	3%	10	16	Yes	No
IV	9	3-8%	3%	10	16	Yes	No
IV/V	10	3-8%	3%	10	16	Yes	No
V	11	3-8%	3%	10	16	Yes	No

Figure 3.2: Overview of gametocyte culture from day -3 to 11. Diagram shows the optimal parasitemia, haematocrit, number of possible flask produced, and when gametocyte gas and NAG should be used for culturing.

Day -3: Trophozoites

When a parasitemia of 2% trophozoites was obtained in at least two flasks, containing 1.5ml in packed cell volume (pcv) of RBCs, 50ml of CM was added (3% haematocrit) and parasites were placed onto a shaker overnight to maximize invasion of the parasites. Parasites must look relaxed and should not be stressed at this stage, which can be seen as the trophozoite stage fills out most of the RBC and does not stain very dark during the staining process. It is best to place parasites at the late trophozoite stage or early schizont stage on the shaker to minimize stress, see figure 3.3 for a late trophozoite stage.

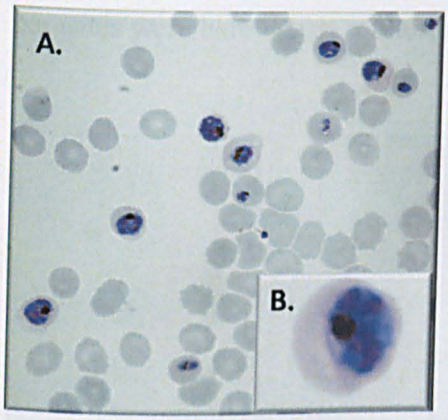


Figure 3.3: Late stage trophozoites. Giemsa stained parasites observed through the light microscope x 1000. Day -3 parasites should not be stressed and placed on the shaker when at the schizont stage. B.) is a close up of A.).

Day -2: Rings

Cultures (ring stages) were kept at 3% haematocrit and between 2-15% parasitemia. Parasites were only synchronized when a large number of trophozoites stages were present (> 5 %). Parasites were fed with 50ml of CM and gassed, see figure 3.4.

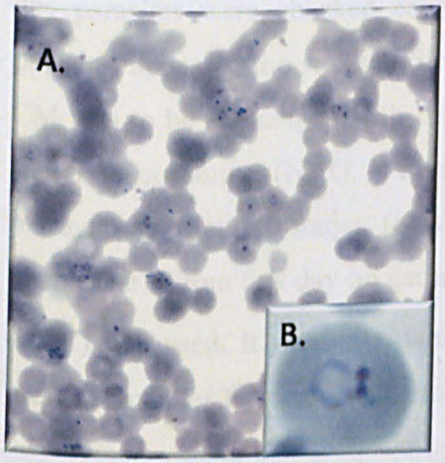


Figure 3.4: Ring stage parasites. High parasitemia is needed for day -2. A.) was taken x 1000 and B.) is a close up of A.).

Day -1: Trophozoites

Parasites (trophozoite stage) were subbed down to a maximum of 3-4% containing 750µl pcv of RBC, 30-40ml of CM and 10-20ml old CM, which was kept from the same flask and filled to a total of 50ml of CM per flask. How much old CM was added was dependent on how stressed the parasites looked (the darker the parasites stain, the less old CM should be added, also the dark colour of the CM of the culture is an indication of whether the parasites are stressed). Parasites were then placed onto the shaker overnight. When having four culture flasks at day -3 it is possible to obtain 16 flasks at day 11. Parasites at this stage must not look stressed (see figure 3.5) and if parasites were below 10%, culturing had to be moved back to day -4.

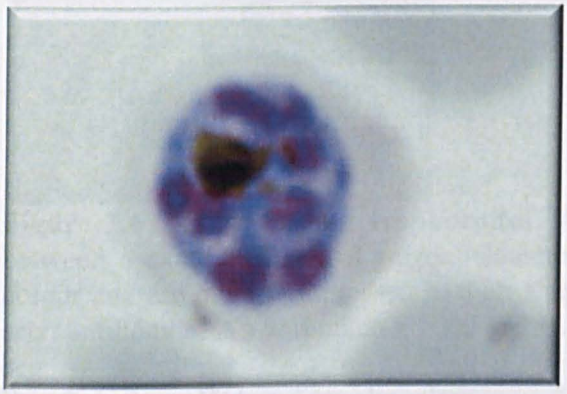


Figure 3.5: Immature schizont. Ideally parasites are placed on the shaker overnight when they are at the schizont stage to maximize the parasitemia for the next day.

Day 0: Ring stage

Parasites (ring stage) were checked for parasitemia and should ideally be >15%. On this day parasites were checked early in the morning and late in the afternoon. When parasites appeared too stressed (depending on parasite shape and colour of media), new CM was to be exchanged with the old media. CM from this day on contained 1 x N-acetyl glucosamine (NAG – Sigma-Aldrich, UK) at a concentration of 50 mM to remove the asexual stages (prevents the merozoites from penetrating the plasma membrane). From this day on gametocyte specific gas containing: 1% O_2, 3% CO_2, N_2 (BOC) was used. It was important from now on to minimize the time of handling to prevent parasites from cooling below 37°C to prevent parasites from becoming stressed.

Day 1: Stage I/Dying trophozoites

Parasitemia was expected to be > 12% consisting of dying trophozoites and stage I parasites. Stage I gametocytes usually appeared smaller and stain dark blue, instead of black, but are difficult to differentiate from dying asexual parasites, see figure 3.6. Parasites are treated the same as described on day 0.

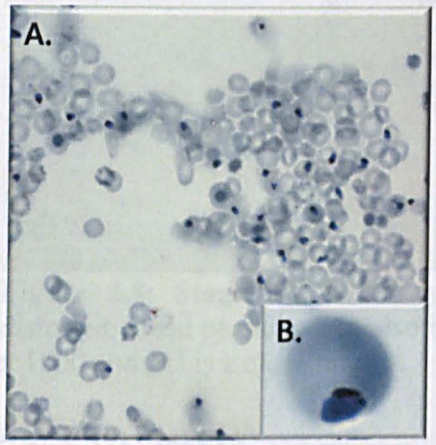


Figure 3.6: Stage I/dying trophozoites. At this stage it is difficult to distinguish between asexual and sexual stages. Gametocytes look stressed, staining dark blue in colour and are more compressed in their shape. Dying asexual parasites can be usually seen until day 5. A.) was taken x 1000 and B.) is a close up of A.).

Day 2-4: StageII/dying trophozoites

Stage II parasites were treated the same as on day 0, see figure 3.7

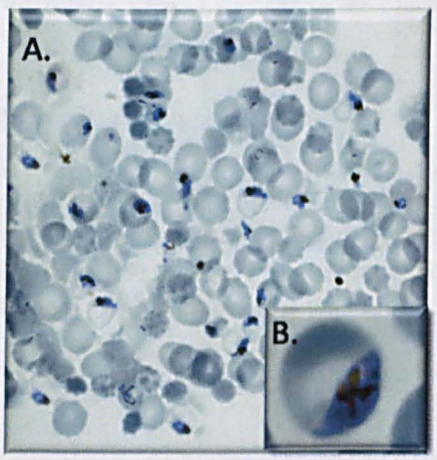


Figure 3.7: Stage II gametocytes. Parasites can be seen to touch one edge of the RBC and to begin to elongate in shape. A.) was taken x 1000 and B.) is a close up picture.

Day 4-6: Stage III

Parasites were treated the same as on day 0, see figure 3.8.

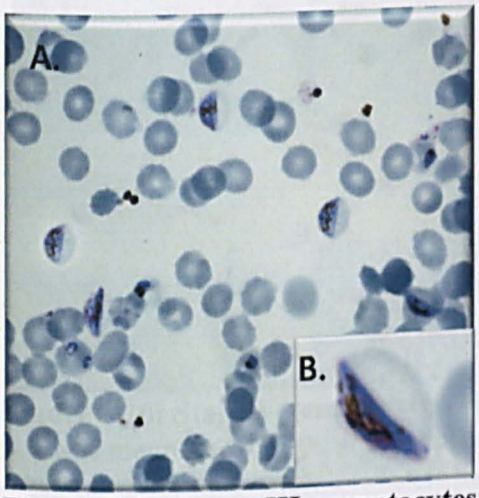


Figure 3.8: Stage III gametocytes. Parasites become D shaped and the RBC is distorted. Dead parasites can also be seen in the background (black dots). A.) was taken x 1000 and B.) is a close up of A.)

Day 6-10: Stage IV

Parasites were treated the same as on day 0, see figure 3.9.

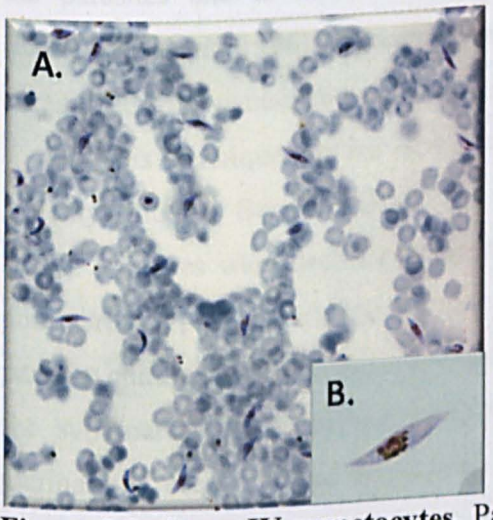


Figure 3.9: Stage IV gametocytes. Parasites reach their elongate and acicular shape typical of this stage. A.) was taken x 1000 and B.) is a close up of A.)

Day 10-11: Stage V

Parasites were treated the same as on day 0, see figure 3.10.

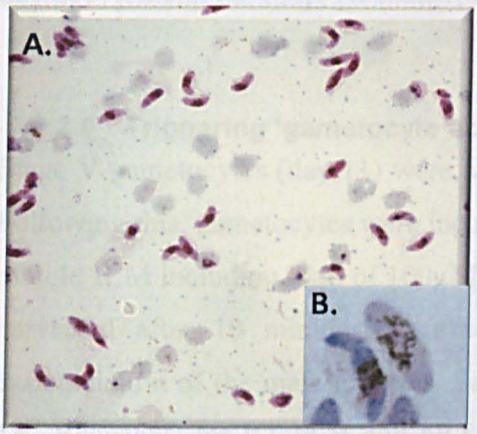


Figure 3.10: Stage V gametocytes. Parasites form into the crescent, or 'sausage' shape. A.) was magnified x 1000 and B.) is a close up of A.)

3.2.8 Parasite harvest

Malaria parasites feed on the erythrocyte cytoplasm by transporting haemoglobin to an acidic food vacuole, where haemoglobin is converted into haemozoin. Haem is toxic to the parasites and is therefore transformed into a crystalline structure haemozoin (Rosenthal *et al.*, 1996), a paramagnetic product, which allows the separation of non parasitized from parasitized cells using magnets.

This technique applied here is called Magnetic activated cell sorting (MACS®; Milentyi BioTec, Bergisch Gladbach, Germany) (Miltenyi *et al.*, 1990; Ribaut *et al.*, 2008). Parasites were used for experiments immediately after the purification process and were not placed back into culture, to prevent the loss of parasites. Before harvest, parasite cultures were washed twice with pre-warmed ICM at 500 x g for 5 minutes and the supernatant was removed. MACS® columns (25CS columns, Miltenyi Biotec, Germany) were preheated by placing them in the incubator, then filling them with warmed ICM (37°C) and columns were washed until no air bubbles remained, to allow the parasites to move through the column.

The pellet was diluted with ICM (at approximately 50% haematocrit, but depending on parasitemia). The parasites were then resuspended in pre-warmed ICM and parasites were transferred into the column with a 1ml pipette, until the whole sample moved through the column. Warm ICM was filled into the column until no RBCs were visible, to wash out the column of any remaining gametocytes. The eluent was then centrifuged at 500 x g for 4 minutes at a minimum of 25°C and the supernatant was removed. The pellet was then resuspended into a smaller volume, proportionate to

the estimated pellet size (5-10ml) and gametocytes were quantified by using a haemocytometer (C-Chip, Neubauer, Germany).

3.2.9 Triggering 'gametocyte activation'

Stage V gametocytes (day 11) were MACS® purified and washed as described before. Following this, gametocytes were incubated at room temperature with 5 x the pellet size of cold ICM including 20µl of 100µM xanthurenic acid (Sigma-Aldrich). Parasites were harvested after 10 minutes, to observe the activation of gametogenesis and the exflagellation of the male gametes. Samples were used for IFA analysis, and stored in Tri- Reagent® for RT-PCR and qRT-PCR analysis. In this study, parasites that were triggered to undergo gametogenesis ('round up') are referred to as 'activated gametocytes'.

3.2.10 Giemsa staining of asexual stages and gametocytes

Asexual parasites and gametocytes were monitored daily by spreading approximately 10µl of the culture at a 50% haematocrit onto a glass slide. Slides were then fixed in methanol for 10 seconds, stained in 10% Improved R66'Giemsa (BHD) in a molarity phosphate buffer (BDH), where the pH was adjusted to 7.4. Giemsa was applied with a syringe containing a 0.45µm pore size filter (Millex), to prevent any impurities affecting the 35 minute staining process. Asexual parasites were frequently stained for much shorter time periods, whereas for sexing gametocytes the timing of the staining had to be exact. Slides were then rinsed with tap water and air dried. Gametocytes were magnified x 100 for observation. See section 1.4.2 for the identification of the male and female gametocytes and see section 1.3.1 for the identification of the five gametocyte stages.

3.3 Isolation of gametocyte RNA

Parasites were harvested, purified and quantified with a haemocytometer (C-Chip, Neubauer, Germany) before they were added to 1.5ml of the pre-warmed Tri-Reagent® (37 °C). Samples were gently shaken, left at room temperature for at least five minutes and were then stored at -80°C. Isolation of RNA was achieved by following the Tri-Reagent® manufacturer's protocol.

Briefly, samples were thawed at 37°C and gently vortexed; 0.1ml of BCP (Sigma-Aldrich) was added per 0.75ml of Tri-Reagent® used and vortexed, left at room temperature for 5 minutes. Samples were centrifuged for 30 minutes at 12,000 x g at 4°C, which allowed the phase separation of the RNA and DNA. The upper aqueous phase containing the RNA was carefully removed, to which 0.5ml of isopropanol (Sigma Aldrich) was added, allowing precipitation of the RNA. Samples were stored at 4°C overnight and then centrifuged at 12,000 x g for 30 minutes, separating the RNA pellet. The pellet was washed once in 75% ethanol (Sigma Aldrich), removing any impurities from the RNA. Once the ethanol was carefully removed, the samples were allowed to air dry until no ethanol remained and 20µl of nuclease free water was added (Promega), gently re-pipetting the solution to allow the RNA to dissolve in the water. The RNA solution was then stored at -80°C for up to three months. All reagents used for this step were sterile and RNAse free, according to the manufacturer's protocol.

3.4 Obtaining cDNA from gametocyte RNA

In order to obtain copy DNA (cDNA) of parasite RNA, a reverse transcriptase step was used to convert RNA into cDNA. The master mix for the reverse transcriptase step was prepared, containing 3µl of random primers, 3µl of 5mM dNTPs (Promega), 3µl of RQ-DNase (Invitrogen), 2µl of RsaI (Invitrogen), 12µl of 5 x first strand RT-buffer (Invitrogen), 3µl of 0.1 DTT (Invitrogen) and 14µl of nuclease free water (Promega). A master mix was added to the 20µl of RNA dissolved in nuclease free water (Promega). Samples were incubated at 37°C for 1 hour, 94°C for 6 minutes and 12°C for 2 minutes. Samples were then split into two, one containing 50µl, to which 1.6µl of superscript III (Invitrogen) were added, the other sample containing 10µl to which 0.33µl nuclease free water (Promega) was added instead of the superscript III, thereby serving as a negative control that will later allow the detection of DNA contamination. All samples were incubated at 50°C for 1 hour and 70°C for 15 minutes; cDNA samples were stored at -20°C. Superscript negative samples are noted as '- ss' and superscript positive samples noted as '+ ss'.

4 INVESTIGATION OF SEXUAL STAGE DYNEINS

4.1 INTRODUCTION

4.1.1 Dyneins in *Plasmodium*

Khan *et al.*, (2005) separated for the first time male and female transgenic *P. berghei* gametocytes using flow cytometry, which allowed the analysis of protein expression profiles using mass spectrometry. In this study a range of novel sexual stage proteins were discovered, of which a large number were described to be associated with the axoneme or flagella. These proteins included a family of dynein isoforms, all of which were isolated solely from male gametocytes, with the exception of one protein, PB000989.00.0, which was found exclusively in female gametocytes. This female specific protein is a putative dynein heavy chain (DHC) and was indicated by Khan *et al.*, (2005) to have an ortholog in *P. falciparum,* which has been assigned the name MAL7P1.162. Dyneins have not yet been studied in gametocytes of *P. falciparum* and should the sex specific dynein heavy chains identified by Khan *et al.*, (2005) have close homologies encoded by the *P. falciparum* genome, as they may have the potential to serve as an ideal target for quantifying male and female gametocytes. *Plasmodium spp.* have a complex life cycle and undergo distinct morphological changes, which mirror the large number of new proteins expressed during each individual stage. Only a few sex specific proteins have been studied in *P. falciparum* and of those known none have been identified where a male specific protein is homologous to a female specific protein and *vice versa.* Using male and female sex specific proteins that are homologous to each other could have the advantage of targeting genes with similar gene expression profiles, ensuring that mRNA levels are present at similar transcription levels and with similar temporal expression patterns.

Dynein, kinesin, and myosin belong to a group of motor molecules and all appear to be similar in structure and function. These motor proteins convert ATP into mechanical energy, but this does not reflect their divergent evolution, as dyneins are unique in belonging to the AAA (ATPase associated with diverse cellular activities) protein superfamily (Neuwald *et al.*, 1999). All three motor molecules have been reported to aid in cell division, organelle and vesicle transport (Schliwa *et al.*, 2003) and have also been described to work as combined forces (Welte *et al.*, 1998).

Dynein (Greek: 'dyne' meaning force, or power and 'ein' coming from protein), compared to other motor molecules, is extremely large, but produces weaker forces than

myosin or kinesin in comparison. Dynein requires accessory molecules and has been observed to work as multimers with multiple dyneins working together in order to increase its strength (reviewed by Mallik *et al.*, 2004).

There are two main types of dyneins; one is involved in the movement of cilia and flagella (axonemal dynein) and the other type of dynein is involved in the transport of cytoplasmic cargo (cytoplasmic dynein) (Gibbons *et al.*, 1965; Gibbons, 1995). Consequently, dynein will be either involved in the direct movement of a cell; such as sperm for example; or the transport of cytoplasmic cargo, used for the nuclear migration, organization of the mitotic spindle, chromosome separation during mitosis, or the transport and function of organelles (Karki *et al.*, 1999). As cytoplasmic dynein moves along to the negative end of the microtubule, it is most commonly associated with transporting cargo towards the cell centre and nucleus (Mallik *et al.*, 2004), see figure 4.1. Dyneins may prove difficult to study, as they often occur in recombinant forms and are large in size (approximately 4600 amino acids). Due to the large number of isoforms that exist, each dynein isoform has been hypothesized to be specialized for a specific function (Asai, 1995). This makes it difficult to study specific dyneins, as frequently, different isoforms may work together in hetero-multimers and therefore co-locate in the cell architecture. Only very few studies exist where dyneins have been investigated in malaria parasites; one study investigated motor proteins in merozoites and suggested that it is likely that dyneins are involved in the merozoite invasion process of the RBC (Fowler *et al.*, 2001). This study reveals one of the vital functions dyneins may play in the malaria life cycle.

Investigating novel malaria proteins, which may play vital roles in the parasite survival, may also be of interest for identifying new parasite targets for improved intervention strategies. *P. falciparum* dyneins investigated in this study will either be involved in the preparation of gamete formation (cytoplasmic dynein) for the male and female gametocytes, or be responsible for the movement of the flagella of the male gamete (axonemal dynein).

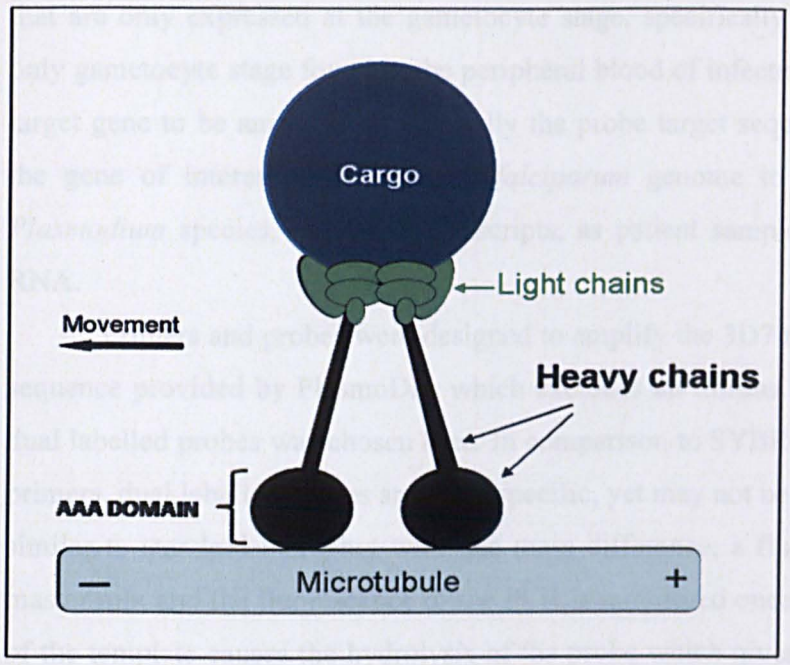


Figure 4.1: Schematic diagram of axonemal dynein. The heavy chains move along the microtubule towards its negatively charged end carrying cargo to be transported within the cell, resulting in ATP hydrolysis. Dynein structures vary and contain variable number of heavy, light and intermediate chains depending on function, but all dyneins share the AAA ATPase domain which binds to the microtubule when moving along. The heavy chains are part of the ATPase motor domain. In axonemal/flagellar dynein, the 'cargo head' attaches itself to another microtubule and the movement of dynein along the microtubule causes sliding of the microtubules, thereby causing the movement of the flagella/cilia. This diagram was adapted from Schliwa *et al.*, 2003.

4.1.2 Development of a qRT-PCR assay for sex ratio determination

In order to study *P. falciparum* sex ratios on a large scale, a molecular assay that is sensitive and time efficient is needed. This qRT-PCR assay should quantify the relative abundance of specific male and female gametocyte transcripts present within a given sample, which can then reflect the number of male and female gametocytes present. This assay was developed with 3D7 cultured parasites; the goal of establishing this assay will be to quantify sex ratios from natural infections.

In gametocytes, gender is determined by differential gene expression (Alano *et al.*, 1990) and not chromosomal differences, therefore cDNA (copy DNA) and not gDNA (genomic DNA) was used, as cDNA allows quantifying the mRNA transcription levels of the male and female gametocytes. By using a reverse transcriptase step, cDNA is produced from RNA, which can then reflect the mRNA products that are transcribed in the parasites. The first step in designing such a qRT-PCR assay that quantifies sexual stage and sex specific transcription levels involved choosing sex specific target genes

that are only expressed at the gametocyte stage, specifically at stage V, as this is the only gametocyte stage found in the peripheral blood of infected patients. The part of the target gene to be amplified, specifically the probe target sequence, must be specific to the gene of interest and to the *P. falciparum* genome to avoid quantifying other *Plasmodium* species, or human transcripts, as patient samples will contain leukocyte RNA.

Primers and probes were designed to amplify the 3D7 mRNA template using the sequence provided by PlasmoDB, which excludes all introns. A qRT-PCR assay using dual labelled probes was chosen here. In comparison to SYBR® green, which only uses primers, dual labelled probes are more specific, yet may not be as sensitive. qRT-PCR is similar to standard PCR, but with one main difference; a fluorophore is added to the master mix and the fluorescence of the PCR is monitored once per cycle. Amplification of the template causes the hydrolysis of the probe which gives off a fluorescent signal. The more template that is present within a given sample, the more rapidly a fluorescent signal accumulates. The rate of signal accrual therefore relates to the amount of initial template created. The amount of the fluorescent signal is measured during each PCR cycle by the qRT-PCR machine, and can be visualized as an exponential curve, see figure 4.2. A threshold value can then be set, to compare relative amounts of mRNA from each gene of interest. The point where fluorescence of a sample crosses the threshold is known as a threshold cycle (Ct). As a rule of thumb Ct values taken from cDNA are only meaningful when taken earlier than approximately 30 cycles or more, as the results will be unreliable otherwise.

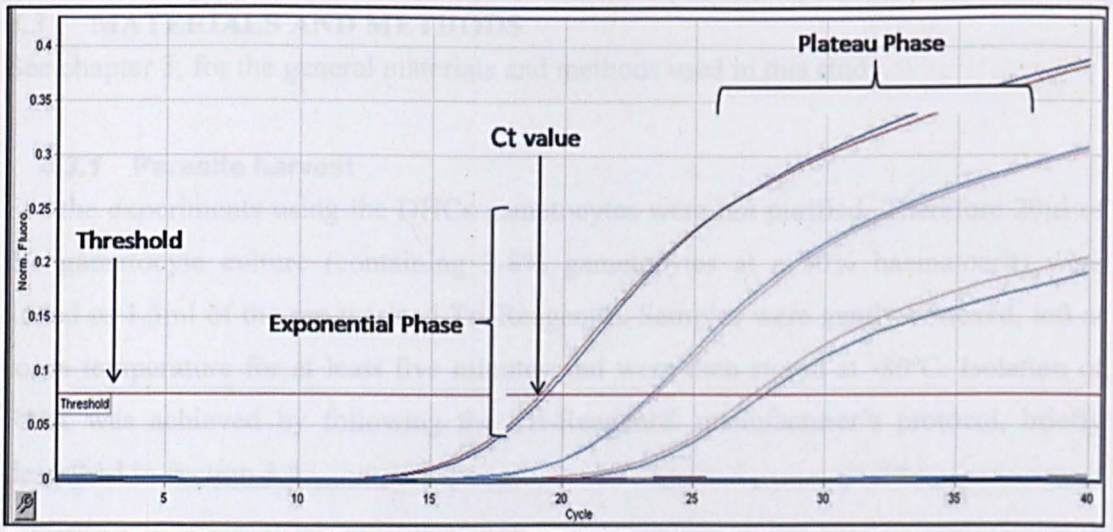


Figure 4.2: Schematic diagram of a qRT-PCR plot. Indicating the location of the threshold that is subjectively set, indicating a Ct (threshold cycle) value when crossing the sample during its exponential phase. The plateau phase can also be seen, occurring when reaction components become limited. Graphics are taken from the Rotorgene 3000 software.

4.2 AIMS AND OBJECTIVE

1. Analysis of orthologs of dynein heavy chains (DHCs) described in *P. berghei* by Khan *et al,*. 2005.

2. Design of primers and probes to develop a qRT-PCR assay that quantifies mRNA levels of the putative sex specific dyneins in *P. falciparum* to allow quantifying sex specific transcription levels, which will reflect the sex ratios of cultured gametocytes established by light microscopy and IFA.

3. Optimization of the assay, allowing the quantification of sex ratios from peripheral blood samples from gametocyte positive individuals.

4.3 MATERIALS AND METHODS

See chapter 3, for the general materials and methods used in this study.

4.3.1 Parasite harvest

For the experiments using the DHCs gametocytes were not purified. Therefore 20µl of the gametocyte culture (containing 3-8% gametocytes at a 50% haematocrit) were added to 1.5ml of the pre-warmed Tri-Reagent®. Samples were gently vortexed, left at room temperature for at least five minutes and were then stored at -80°C. Isolation of RNA was achieved by following the Tri-Reagent® manufacturer's protocol, briefly described in section 3.4.

4.3.2 Design of primers and probes for the DHCs (dynein heavy chains) and Pfs16

Information concerning the design of DHC primers and probes are found in the results section; see section 4.4.1 and section 4.4.2. Primers were also designed to target part of the Pfs16 sequence (PlasmoBD Gene ID: PFD0310w). Pfs16 is the earliest known marker for sexual commitment (Dechering *et al.*, 1997) and will serve as a control for the RT-PCRs. The SA07 forward primer: 5'-TTCTTCGCTTTTGCAAACCT-3' and SA08 reverse primer 5'-AAAGGCATTTTGTCAGCAGAA-3' were designed by retrieving mRNA sequences from PlasmoDB and identifying the optimal primer regions with the help of the Primer 3 (v. 0.4.0) software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers (see table 4.1) were obtained through MWG-Biotech (Germany), all probes except PfDF2 (Applied Biosystems) were obtained through MWG, see table 4.2 for the list of primers used.

Name	Sequence	Target
SA01	5'-GACCAGCTGGAACAGGAAAA-3'	DHC
SA02	5'-AAATTCATCAAAACA(A/T)C(A/C)CC-3'	DHC
SA03	5'-G(A/G)CCAGCKGG(A/T)ACAGGAAAA-3'	DHC
SA04	5'-AAATTCATCAAAACA(A/T)CCCCA-3'	DHC
SA07	5'-TTCTTCGCTTTTGCAAACCT-3'	Pfs16
SA08	5'-AAAGGCATTTTGTCAGCAGAA-3'	Pfs16

Table 4.1: List of the DHCs and Pfs16 primers. SA01/SA02 and SA03/SA04 were designed to target multiple DHCs, SA07 and SA08 were designed to target Pfs16.

Name	Sequence	Target
PfDF	JOE-5'- CCTTAGGTGCACAATTAG-3' -BHQ1	DHC (Female)
PfDF2	JOE-5'-CCTTAGGTGCACAATTAG-3'-MGB	DHC (Female)
PfDM	FAM-5'-ATTTAGGTAGAACCTTAG-3'-BHQ1	DHC (Male)

Table 4.2: List of the DHC probes. The probes PFDF and PFDF2 were designed to target the female specific DHCs and the male probe PfDM was designed to target the male specific DHC sequence.

4.3.3 RT-PCR of the DHCs and Pfs16 during gametocytogenesis

Parasites were harvested on day 1, 3, 5, 7, and 11 as previously described. The master mix for the PCR was made with the following reagents for one 25µl reaction: 2.5µl of 10 x NH_4 buffer (Bioline), 0.5µl of 2.5µM dNTPs (Promega), 0.5µl of 50 mM $MgCl_2$ (Bioline), 1µl of 5µM for the forward and reverse primer (see table 4.1), 0.2µl of 50 units/µl of *Taq* (Bioline), and 3µl of cDNA and 13.8µl of nuclease free water (Promega). PCR cycling conditions are shown in the appendix (see section 8.2). Bands were analyzed with ultraviolet light after running a 1.2% (0.5 x TBE) agarose gel (Sigma-Aldrich), containing 2.5µl of ethidium bromide (10mg/l) (Sigma-Aldrich), at 100 V for 1 to 1.5 hours.

4.3.4 Sequencing of gametocyte gDNA and cDNA of the DHCs

Sequencing was done with gDNA and cDNA from *P. falciparum* (3D7) using the Big Dye™ terminator cycle sequencing kit according to the protocol (Applied Biosystems). This was done after running a PCR and RT-PCR with SA01 and SA02 under the same conditions as previously described. Following this, PCR products were purified with the QIAquick Kit (Qiagen) according to manufacturer's instructions. Five volumes of PI buffer to one volume of the PCR sample (PCR products ranging from 19-36µl) was added.

Samples were placed in the QIAquick spin column, centrifuged for 60 seconds at 10,000 x g. The liquid collected from the column was discarded and 0.75ml PE buffer was added and centrifuged under the same condition. The liquid was discarded again and the sample was centrifuged. The QIAquick column was then placed into a new Eppendorf tube, 15µl of EB buffer was added onto the columns and left for 5 minutes at room temperature and the samples were centrifuged again and stored at -20°C. For the next step, a master mix was prepared that included 3.5µl of Big Dye® (Applied

Biosystems), 1.75µl of 50 x sequencing buffer (Applied Biosystems), 2µl of 5µM SA01 or SA02, 3µl of cDNA and 2.75µl of nuclease free water per 10µl reaction.

Following the sequencing reaction on the PCR machine, samples were spun for 5 seconds, 3µl of 3M NaAc (pH 4.5), 62.5µl of 100% ethanol and 24.5µl of nuclease free water (Promega) were added to each sample. Samples were vortexed briefly and placed on ice for 20 minutes. Samples were centrifuged at 20,000 x g for 30 minutes, the supernatant was carefully removed and 200µl of 70% ethanol was added to each sample. Samples were spun for 10 minutes at 20,000 x g. The supernatant was discarded and samples were air dried in the dark for at least 3 hours and stored at -20°C until samples were ready for sequencing. Samples were sequenced using an Applied Biosystems 3730 Genetic Analyzer and results were analyzed with the Chromas program (www.technelysium.com.au). Sequence results were blasted against published sequence databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.3.5 qRT-PCR of the DHCs

qRT-PCR was performed on the Rotor-Gene 3000 (Corbett, Australia). The superscript positive master mix contained 2.5µl of NH_4 (Promega), 0.5µl of 50mM $MgCl_2$ (Bioline), 2µl 2.5µM of dNTPs, 1µl of 5µM of either SA01 and SA02 or SA03 and SA04, 0.2µl of 50 units/µl of *Taq* (Bioline), 0.5µl of 5µM of the male PfDM probe and 0.5µl of 5µM of the female PfDF probe, 2-3µl of cDNA, and nuclease free water (Promega) was added for a 20µl reaction. For the negative controls, nuclease free water (Promega) was used instead of cDNA and DNA was used for the positive control. Superscript negative samples were also included to observe if any DNA was present in the cDNA samples. The cycling conditions for the qRT-PCR were 96°C for 6 minutes, 96°C for 15 seconds, 51°C for 60 seconds and 70°C for 15 seconds, then 40 cycles of step two to four.

4.4 RESULTS

4.4.1 Identification of *P. falciparum* DHCs as targets for the qRT-PCR assay

Khan *et al.,* (2005) reported in his study a female specific protein as a putative dynein heavy chain (PB000989.00.0), which was indicated to have an ortholog in *P. falciparum* (MAL7P1.162). No further details were provided concerning the homology between the two proteins; therefore it was necessary to investigate the sequences of the two proteins first. This was done by blasting the whole amino acid sequence of PB000989.00.0 (*P. berghei*) using blastp into the PlasmoDB database (http://plasmodb.org). The search results showed that MAL7P1.162 (*P. falciparum*) was the closest match and was found to be 73% identical to PB000989.00.0, which indicates these proteins are likely to be orthologous to each other. The three next closest matches selected by the blast search were PFI0260c, PF10_0224 and PF11_0240, which are all male specific putative dynein heavy chains found in *P. falciparum*, see table 4.3.

The corresponding *P. falciparum* amino acid sequences of the three male specific proteins (PFI0260c, PF10_0224, PF11_0240), all named putative DHCs, were retrieved from PlasmoDB and analyzed for homology and searched for a consensus (shared) sequence. The identification of a consensus sequence allowed observing the shared regions of the DHCs. This region will later be used to amplify multiple DHC sequences at the same time for the simultaneous quantification of male and female specific gametocyte transcription levels. The consensus sequence 'GPAGTGKTE' was identified by boxshade analysis (http://www.ch.embnet.org). Next, the amino acid sequence was converted into its corresponding nucleotide sequence, allowing the design of primers and probes, see figure 4.3.

Gene ID	*P. falciparum*	Protein name	MG or FG
PB000989.00.0	MAL7P1.162	DHC, putative	FG
PB001056.02.0	PF10_0224	DHC, putative	MG
PB000791.03.0	PFI0260c	DHC, putative	MG
PB000733.03.0	PF11_0240	DHC, putative	MG

Table 4.3: List of the DHCs identified in *P. berghei.* The corresponding orthologs of *P. falciparum* are shown, showing the given gene ID (Khan *et al.,* 2005). All four proteins were identified as hypothetical DHCs and were either only found in the male gametocyte (MG), or only in the female gametocyte (FG).

Figure 4.3: Boxshade analysis of the amino acid sequence of the DHCs. Sequences of the female specific DHCs PB000989_00_0, MAL7P1_162 and the male specific DHCs PFI0260c, PF10_0224, PF11_0240. Letters highlighted in black reveal amino acids that are identical within all sequences, letters highlighted in grey represent where some amino acids are shared, letters not highlighted indicate amino acids that are not shared. The consensus sequence reveals the shared conserved region 'GPAGTGKTE', which is highlighted in yellow.

4.4.2 Design of primers and probes for the DHCs and Pfs16

Primers and probes were designed to target the consensus sequence 'GPAGTGKTE', which was converted back into its corresponding nucleotide sequence. A total of three sets of primers and three probes were designed for the investigation of the putative dynein heavy chains as tools for quantifying sex specific transcription levels. The first set of primers SA01 and SA02 were designed to amplify transcripts of three proteins, see figure 4.4, whereas SA03 and SA04 were designed afterwards to amplify transcripts levels of two sequences, see figure 4.5.

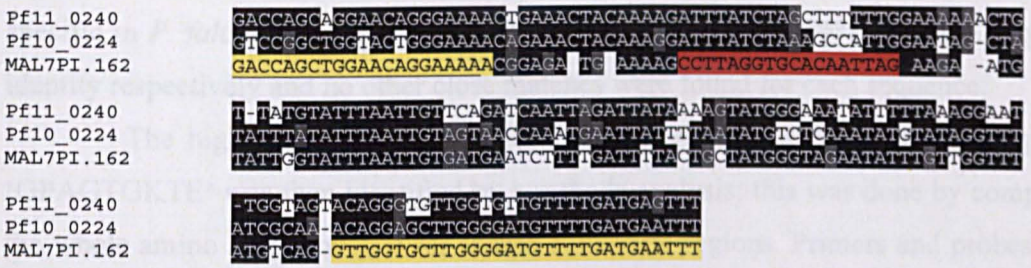


Figure 4.4: Boxshade analysis of the DHCs for SA01 and SA02. Highlighted in yellow is the first set of primers that were designed (SA01/SA02) and the PfDF probe is highlighted in red. Primers were designed to amplify three DHC sequences; PCR products were predicted to be 158bp long.

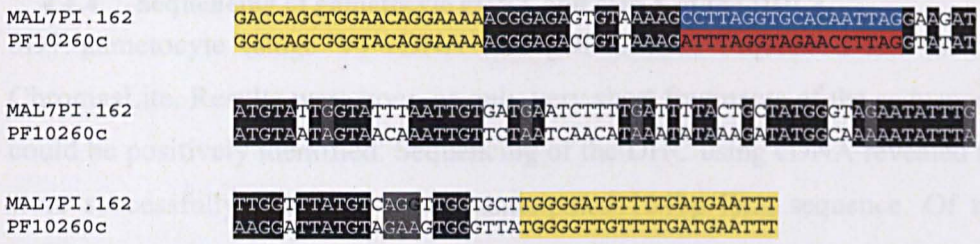


Figure 4.5: Boxshade analysis of the DHCs for SA03 and SA04. Boxshade analysis highlighting the second set of primers designed, SA03 and SA04, highlighted in yellow. Location of the female probe PfDF can be seen highlighted in red, the male probe PfDM is highlighted in blue. This time primers were designed to amplify two sequences only and the PCR product was expected to be 164bp long.

4.4.3 Investigation of the DHCs in *P. falciparum*

It was confirmed that the DHC PB000989.00.0 in *P. berghei* is an ortholog to the DHC MAL7P1.162 in *P. falciparum*. The complete amino acid sequence of PB000989.00.0 was blasted into PlasmoDB, revealing that the *P. berghei* and *P. falciparum* peptide sequences are 73% identical to each other. As no other close matches were found in PlasmoDB, it was hypothesized that MAL7P1.162 is a female specific sexual stage protein in *P. falciparum,* due to the sequence similarities. The three orthologs (PF10_0224, PFI0260c, and PF11_0240) of the putative DHCs in *P. berghei* (PB001056.02.0, PB000791.03.0, PB000733.03.0) were hypothesized to be male specific in *P. falciparum,* as Blast results revealed 73%, 84%, 71% and 80% sequence identity respectively and no other close matches were found for each sequence.

The highly conserved and shared region encoding the amino acid sequence 'GPAGTGKTE' was then identified by boxshade analysis; this was done by comparing the whole amino acid sequence for shared conserved regions. Primers and probes were then designed to target the conserved region 'GPAGTGKTE'. The probes PfDF and PfDM were both blasted in PlasmoDB with blastn, highlighting the search among all *Plasmodium* species, which showed that both probes are unique to the *P. falciparum* sequences targeted.

4.4.4 Sequencing of gametocyte gDNA and cDNA of the DHCs

3D7 gametocyte (stage V) cDNA and gDNA were sequenced and analyzed with ChromasLite. Results were poor, as only very short fragments of the sequences targeted could be positively identified. Sequencing of the DHC using cDNA revealed that 105bp were successfully identified of the anticipated 164bp long sequence. Of the 105bp, 102bp were identical to the MAL7P1.162 sequence. Two of the nucleotides were not present in the Mal7P1.162 sequence, but were found in the PF11_0240 and Pf10_0224 sequence.

The cDNA sequencing results have shown that all three sequences targeted were amplified by the primers SA01 and SA02. This was observed by identifying double and triple fluorescent peaks in the sequencing results. Interestingly, the female specific sequence was found to be dominant at times, as it revealed higher fluorescent peaks at some points of the sequence, suggesting that this sequence was present in larger quantities, as it would be expected with a female biased sex ratio (contain more female specific transcripts).

Sequencing results from the gDNA revealed that another male specific sequence was amplified, that did not match any of the sequences targeted. This gDNA sequence was blasted in PlasmoDB and it was revealed that another male specific DHC, PFI.0260c, was picked up by the primers. The homology of MAL7.P162 and PF1.0260c was compared by boxshade analysis see figure 4.6, both sequences were found to be homologous to one another.

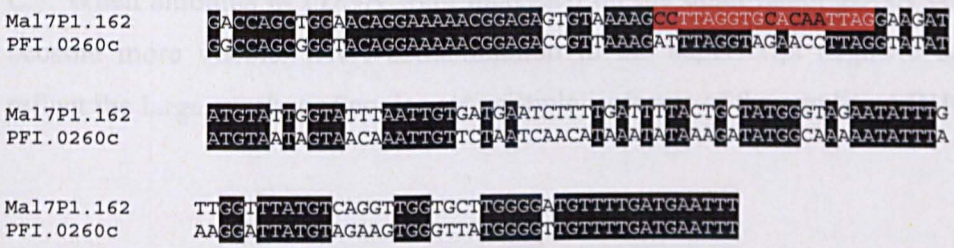


Figure 4.6: Boxshade analysis of Mal7P1.162 and PFI.0260c. Regions highlighted black indicated shared base pairs; the location of the female specific probe (PfDF) is highlighted in red.

The PfDF probe was blasted and found to be sequence specific and not likely to have hybridized with PFI.0260c, as seen in the boxshade analysis. However, the primer efficiency is questionable when four sequences are targeted, as only the individual

amplification efficiency can be measured (See chapter 6 for further information). New primers, SA03 and SA04, were therefore designed, where only one male (PFI.0260c) and one female (Mal7P1.162) specific DHC sequence was targeted.

4.4.5 RT-PCR of the DHCs

RT-PCR products of SA01 and SA02 showed that the DHCs are transcribed from stage I-V of gametocyte development from cultures, see figure 4.7 A.) Based on the sequencing results it is likely that multiple sequences were amplified. Also transcription levels do not reflect any real quantitative value, as different amounts of gametocytes were used, see table 4.4. It should also be noted that the gametocyte culture was not 100% synchronous, which makes it difficult to conclude at which stages during gametocytogenesis the DHCs are transcribed from.

gDNA contamination has been found consistently in the superscript negative control samples of the DHCs, particularly in stage III and stage IV gametocytes (see figure 4.7 A.), and see figure 4.8 A.). However, amplification of the superscript negative samples was not observed when Pfs16 was amplified, see figure 4.7 B.) and 4.8 B.) and the same cDNA was used.

When using cDNA encoding Pfs16 as a comparator, it became evident that gDNA contamination in the superscript negative samples was specific to dyneins. When amounts of cDNA were reduced (either in the dilution of RNA, or the amount of cDNA used in a PCR), the RT-PCR products of the DHCs were usually faint, see figure 4.8 C.). When amounts of cDNA were increased on the other hand, gDNA contamination became more visible. DNA contamination in the superscript negative samples may reflect the large number of copies, or multiple isoforms of the amplified DHCs.

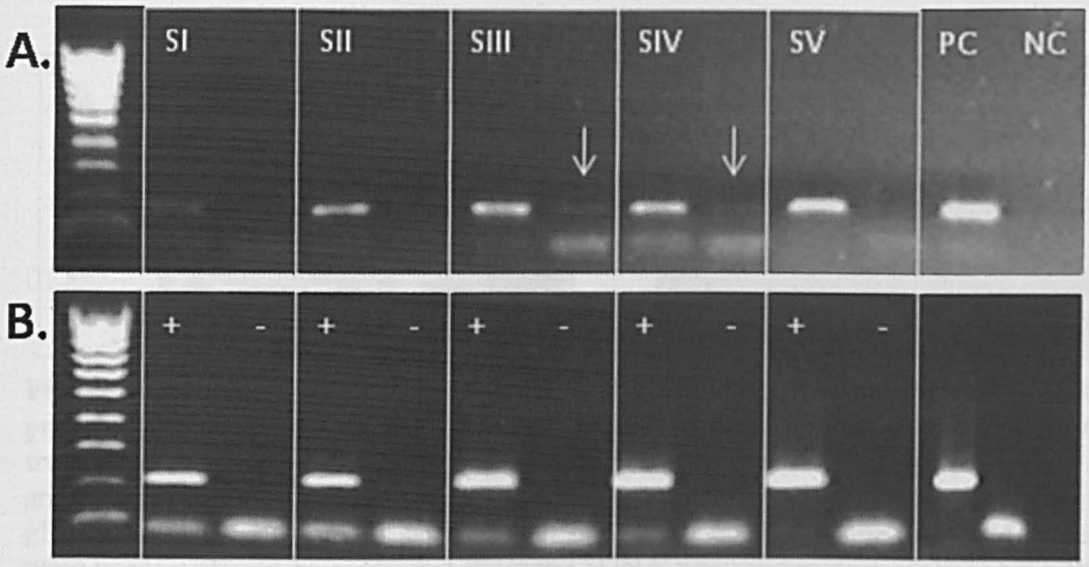


Figure 4.7: RT-PCR of the DHCs and Pfs16. A.) The primers SA01& SA02 were used and in B) the SA07/SA08 primers were used. Parasites were harvested on day 1, 3, 5, 7 and 11. Samples either included reverse transcriptase (+), or excluded reverse transcriptase (-), which served as a negative control to pick up DNA contamination. The PC (positive control) contained gDNA from 3D7 parasites. A Hyperladder IV (Bioline) was used, which shows bands at 100bp intervals. A.) RT-PCR products amplified with SA01/SA02 (DHC) were expected to be 164bp, B.) RT-PCR product of SA07/SA08 (Pfs16) were expected to be 191bp long. Arrows indicate DNA contamination.

Day (Stage)	SI/dying trophozoites	SII	SIII	SIV	SV	R
1 (SI)	88.5	0	0	0	0	11.5
3 (SII)	46.5	41	4.5	0	0	8
5 (SIII)	63.5	0.5	31	3	2	8
7 (SIV)	22	0	10	68	0	0
11 (SV)	0	0	0	11	89	0

Table 4.4: Percentage of gametocytes identified per stage. Different quantities of gametocytes were harvested at each stage; therefore RT-PCR transcription levels do not represent quantitative transcription levels. R stands for ring stage parasites.

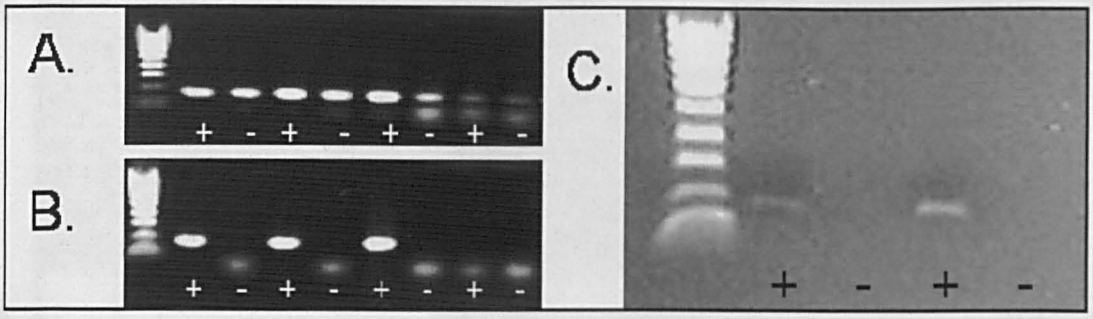


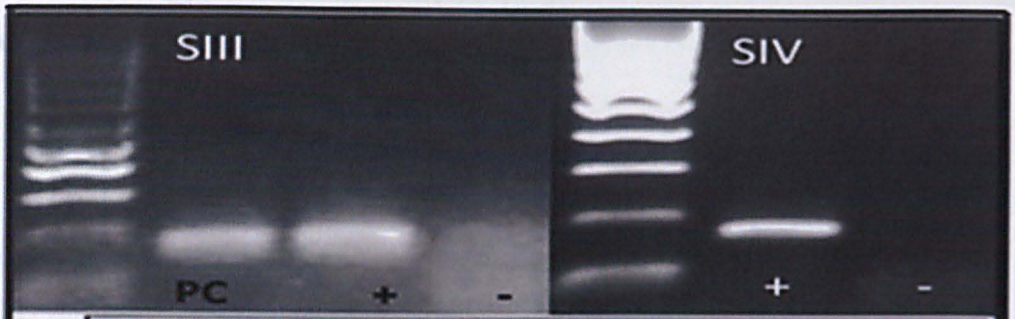
Figure 4.8: Comparison of RT-PCR products of the DHCs and Pfs16. A.) RT-PCR product of dynein (using SA03/SA04) and B.) RT-PCR product of Pfs16 (SA07/SA08) from stage IV gametocytes; Hyperladder IV (Bioline) was used. Superscript positive (+) and superscript negative samples are seen respectively. Dynein PCR products revealed gDNA contamination in all samples that appeared negative when the Pfs16 primers were used, it should be noted that the same cDNA was used in each well. C.) RT-PCR products amplified with dynein primers SA03/SA04. Products were faint, when increasing amounts of cDNA per reaction, gDNA contamination often becomes visible. The successive lanes in A and B are all stage IV gametocyte cDNA from the same culture.

4.4.6 qRT-PCR of the DHCs

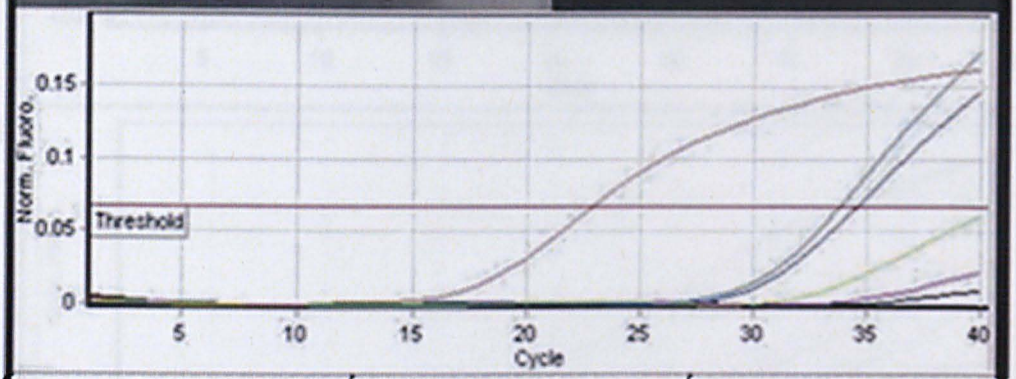
The qRT-PCR results revealed that cDNA obtained from gametocyte RNA was consistently contaminated with gDNA, even when it was not detectable in superscript negative samples of the RT-PCR targeting the DHC sequences see figure 4.9 A.) - C.). Increasing the amounts of cDNA within a reaction was necessary to reach lower, more consistent Ct values within a range of 15-25 cycles, but increasing the amounts of cDNA also led to stronger gDNA contamination, see figure 4.10B and C. It was also difficult to set threshold values for calculating Ct values, as no exponential curve was observed during the amplification of the DHCs. The threshold must be placed where the exponential phase begins, to interpret quantitative data. In comparison, the control containing gDNA showed a clear exponential phase, suggesting appropriate conditions were present for the primers and probes to function.

Fluorescence observed from both probes was low, yet the fluorescence of the PfDM probe was minimal, suggesting poor amplification of the primers, or hybridization of the probe to the primers. Temperatures of the amplification step were adjusted from 50 to 60°C and it was found that the male probe worked best on gDNA at an amplification temperature of 51°C.

A.



B.



C.

Colour	Sample	CT
	PC	22.97
	NC	
	SIII+	34.58
	SIII-	
	SIV+	33.88
	SIV-	

Figure 4.9: Stage III and IV RT- and qRT-PCR run. Three figures are linked: A.) RT-PCR of the DHCs, B.) output from the Rotorgene 3000, C.) Ct values of the qRT-PCR run. Representation of two gametocyte cDNA preparations (stage III and IV) used in RT-PCR (A.) and qRT-PCR (B. and C.). A.) Shows the RT-PCR products of SA03 and SA04, expected to be 164bp long. Positive control (PC) and superscript positive and superscript negative samples are indicated with '+' and '–' respectively. B.) shows the qRT-PCR results obtained using the Corbett software, revealing fluorescence and the Ct values obtained, which are listed in C.). It can be seen that superscript negative samples are amplified, even though no RT-PCR product was visible.

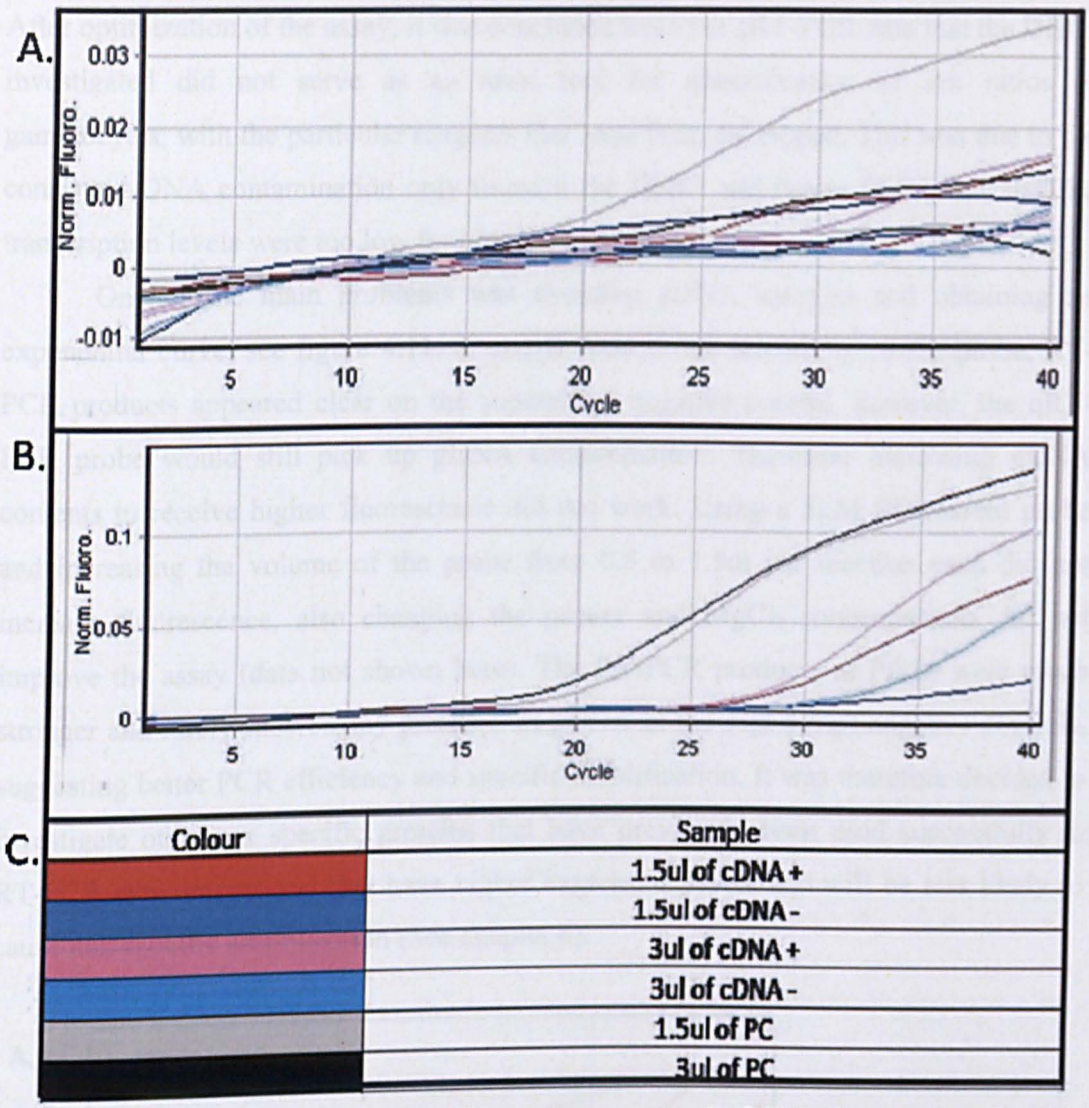


Figure 4.10: qRT-PCR run with different amounts of cDNA. Illustration of qRT-PCR of the male specific probe A.) PfDM and B.) the female specific probe PfDF. C.) shows the cDNA volume used in each reaction according with the representative colours of each sample C.). Superscript positive samples are indicated with a '+', whereas superscript negative samples are indicated with a '-'. Amounts of cDNA were doubled and it can be seen that superscript negative samples also showed an increase in fluorescence when the volume was increased.

After optimization of the assay, it was concluded from the qRT-PCR data that the DHCs investigated did not serve as an ideal tool for quantification of sex ratios of gametocytes, with the particular reagents that have been developed. This was due to the consistent DNA contamination only found n the DHCs and not in Pfs16, but also the transcription levels were too low for obtaining consistent results.

One of the main problems was avoiding gDNA samples and obtaining an exponential curve, see figure 4.11. A and B. Due to the sensitivity of the probe, RT-PCR products appeared clear on the superscript negative control, however, the qRT-PCR probe would still pick up gDNA contamination. Therefore increasing gDNA contents to receive higher fluorescence did not work. Using a 5µM fluorescent probe and increasing the volume of the probe from 0.5 to 1.5ul per reaction used did not increase fluorescence, also changing the primer and $MgCl_2$ concentrations did not improve the assay (data not shown here). The RT-PCR products of Pfs16 were much stronger and rarely showed any presence of gDNA in the superscript negative controls, suggesting better PCR efficiency and specific amplification. It was therefore decided to investigate other sex specific proteins that have previously been used successfully in RT-PCR experiments and that have higher expression levels and will be less likely to cause non specific amplification (See chapter 6).

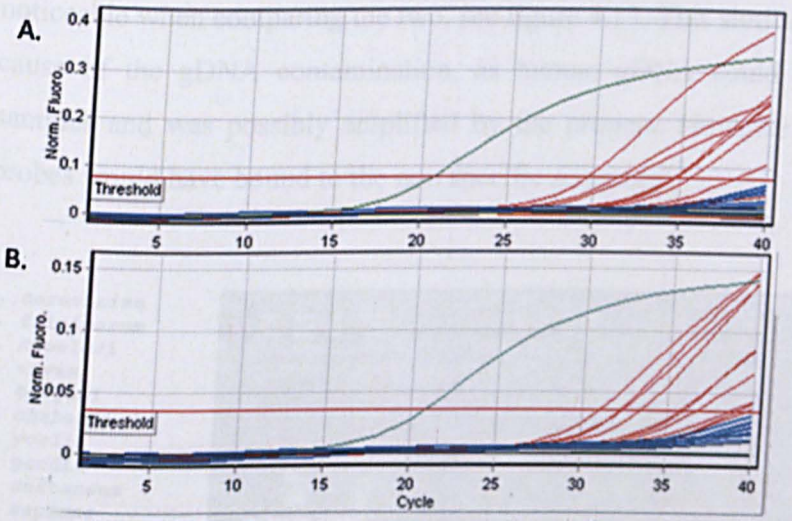


Figure 4.11: DHC qRT-PCR results. A.) and the male probe PFDM B.) the female probe PFDF. Highlighted in red are the superscript positive samples, highlighted in blue are the superscript negative samples. It can be seen that gDNA contamination is present in the superscript samples, as some fluorescence is detected. Only the PC (positive control), highlighted in green shows exponential amplification reaching a plateau.

4.4.7 Identification and characterization of the DHCs in *P. falciparum*

The function of DHCs has not been previously investigated in the sexual stages of malaria parasites and whether the DHCs investigated here are involved in the movement of cytoplasmic cargo, or axonemal/flagellar movement is yet unclear. Compiling a boxshade analysis of sex specific DHCs revealed close homology of the DHCs found in apicomplexan parasites and humans, see figure 4.12. These conserved regions suggest that these dyneins are likely to play a vital biological role, as they have been preserved throughout the course of evolution.

Eshel *et al.,* 1993 analyzed the DHCs (Gene ID: DYN1) in *Saccharomyces cerevisiae*, a budding yeast. Dyn1 is homologous to the DHCs investigated in this study, and also contains the highly conserved region 'GPAGTGKTE'. The DYN1 gene was identified and characterized, suggesting it encodes cytoplasmic DHCs (Eshel *et al.*, 1993). In this study it was found that disrupting this gene caused a misalignment of the mitotic spindle, affecting nuclear segregation during mitosis. It is likely that the function of MAL7PI.162 and PFI0260c are similar to the function of the DHC observed in yeast. It is possible that the DHCs investigated here are involved the nuclear organization of the cell in preparation for fertilization. It is also possible that the knock out gene described by Eshel contains other similar isoforms of the DHCs (Asai, 1995). The close homology between the human and malaria parasite DHCs sequence is noticeable when comparing the two, see figure 4.13. This similarity could have been the cause of the gDNA contamination, as human gDNA could have contaminated the samples and was possibly amplified by the primers. However, it is unlikely that the probes would have bound to the non specific sequence.

Figure 4.12: Boxshade analysis of MAL7P1.162 and its orthologs. Revealing close homology of the DHC amino acid sequences investigated within the animal kingdom, as regions highlighted in black show the shared amino acids, whereas the ones seen in white show the non shared regions.

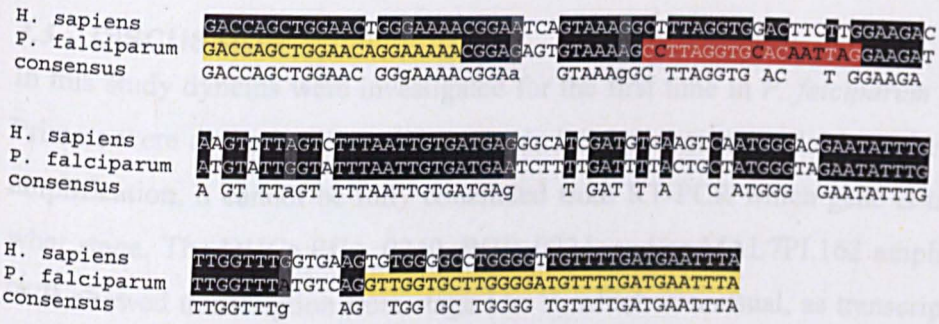


Figure 4.13: Boxshade of the DHCs in human and malaria parasites. Boxshade analysis of the female specific DHC Mal7P1.162 and the corresponding sequence found in *Homo sapiens* (79659 DYNC2H1). The consensus sequence reveals the close homology between the two sequences. Primers SA03 and SA04 are highlighted in yellow and the female probe PfDF is highlighted in red.

4.5 DISCUSSION

In this study dyneins were investigated for the first time in *P. falciparum* gametocytes. Primers were designed to amplify multiple DHC sequences and due to the non-specific amplification, it cannot be fully concluded from RT-PCR which gene is transcribed at what stage. The DHCs Pf11_0240, Pf10_0224, and/or MAL7PI.162 amplified by RT-PCR showed transcription from stage I to V, which is unusual, as transcription has not yet been reported from any sex specific protein before stage III (Alano *et al.*, 1995a; Baker *et al.*, 1995; van Schaijk *et al.*, 2006). This would suggest that DHCs are therefore the earliest transcribed sex specific proteins to be reported, or DHCs may also be transcribed in asexual parasites, as these were still present in day 1 to 5 of the gametocyte cultures. Since gametocyte cultures are never 100% synchronous, it is also possible that transcription levels of more mature gametocytes were picked up and amplified. If the DHCs are transcribed from stage I onwards, they may have the potential to serve as a novel marker to detect male and female gametocytes from stage I onwards. This would further allow differentiating and quantifying between generations of gametocytes present within natural infections and allow studying sex ratios before and after sequestration, which would provide further information on how the host's immune system affects the sex ratio.

The main problem encountered with dyneins here was persistent human or parasite gDNA contamination and low transcription levels, which was not found to be the case when targeting Pfs16, which was used as a control. Pfs16 was observed to be highly expressed during gametocytogenesis and served as a useful tool in this study to observe the cDNA quality, as it is transcribed at consistent levels throughout gametocytogenesis. From these observations it was concluded that the gDNA contamination problems were specific to the DHCs investigated in this study. Having a human gDNA and cDNA control would have helped to identify the source of contamination. It is not clear whether contamination may have resulted from the amplification of the large number of identical, or possibly of the several isoforms of the *P. falciparum* or human DHCs. Since the DHCs investigated did not contain introns, it was difficult to identify gDNA contamination by RT-PCR, as frequently no gDNA contamination was visible, yet gDNA was picked up by the qRT-PCR assay due to its increased sensitivity. If human DNA was picked up, it is likely that the buffy coat,

which separates the leukocytes from the RBCs, was not fully removed, when the blood was washed, as leucocytes contain DNA.

It is also possible that the DNA contamination occurred during the RNA extraction. During this process the RNA, which is in the form of an aqueous layer, has to be removed from the DNA and proteins. When the aqueous layer is removed it is possible that small amounts of DNA were picked up, leading to the transfer of DNA into the cDNA sample. It is possible that such high quantities of DNA was transferred that the DNase step was not able to destroy the remaining DNA.

More costly and time efficient procedures exist to produce cDNA, yet our assay was designed for use in countries affected by malaria and keeping laboratory costs to a minimum played a role in the choice of our protocol for the synthesis of cDNA. Therefore, future investigations of dynein may benefit from using other methods for cDNA preparation, such as more costly columns that may facilitate RNA purification.

When comparing the expression percentile on PlasmoDB of the DHCs and Pfs16, it becomes evident that expression levels of dyneins are much lower, ranging from approximately 20-80% (maximum 20% for PFI0260c, and maximum of 80% for MAL7P1.162), in comparison to Pfs16, where protein expression is approximately 100% from stage I to V of gametocytogenesis. When considering that gametocyte sex ratios are most frequently observed to be female biased, transcription levels of male mRNA should have higher expression levels than that of female gametocytes, as male gametocytes and therefore also their transcripts will occur in lower numbers. It may therefore be advisable to investigate other DHC genes that have higher expression profiles, particularly at stage V, to evaluate sex ratios from mature gametocytes from natural infections.

Previous bioinformatics studies have shown that certain domains of dynein light chains, for example, reveal high sequence similarities among *Plasmodium* species, yet some sequences are distinct from the human orthologs (Githui *et al.*, 2008). It is therefore advisable to carefully identify regions of target genes that are specific to the target species so that the primers/probes only target a single *Plasmodium* species. Targeting polymorphic regions should also be avoided, as some genotypes may not be recognized by the probe due to differences in the nucleotide sequence. Sequencing different genotypes would allow evaluating whether certain regions of the sequence are polymorphic.

The amino acid sequence 'GPAGTGKTE' which was targeted here, is also recognized as the motif GXXGXGK [TS] (X = any amino acid), known as the Walker loop, or p-loop (phosphate binding loop) and is an ATP binding site found in many nucleotide binding proteins (Walker *et al.*, 1982; Gibbons *et al.*, 1991). This region is highly conserved in the animal kingdom, shown by the boxshade analysis, see figure 4.12.

DHCs have not been well studied in malaria parasites, but are much better understood in other organisms, such as yeast. Eshel *et al.*, 1993, showed disrupting the DYN1 gene in yeast targeting the DHC and amino acid sequence 'PAGTGKT' had no effect on growth or survival in yeast. It is likely that defective DHCs targeting the same amino acid in malaria parasites would not be fatal to the parasite. In this study it was suggested that cytoplasmic dynein in yeast plays a role by walking along the microtubule, towards its minus end, thereby pulling the mitotic spindle in preparation for mitosis. It is possible that the DHCs play a similar role in *P. falciparum* gametocytes, where the chromosomes are rearranged in preparation for three rounds of mitosis, in the case of the male gametocyte, meiosis occurring when the male gamete fuses with the female gamete.

The multi dynein hypothesis (Gibbons, 1988) states that dynein isoforms work together and that many isoforms exist, therefore the disruption of one DHC may not have an effect on the organism (Eshel *et al.*, 1993), which may therefore also be the case when disrupting dyneins in malaria parasites. One study has shown that dynein inhibitors have reduced the number of *P. falciparum* ring stage parasites (Fowler *et al.*, 2001). However, motor molecules have been suggested to compensate for the loss of another motor molecule (Brendza *et al.*, 2000), just as Eshel *et al.*, (1993) suggested. It is therefore possible the dynein knock out was not lethal, as other motor molecules could replace the function of the dynein.

Dyneins are likely to play important, specialized roles in gametocytes, as they are diverse and present in large numbers, many are highly conserved and some serve sex specific functions. It is likely that many isoforms of dyneins have not yet been identified and that studying the roles dyneins play may help to better understand the development of the sexual stages of malaria parasites.

5 SEX RATIO ANALYSIS USING MICROSCOPY

5.1 INTRODUCTION

5.1.1 Sex specific proteins in *P. falciparum*

Currently there are only two proteins that have been used together to distinguish between the sexes when using IFA; Pfg377 (PFL2405c) was used to identify female gametocytes and Alpha-tubulin II (PFD1050w) was used to identify male gametocytes (Severini *et al.*, 1999; Silvestrini *et al.*, 2000b), see figure 5.1 and 5.2. Alpha-tubulin II was previously described as a male specific protein (Rawlings *et al.*, 1992; Guinet *et al.*, 1996; Severini *et al.*, 1999; Silvestrini *et al.*, 2000a; Smith *et al.*, 2002), but has now been shown to be expressed at lower levels in asexual parasites and female gametocytes (Khan *et al.*, 2005; Kooij *et al.*, 2005; Fennell *et al.*, 2008), see table 5.1. However, Khan *et al.*, 2005 was able to use Alpha-tubulin II in transfected *P. berghei* parasites to separate male from female gametocytes using fluorescent flow cytometry, which was possible due to the higher expression levels found in male compared to female gametocytes. Alpha-tubulin II was found not to be strictly sex specific, but still allowed distinguishing between male and female gametocytes when using flow cytometry.

Tubulins belong to the globular proteins, where Alpha-tubulin and Beta-tubulin dimers make up microtubules by forming long filamentous polymers. These tubes assemble by the polymerization of Alpha and Beta dimers; known to aid in chromosome separation, movement of cells and cytoskeletal support (Howard *et al.*, 2003). The importance of microtubules becomes evident when their function is disrupted. Many anti-microtubule agents have shown to cause cell death, killing cancer cells for example, and have also been used as possible antimalarial drug candidates (Fowler *et al.*, 1998; Attard *et al.*, 2006). Three different functional classes of microtubules have been identified in *Plasmodium*; axonemal and flagellar -involved in the movement of the male gamete; subpellicular microtubules, -involved in the cell structure; or motility, and spindle – associated microtubules, -involved in cell division (Bell, 1998). Scanning electron microscopy (SEM) and IFA images have reported that male gametocytes contain all classes of microtubules, whereas female gametocytes have been reported to only have subpellicular microtubules. However, female gametes were reported not to contain any microtubules and male gametes were reported to only contain axonemal microtubules (Sinden, 1982; Bell, 1998).

In *Plasmodium*, two Alpha-tubulins (Alpha-tubulin I and Alpha-tubulin II) (Holloway *et al.*, 1989; Holloway *et al.*, 1990) and one Beta-tubulin are present (Delves *et al.*, 1989). Tubulins are highly conserved proteins, where Alpha-tubulin I and II share 95% of the amino acid sequence (Holloway *et al.*, 1990). Gene knockouts of Alpha-tubulin II revealed that this protein plays a crucial role in the development of asexual parasites, as no viable parasites were produced from these knockouts (Kooij *et al.*, 2005). Yet it is important to note that the transcripts from asexual parasites and gametocytes differed in size, suggesting that Alpha-tubulin II probably does not play an exclusive function in the formation of microtubules of the axoneme (Kooij *et al.*, 2005). Post-translational modifications in Alpha-tubulin II, such as glutamylation, suggest that the same protein may serve different functions (Fennell *et al.*, 2008). Many isoforms may exist, having the potential to cause rapid changes and generate highly dynamic subgroups within the tubulin population (Fennell *et al.*, 2008).

Author	Year	Species	Method	Sex Specific
Rawlings *et al.*,	1992	*P. falciparum*	IFA	Yes
Guinet *et al.*,	1996	*P. falciparum*	IFA	Yes
Severini *et al.*,	1999	*P. falciparum*	IFA	Yes
Silvestrini *et al.*,	2000	*P. falciparum*	IFA	Yes
Smith *et al.*,	2000	*P. falciparum*	IFA	Yes
Kooji *et al.*,	2005	*P. berghei*	Transfection	No
Khan *et al.*,	2005	*P. berghei*	Transfection	No
Fennel *et al.*,	2008	*P. falciparum*	IFA & SDS page	No

Table 5.1: List of publications investigating the sex specificity of Alpha-tubulin II. List of Authors, year of publication, organism studied, and the method used to study Alpha-tubulin II are noted.

Name	Gene ID	Gender	Associated with	Chromosome	Intron	bp
Pfg377	PFL2405c	FG	Osmiophilic bodies	4	No	9360
Alpha-tubulin II	PFD1050w	MG FG	Axoneme, flagella Cytoskeleton?	12	2	1680

Table 5.2: Overview of Pfg377 and Alpha-tubulin II in *P. falciparum*. Pfg377 has only been reported to be present in female gametocytes (FG), whereas Alpha-tubulin II has been reported to be found in male gametocytes (MG) and female gametocytes.

Pfg377 is the only protein that has been associated with osmiophilic bodies and has been reported to be only expressed in *P. falciparum* female gametocytes (de Koning-Ward *et al.*, 2008). These intracellular bodies are membrane bound vesicles and are found beneath the subpellicular membrane from stage III gametocytes onwards,

occurring in fewer numbers in male than female gametocytes (Rudzinska *et al.*, 1968; Sinden, 1983). Osmiophilic bodies are approximately 100-300 nm in diameter and are believed to originate from the Golgi apparatus (Sherman, 2005) and received their name due to the fact that they stain densely with osmium tetroxide. It is believed that by releasing the osmiophilic bodies into the parasitophorous vacuole, the lysis of the RBC membrane is triggered to aid the emergence of the macrogamete (Sinden, 1982; de Koning-Ward *et al.*, 2008). The function of osmiophilic bodies was confirmed when Pfg377 was disrupted and the emergence of the macrogametes from the RBC was reduced, thereby almost entirely blocking all laboratory induced mosquito infections (de Koning-Ward *et al.*, 2008). Osmiophilic bodies have been reported to be specific to the *Plasmodium* lineage and are believed to be solely involved in the process of gametogenesis of these parasites (Hayton *et al.*, 2008).

5.2 AIMS AND OBJECTIVE

Objective: Establish sex ratios from MACS® purified gametocytes using light microscopy and IFA from cultured parasites.

1. Obtain sex ratios of using microscopy from purified stage V gametocytes.
2. Use IFA to observe localization of sex specific proteins of purified gametocytes from stage I to V and activated gametocytes.
3. Establish sex ratios using IFA from purified gametocytes during gametocytogenesis and of activated gametocytes.
4. Compare sex ratios established from light microscopy and IFA.

5.3 MATERIALS AND METHODS

See chapter 3, for general material and methods.

5.3.1 Sex ratio quantification using light microscopy

Mature gametocytes were obtained after 11 days of culturing and parasites were stained, as described in section 3.2.10. Parasites were magnified x 1000 and the sex of the gametocytes was identified based on the five characters described by Carter *et al.*, 1988, see section 1.4.2. The sex ratio was then calculated by dividing the total number of male gametocytes observed, by the total number of male and female gametocytes observed. The sex ratios were calculated when 5 out of 5 or at least 4 out of 5 characters could be identified; the number of undetermined parasites was also noted.

5.3.2 IFA-Immuno Fluorescent Antibody Test

Gametocytes were harvested on day 3 (stage II), 5 (stage III), 7 (stage IV) and 11 (stage V). The sexual stages were MACS® purified as previously described (See section 3.2.8). Following this, gametocytes were washed with ICM; ICM was then added to dilute the resuspended pellet and the amount of ICM added depended on the size of the pellet. To achieve the desired parasite density, a thin film was prepared on a glass slide and analyzed under a light microscope (magnified x 1000) and parasites were either further diluted down with ICM, or washed again and less ICM was added to the pellet until the optimal gametocyte density was achieved. For the observation of activated gametocytes, xanthurenic acid was added onto the slides (see section 3.2.9) and slides containing activated gametocytes were handled in the same way from here on.

The washed parasites were pipetted onto multiwell slides (Hendley Essex); air dried and stored at -20°C in a sealed box, containing silica gel (Sigma-Aldrich) for long term storage. Slides were removed from the freezer, air dried in a 'dry box' containing silica gel for 15 minutes, slides were then fixed in ice cold anhydrous acetone (BDH) for 30 minutes. The slides were carefully washed with PBS (Sigma Aldrich) and were dried between the wells. Next, PBS-1% BSA, 0.1% Tween®20 was added to each well to prevent non specific binding of the antibodies, slides remained for 30 minutes in a humidity box, containing moist paper towels to prevent the wells from drying out.

Next, slides were washed again in PBS and the primary polyclonal antibody Alpha-tubulin II (1:7000 dilution in PBS-1% BSA, 0.1% Tween®20) was added to the wells and incubation lasted for 1 hour. The slides were washed with PBS and the red fluorescent secondary antibody (1:400 dilution in PBS) Jackson Immuno Research

(TRITC)-AffiniPure Donkey Anti-Rabbit IgG (H+L) was added for 30 minutes. The slide was washed again and another primary monoclonal antibody Pfg377 (1:400 dilution in PBS-1% BSA) was added for 1 hour. Slides were washed and the green fluorescent secondary antibody (Jackson Immuno Research, FITC-AffiniPure Donkey Anti-Rat IgG (H+L) was added for 45 minutes.

Briefly, the slides were washed with PBS and 40µl of Vectashield® containing DAPI (Vector Laboratories) was added to the slide, a large coverslip was mounted and slides were sealed and stored at 4°C for a maximum of 2 days until visualized and photographed by confocal microscopy (Zeiss Axioplan LSM510). Parasites were analyzed using a 488nm wavelength for FITCI (Pfg377-female gametocytes), 543nm wavelength for rhodamine (Alpha-tubulin II-male gametocytes), and 403nm wavelength for DAPI (staining the nuclear material). Parasites were photographed and analyzed with the help of the Zeiss LSM software and parasites were magnified x 1000 using immersion oil. The anti-Pfg377 antibody (anti-rat) was a kind gift of Pietro Alano and anti-Alpha-tubulin II (anti-rabbit) was obtained by the MR4 (MRA-37 MR4, Manassas, VA.), courtesy of David Baker.

To observe the activated gametocytes Alpha-tubulin II and Pfs16 antibodies were used together. Procedures were the same as described above, apart from using Pfs16 instead of Pfg377. Pfs16 (1:400 dilution in PBS-1% BSA) was incubated for an hour and used with the green fluorescent secondary antibody (1:400 dilution in PBS) Jackson Immuno Research (TRITC)-AffiniPure Donkey Anti-Rabbit IgG (H+L). The secondary antibody was incubated for 30 minutes and slides were prepared as described above.

5.3.3 Application of Image ProPlus 6.3 for sexing gametocytes using IFA

IFA images were analyzed with the Image-ProPlus 6.3 software, which allowed tagging individual gametocytes according to stage and colour. From stage III onwards both antibodies were detectable and sex ratios could be measured. Parasites staining only red (Alpha-tubulin II) were identified as males, whereas parasites staining with red and green (Pfg377 and Alpha-tubulin II) were counted as females (reasoning for sex ratio determination is described in the results section, see section 5.4.4). Gametocytes that only stained with DAPI were also noted. The use of this software allowed noting 'sex ratios' in regard to the maturity of the gametocytes and allowed to tag individual gametocytes, which facilitated sex ratio quantification using IFA.

5.4 RESULTS

5.4.1 Sex ratios established using light microscopy

Giemsa stained stage V (day 11) purified parasites were sexed based on the five characters stated by Carter *et al., 1988,* described in section 1.4.2. MACS® purification allowed observing high densities of gametocytes on a single slide which facilitated sex ratio quantification, see figure 5.2 A.) and B.). Purification allowed on average to see approximately 20 x more gametocytes per field of view, compared to observing non-purified parasites taken from cultures. Sex ratios were found to be female biased, having a sex ratio of 0.12 to 0.14, when at least 4 out of five characters were identified, see table 5.1. Characters that were frequently difficult to differentiate in male and female gametocytes include the size of the cell (males are smaller than females) and the shape of the cell (the ends of the cells are more round in male than in females). Characters that were more reliable for the identification of the sex included the colour (males stain pink and females purple), and the size and localization of the nuclear material (the pigments are more centrally located in females and the nucleus is larger in males). Sex ratios were measured from three separate rounds of culturing.

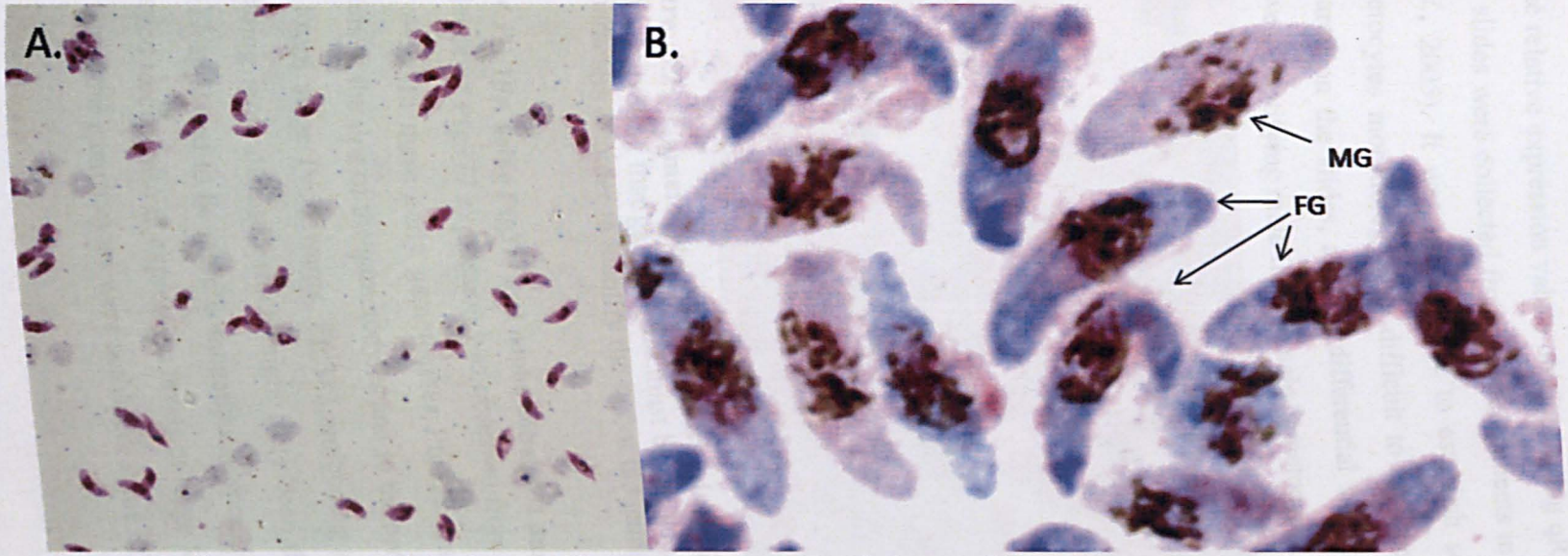


Figure 5.1: Sex ratios established using light microscopy. Purified stage V gametocytes stained with Giemsa. A.) 3D7 MACS® purified gametocytes magnified x 1000. B.) Male gametocytes (MG) can be seen to stain pink and female gametocytes (FG) can be seen to stain purple, when magnified x 1000.

Culture	N	SR: 5/5 characters	SR: 4/5 characters
1	110	0.14 (57%)	0.13 (92%)
2	114	0.12 (65%)	0.14 (95%)
3	116	0.14 (66%)	0.12 (96%)
Total	**340**	**0.14**	**0.13**

Table 5.1: Sex ratio established using light microscopy. Thin film slides were prepared from three separate cultures. Gametocytes counted (N), and total percentage of parasites identified depending whether four or five characters were noted are shown. The average sex ratio (SR) depending on the total number of characters identified was also calculated.

5.4.2 Sexing ratios established from natural infections

In this study it was planned to compare sex ratios obtained from cultures and from naturally infected hosts using light microscopy first, then later compare these sex ratios to the relative expression values obtained using qRT-PCR, discussed in chapter 6. Thin film slides were collected from infected patients in The Gambia from 2001 (Sutherland *et al.*, 2005). It was not possible to establish sex ratios from these slides, as the gametocytes morphology was difficult to see due to way the gametocytes had been prepared on the slides, also no differential staining was observed which would have allowed identifying male from female gametocytes, see figure 5.3.

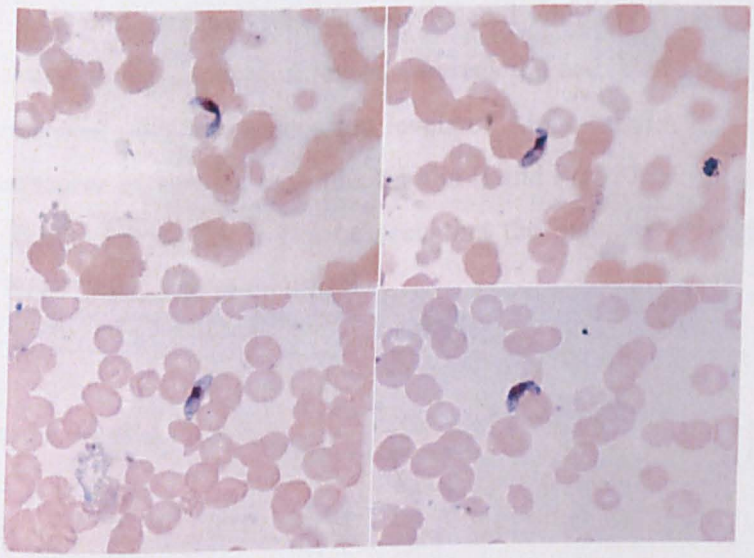


Figure 5.2: Gametocytes from natural infections. Four gametocyte images are shown, revealing that gametocytes must be prepared carefully to allow the visualization of characters in order to identify the sex of the gametocyte.

5.4.3 IFA using Pfg377 and Alpha-tubulin II during gametocytogenesis

Evidence of Pfg377 expression was observed from early stage III gametocytes onwards (day 4-5). In these early gametocytes Pfg377 antibodies were appearing as granular clusters at the tips of the gametocytes and in the centre of the cell, see figure 5.4 A.) and B.). From stage IV onwards, Pfg377 antibodies appeared to fill out the gametocyte, appearing more diffuse, which might have been due to the change of localization, as Pfg377 appeared to be more homogenously spread throughout the parasite and was not observed to decrease in expression levels of stage V gametocytes, see figure 5.4 C.) and D.). Once the gametocytes were activated, some of the Pfg377 clusters reappeared near

the circumference of the parasitophorous vacuole, see figure 5.4E.) and F.) and figure 5.6 (1A.-E. and 2 A.-E.).

Expression of Alpha-tubulin II was detected as early as stage I, even though expression appeared very weak at this stage, compared to the later stages, see figure 5.5 A.). Overall, expression was found to increase as the gametocytes matured, see figure 5.5 (A.- G.). From stage II to III Alpha-tubulin II was found to be expressed along the straight edges of the cell, and striations were seen throughout the cell, see figure 5.5 B.), C.) and D.). From stage I to III, Alpha-tubulin II was found to be expressed equally in all gametocytes, yet at stage IV gametocytes reacting with anti-Pfg377 revealed much lower expression levels than those gametocytes not expressing Pfg377. Particularly, the activated gametocytes that only reacted with anti-Alpha-tubulin II antibodies revealed very strong expression levels, whereas the activated gametocytes reacting with Pfg377 showed very low expression levels of Alpha-tubulin II, see figure 5.5 F.) and G.). To observe expression of Alpha-tubulin II and Pfg377 during gametocyte development (see figure 5.6 A.-E.), it can be seen that the majority of mature gametocytes react with Pfg377 and Alpha-tubulin II.

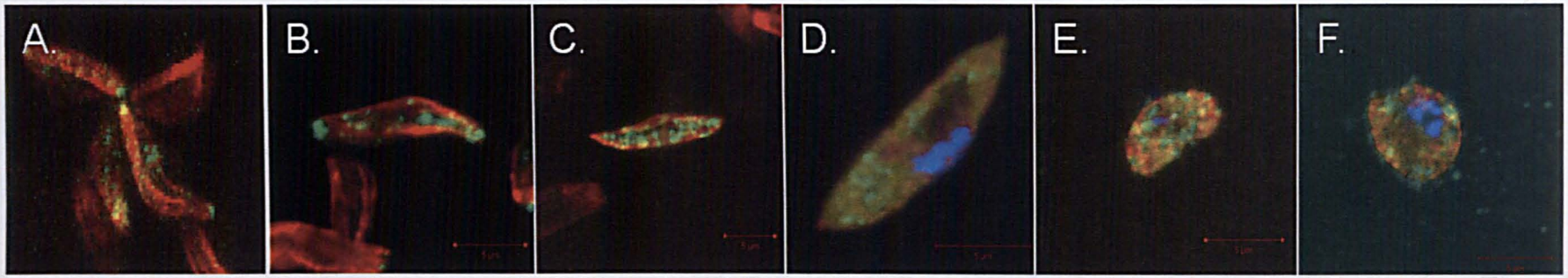


Figure 5.3: Gametocytes reacting with anti-Pfg377 and anti-Alpha-tubulin II antibodies. Gametocyte revealing expression of Pfg377 (green) from stage III onwards stage III A.), late III B.), IV C.), late IV D.), V E.), activated gametocyte F.). Pfg377 appears more granular in stage V gametocytes again and the granular appearance of Pfg377 can be seen again on the circumference of the cell of the activated gametocyte. Nuclear material was stained with DAPI, appearing blue in colour. Parasites were magnified x 1000.

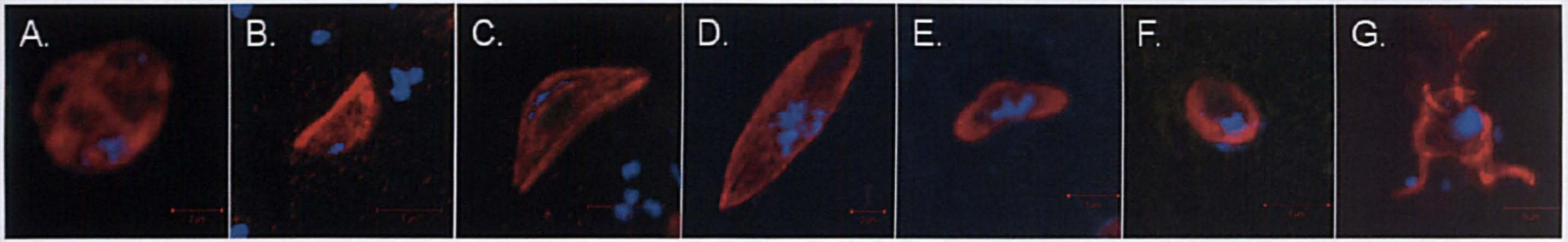


Figure 5.4: Gametocytes reacting with anti-Alpha-tubulin II antibodies. Gametocytes revealing expression of Alpha-tubulin II (red) during gametocytogenesis. Alpha-tubulin II was found to be expressed from stage I A.), II B.), III C.), IV D.), V E.) and in activated gametocytes F.), and during exflagellation G.). Striations can be seen throughout the cell, particularly at stage III C.), suggesting that Alpha-tubulin II could be involved in the structural support of the cell. Nuclear material was stained with DAPI, appearing blue in colour. Parasites were magnified x 1000.

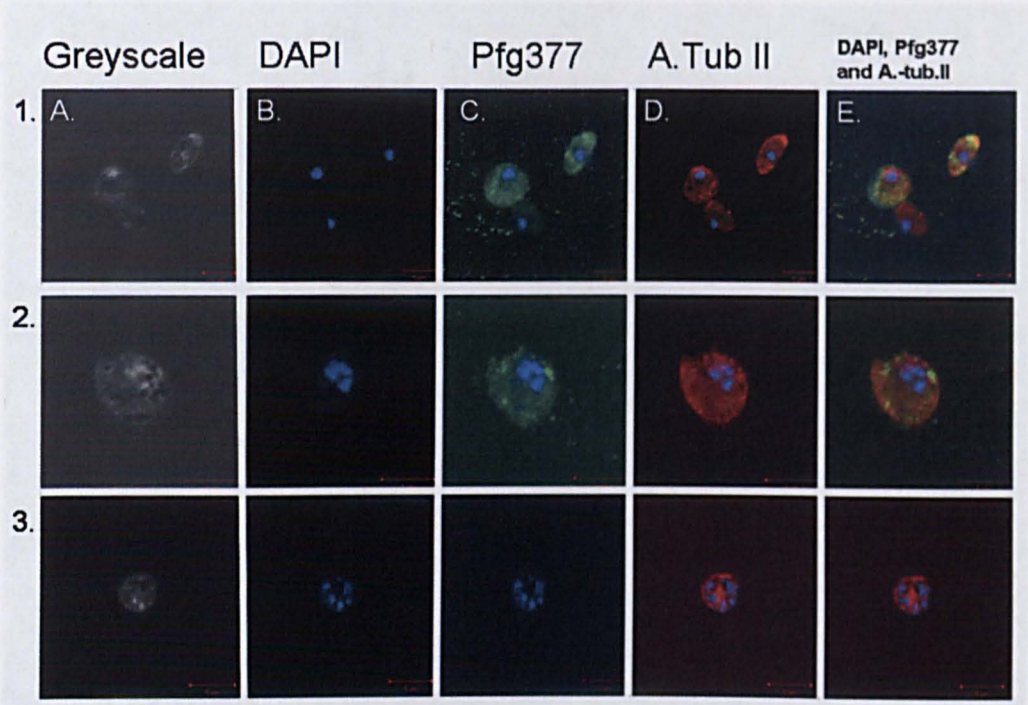


Figure 5.5: Expression of Alpha-tubulin II and Pfg377 in activated gametocytes. A) showing gametocytes in the greyscale, B.) only DAPI is visualized, C.) only FITCI is visualized (Pfg377), D.) only rhodamine is visualized (Alpha-tubulin II), E.) all three fluorophores are visualized. Release of Pfg377 can be seen in the images 1 and 2 C.) and 1 and 2 E.). In 2 C.) and E.) accumulation of Pfg377 can be seen at the circumference of the activated gametocyte. Possibly six nuclei can be seen in an activated male gametocyte (A.-E.). The size difference between activated gametocytes becomes evident when comparing images 2 and 3. Parasites were magnified x 1000.

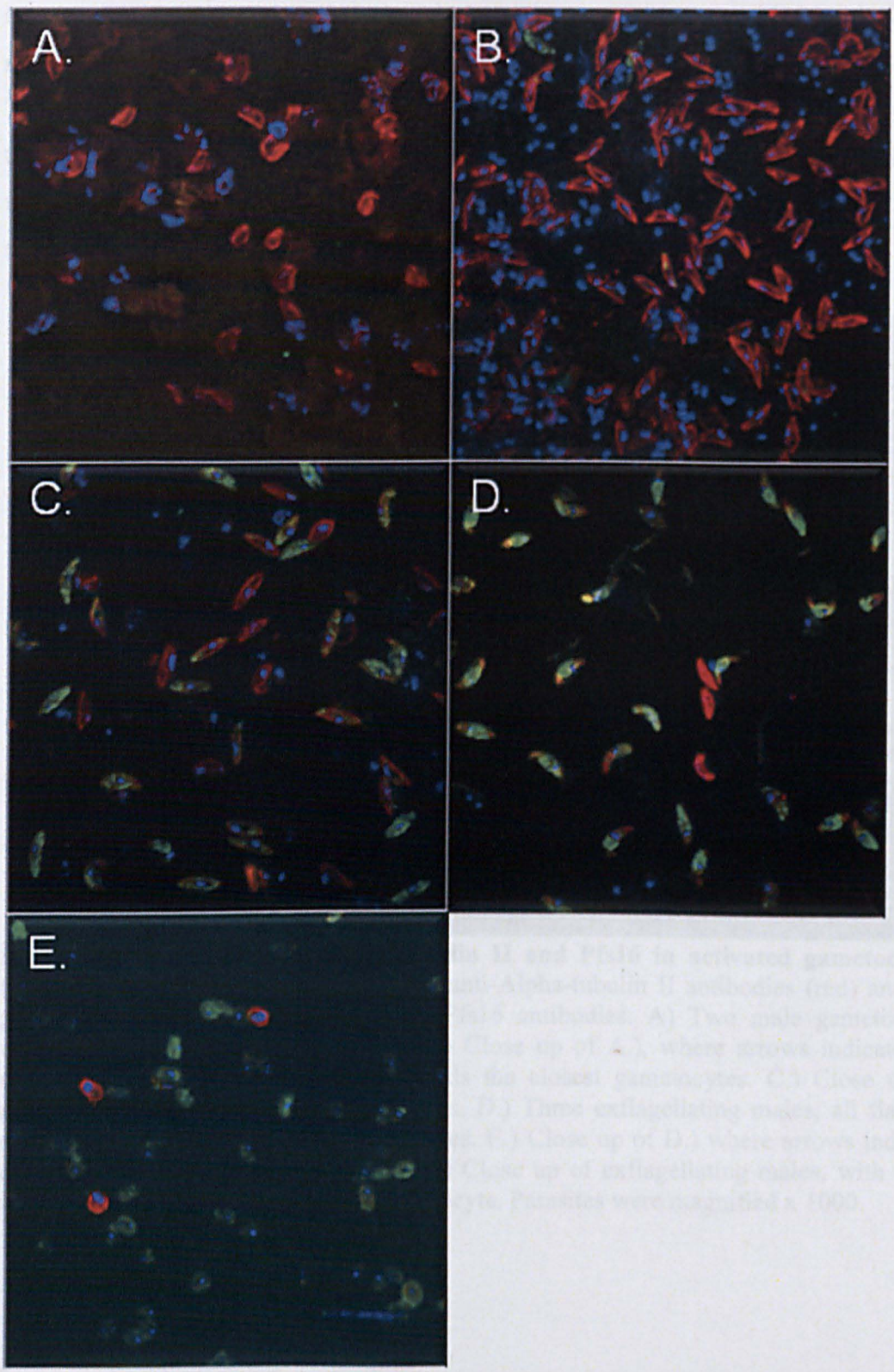


Figure 5.6: Differential staining of gametocytes during gametocytogenesis. IFA slides were prepared on A.) day 2 (stage II), B.) day 5 (stage III), C.) day 7 (stage IV); D.) day 11 (stage V), E.) and after gametocyte activation (AG). Parasites were magnified x 1000. B) reveals many parasites that were damaged during the purification process, as they only stained with DAPI.

One interesting observation was noted during gametocyte activation. The flagella of the microgametes were frequently observed to be directed towards the closest gametocytes. This could be due to the sticky nature of the flagella, but when no gametocytes were in the vicinity the flagella were frequently found not to be extended, raising the possibility that male gametes may be attracted by the nearby gametocytes and can direct their flagella towards them, see figure 5.8 A.-F.).

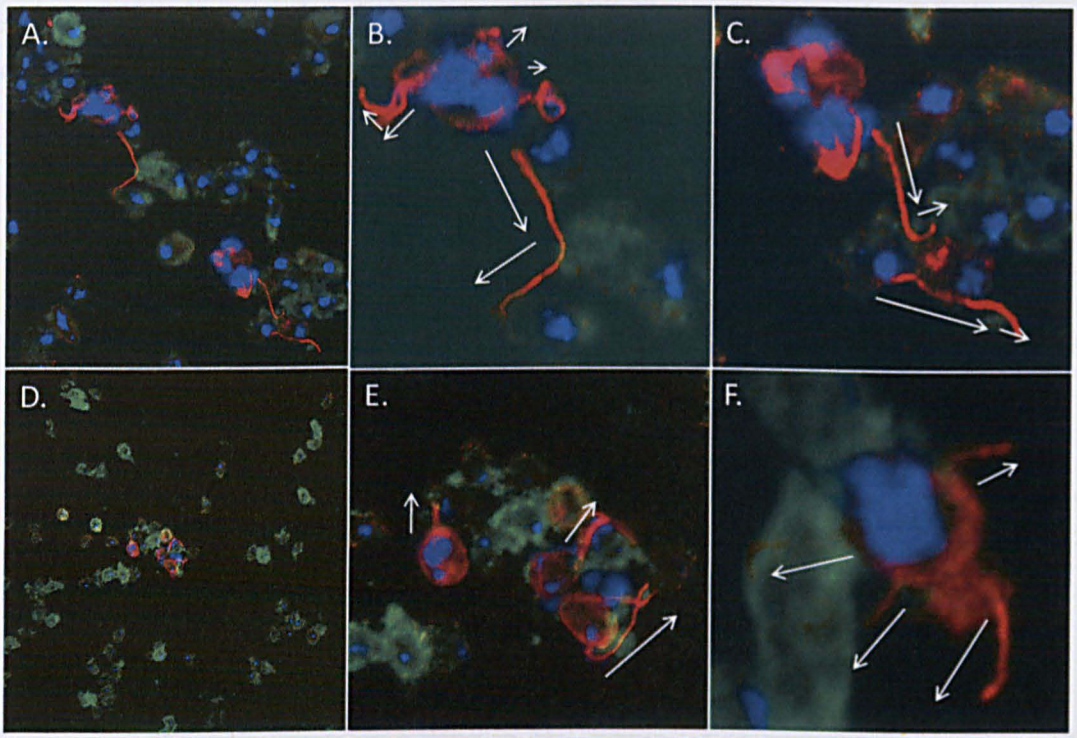


Figure 5.7: Expression of Alpha-tubulin II and Pfs16 in activated gametocytes. Male flagella can be seen to react with anti-Alpha-tubulin II antibodies (red) and the parasitophorous vacuole reacting with Pfs16 antibodies. A) Two male gametocytes undergoing exflagellation are shown B.) Close up of A.), where arrows indicate the direction of the flagella pointing towards the closest gametocytes. C.) Close up of flagella pointing at close by gametocytes. D.) Three exflagellating males, all flagella can be seen to contact nearby gametocytes. E.) Close up of D.) where arrows indicate directions the flagella are pointing at F.) Close up of exflagellating males, with three flagella pointing towards a nearby gametocyte. Parasites were magnified x 1000.

5.4.4 Distinguishing male and female gametocytes using IFA

When counting stage V gametocytes reacting with anti-Alpha-tubulin II and anti-Pfg377 antibodies as females and gametocytes reacting only with Alpha-tubulin II as males, a female biased sex ratio was observed, similar to what was found by using light microscopy. It was therefore concluded that Pfg377 must be female specific and anti-Alpha-tubulin II was found to react with both sexes, as previously suggested by Kooij, *et al.,* (2005). However, Alpha-tubulin II showed far higher expression levels in male gametocytes than in female gametocytes from stage IV onwards, which allowed distinguishing between male and female gametocytes, see figure 5.9.

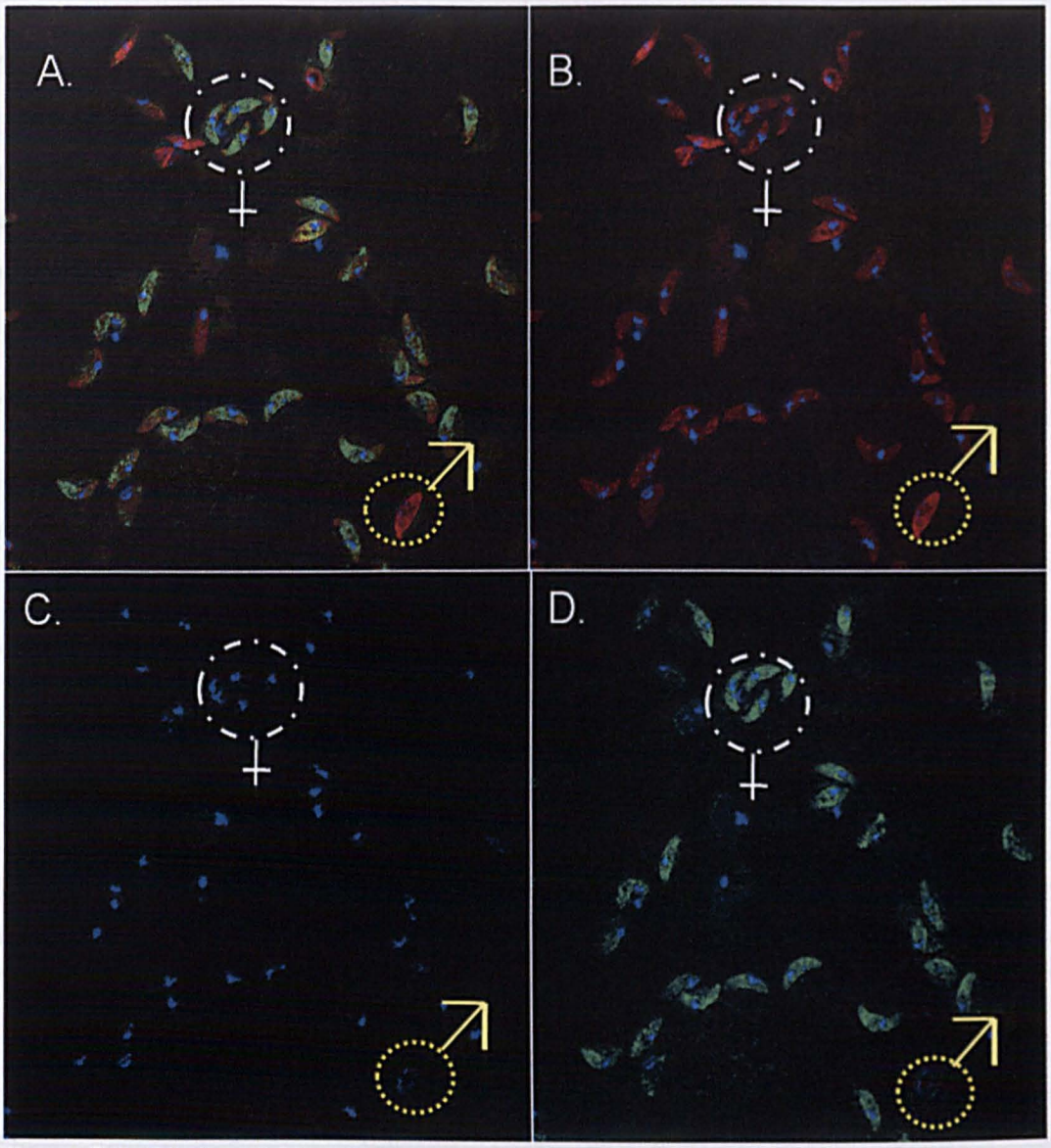


Figure 5.8: Distinguishing between male and female gametocytes. A.) IFA image of stage V gametocytes showing all fluorophores from the secondary antibodies and DAPI (red, green and blue). B.) Only rhodamine (red) is visualized, which reacts with anti-Alpha-tubulin II antibodies. C.) Only the DAPI (blue) is visualized, reacting with the nuclear material. D.) Only FITCI (green) is visualized, reacting with anti-Pfg377 antibodies. The white dotted circle shows gametocytes staining with anti-Pfg377 and anti-Alpha-tubulin II (females), whereas the yellow dotted circle reveals one gametocyte that only reacts with anti-Alpha-tubulin II antibodies (male). It can be seen that gametocytes which reacted with anti-Pfg377 antibodies, also reacted with anti-Alpha-tubulin II antibodies, when comparing B.) and D.). Parasites were magnified x 1000.

5.4.1 Percentage of gametocytes reacting with antibodies during gametocytogenesis

Not all gametocytes observed with DAPI reacted with any antibody. The proportion of gametocytes reacting with antibodies was found to increase over time, see figure 5.10 and table 5.2. Gametocytes not reacting with antibodies may be an artefact from the slide preparation. However, dying asexual parasites were frequently observed until stage III (days 5-6) and Alpha-tubulin II expression was very low from stage I-II and may not have been visualized at these early stages.

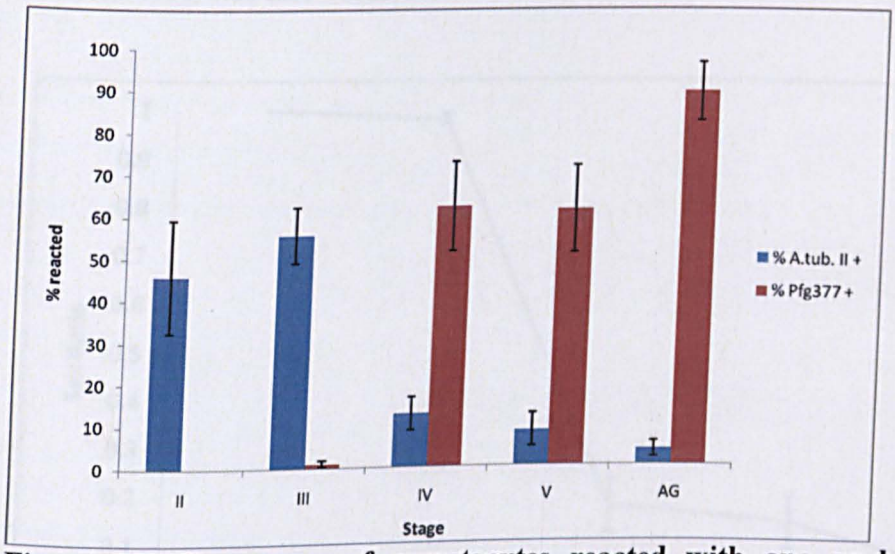


Figure 5.9: Percentage of gametocytes reacted with one or both antibodies. Representation of gametocytes reacted with either Alpha-tubulin II (blue), or Pfg377 (red) during gametocyte development, the error bars are also shown.

Stage	II	III	IV	V	AG
% Alpha-tubulin II +	46.06	55.74	12.80	8.48	3.69
r* of Alpha-tubulin II	0.2875	0.2632	0.5324	0.6664	0.9500
% Pfg377 +	0.00	1.14	62.28	61.44	89.62
r* of Pfg377 +	-	-	0.2480	0.2500	0.2027
S. E. of Alpha-tubulin II	13.40	6.56	3.93	4.01	1.92
S. E. of Pfg377	0.00	0.76	10.71	10.48	6.94

Table 5.2: Percentage of gametocytes which reacted with one or both antibodies. Numbers reveal that in early stages only around 50% of gametocytes reacted with the antibodies, compared to more than 90% of the gametocytes reacting with antibodies when activated gametocytes were observed. *$p < 0.5$

5.4.2 Measuring sex ratios using IFA

The 'apparent' sex ratio calculated using IFA, is not a true sex ratio, but an observed sex ratio, based on the expression of Pfg377 and Alpha-tubulin II. When measured by IFA, the 'apparent sex ratio' decreased from being 100% 'male' to almost all 'female' at the later stages, see figure 5.11. Parasites started reacting with Pfg377 from late stage III to early stage IV onwards; therefore it was only possible to measure sex ratios from stage IV onwards using IFA. A further decrease in the sex ratio after gametocyte activation was induced, was also observed.

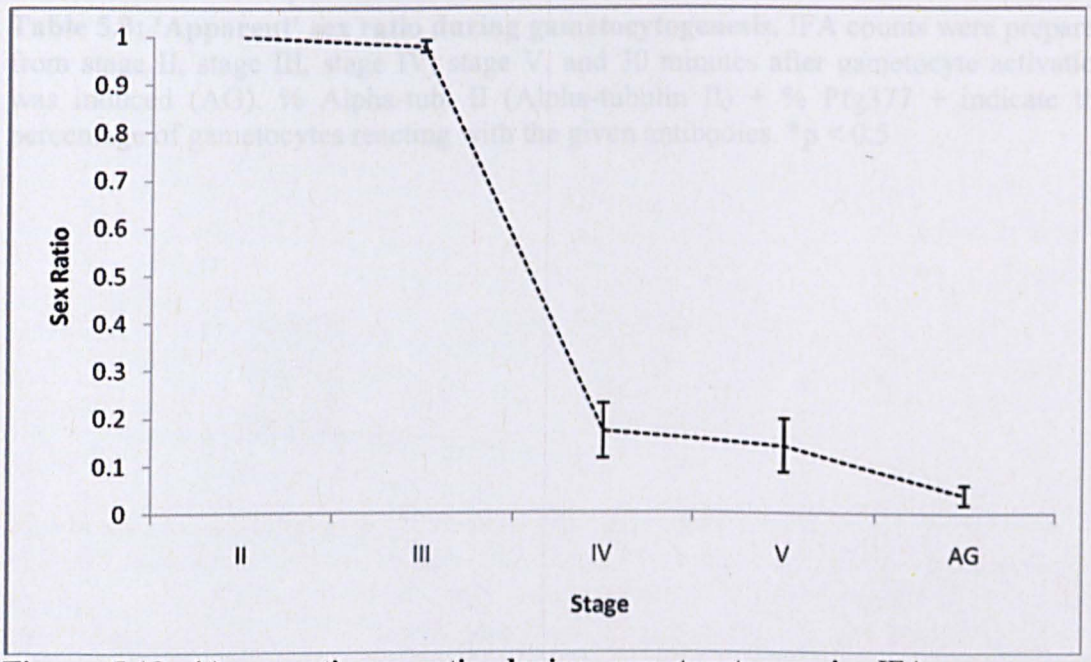


Figure 5.10: 'Apparent' sex ratio during gametocytogenesis. IFA counts were prepared from stage II, III, IV, V, and 10 minutes after gametocyte activation was induced. The standard error bars are also shown, being largest in stage IV gametocytes.

Stage	II	III	IV	V	AG
DAPI	129	611	207	1916	350
r * of DAPI	0.1723	0.1381	0.1381	0.1381	0.1381
% sexed	45.0	57.1	72.9	81.7	88.6
Alapha-tub.II +	58	342	26	221	12
r * of A.-tub.II	0.2564	0.1381	0.3809	0.1381	0.5529
Pfg377/A.tub. II +	0	7	125	1344	298
r* of Pfg377/A.tub.II	-	0.7067	0.1750	0.1381	0.1381
% Alpha-tub. II +	45	56	13	12	3
% Pfg377 +	0	1.1	60.4	70.15	85.1
Sex ratio	1	0.980	0.17	0.14	0.039
Standard error	0	0.011	0.06	0.056	0.021

Table 5.3: 'Apparent' sex ratio during gametocytogenesis. IFA counts were prepared from stage II, stage III, stage IV, stage V, and 30 minutes after gametocyte activation was induced (AG). % Alpha-tub. II (Alpha-tubulin II) + % Pfg377 + indicate the percentage of gametocytes reacting with the given antibodies. *$p < 0.5$

5.4.3 Comparison of the activation of male and female gametocytes

During normal slide preparation (non induced activation), it was noted that a large percentage of male gametocytes were activated (30%), compared to the few female gametocytes that were activated (1%). Interestingly, when gametocytes were activated with xanthurenic acid (active induction), 100% of the male gametocytes were found to be activated, compared to 94% of female gametocytes, see figure 5.12 and table 5.4.

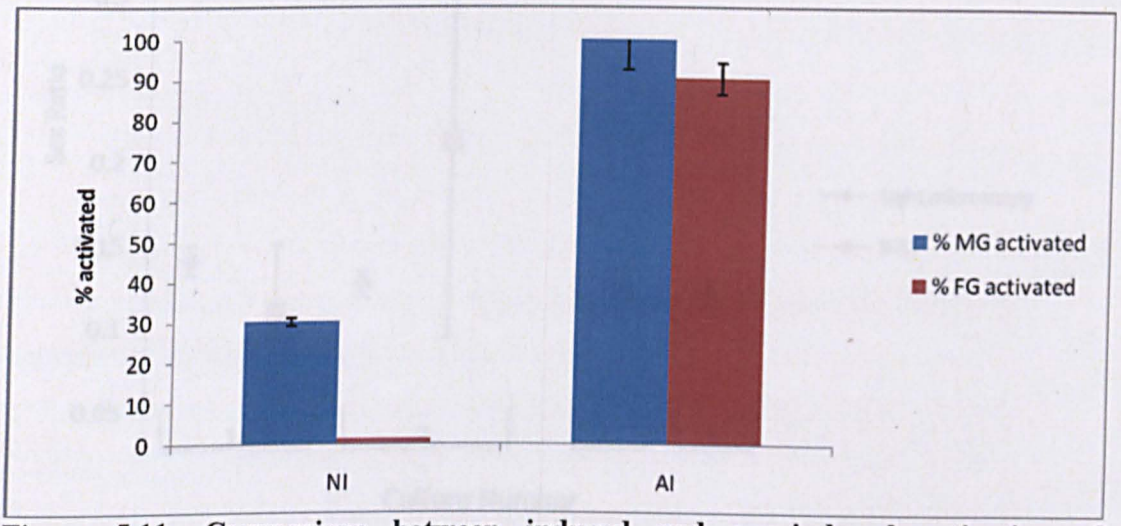


Figure 5.11: Comparison between induced and non-induced activation of gametocytes. Representation of gametocytes reacted with either Alpha-tubulin II (blue), or Pfg377 and Alpha-tubulin II (red) during gametocyte development. SE (Standard error) bars are shown. NI means non induced activation and AI stands for active induction.

	NI	AI
DAPI	974	350
r* of DAPI	0.1381	0.1381
A.tub.II +	103	12
r* of A.tub.II +	0.1927	0.5529
A.tub.II+/Pfg377 +	771	298
r* of A.tub.II+/Pfg377 +	0.1381	0.1381
% MG activated	30.1	100
Standard error	1.08	7.45
% FG activated	1.0	90.6
% sexed	89.7	94
Standard error	0.07	3.82

Table 5.4: Comparison between induced and non induced gametocytes. Percent of gametocytes found activated, not being induced (NI) and 10 minutes after activation was induced (AI). *$p < 0.05$

5.4.4 Comparing the sex ratios using light microscopy and IFA

When comparing the sex ratios from stage V cultured gametocytes, obtained from IFA and light microscopy, it was found to range from 0.11 to 0.20, see figure 5.13 and table 5.5. The largest variation of sex ratios observed was found when using IFA.

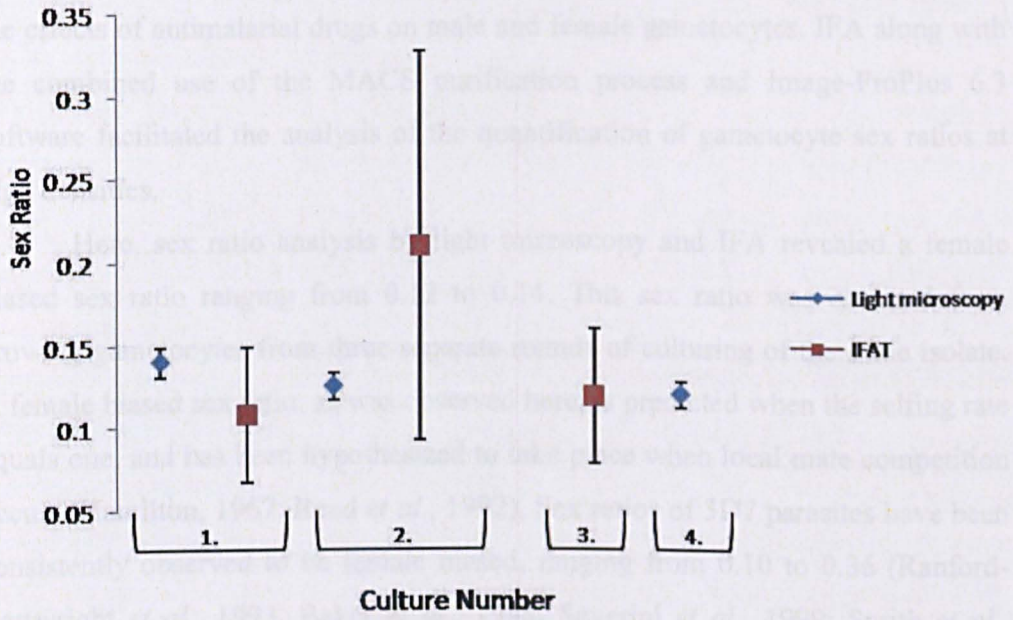


Figure 5.12: Comparison of the sex ratios from light microscopy and IFA. Sex ratios from a total of four separate rounds of culturing are shown with the respective error bars.

Culture	1	2	3	4
IFA	**0.108**	**0.210**	**0.119**	*
Standard error	0.041	0.118	0.041	*
Light microscopy	**0.140**	**0.125**	*	**0.120**
Standard error	0.009	0.008	*	0.008

Table 5.5: Comparison of sex ratios using light microscopy and IFA. Comparison of sex ratios from four separate rounds of culturing. Sex ratios were measured from stage V (day 11) gametocytes from light microscopy and IFA counts. For light microscopy counts, sex ratios were established from at least four out of five characters.

5.5 DISCUSSION

This study is one of the first to establish sex ratios by observing the expression and localization of proteins both in activated gametocytes and during gametocyte development at high densities. Such observations of morphological characters during gametocytogenesis and the establishment of sex ratios using IFA may be of interest in future studies, for example, in the high throughput screenings to study the effects of antimalarial drugs on male and female gametocytes. IFA along with the combined use of the MACS purification process and Image-ProPlus 6.3 software facilitated the analysis of the quantification of gametocyte sex ratios at high densities.

Here, sex ratio analysis by light microscopy and IFA revealed a female biased sex ratio ranging from 0.12 to 0.14. This sex ratio was obtained from growing gametocytes from three separate rounds of culturing of the same isolate. A female biased sex ratio, as was observed here, is predicted when the selfing rate equals one, and has been hypothesized to take place when local mate competition occurs (Hamilton, 1967; Read *et al.*, 1992). Sex ratios of 3D7 parasites have been consistently observed to be female biased, ranging from 0.10 to 0.36 (Ranford-Cartwright *et al.*, 1993; Baker *et al.*, 1995; Severini *et al.*, 1999; Smith *et al.*, 2000). Such a female biased sex ratio may be genetically predisposed in 3D7 parasites, and/or may be present because parasites are of a single genotype. Slight variation of the observed 3D7 sex ratio from several studies may be due to the inaccuracy of sexing gametocytes by eye, or to the ability of clones to change their sex ratio (Graves *et al.*, 1984). Sex ratios in this study from non-purified gametocytes revealed a similar female biased sex ratio to the purified gametocytes, suggesting that the purification process did not affect one sex more than the other.

IFA results of this study supported the findings of Kooji *et al.*, 2005, Khan *et al.*, 2005 and Fennel *et al.*, 2008, as it was observed that Alpha-tubulin II is not strictly sex specific, but it is also expressed at lower levels in female gametocytes. However, due to large differences in expression levels, Alpha-tubulin II can be used for IFA to discriminate between the sexes. Earlier studies reported that Alpha-tubulin II was only expressed in male gametocytes (Rawlings *et al.*, 1992; Guinet *et al.*, 1996; Severini *et al.*, 1999; Silvestrini *et al.*, 2000a; Smith *et al.*, 2000) and that only between 2-6% of gametocytes reacted with Alpha-tubulin II

and Pfg377 (Silvestrini *et al.*, 2000a; Smith *et al.*, 2000). It is likely that these studies attributed background staining to the female gametocytes that showed low expression levels, which can be difficult to discriminate when the secondary antibodies bind to 'non specific' material. These studies also reported that small fractions of Pfg377 were reported to be expressed in male gametocytes (Silvestrini *et al.*, 2000; Smith *et al.*, 2000), which was not observed in this study. IFA sex ratio analysis allowed sexing gametocytes from stage IV onwards, as Pfg377 was only expressed in late stage III gametocytes and gametocyte cultures which were not 100% synchronous.

Approximately 70% of the gametocytes were sexed using IFA, compared to almost 95% when at least 4/5 characters were identified using light microscopy using Giemsa staining. This suggests that light microscopy may be a more accurate method for sexing gametocytes; but it should be noted that after gametocyte activation up to 95% percent of gametocytes were able to be sexed using IFA. This difference in the ability to sex gametocytes at stage IV/V and after gametocyte activation could be explained by the differences in the purification processes and slide preparation. As in the case of some stage IV/V gametocytes, some samples contained a greater number of gametocytes; and due to the technical difficulties in obtaining large amounts of activated gametocytes, these purified samples had far fewer parasites. From this study it can be concluded that IFA analysis can be used to establish accurate sex ratios, just as light microscopy, when purification and slide preparation are performed carefully and with a trained eye. Particular care needs to be taken not to wash the MACS® columns too many times and to prepare slides with an optimum number of gametocytes per field of view, e.g. not more than 100 parasites per field of view.

When sexing Giemsa stained gametocytes using light microscopy, only mature stage V gametocytes could be sexed, mainly due to the lack of differential staining and the difficulties identifying the characteristics allowing for differentiation between male and female gametocytes. Since cultures are never fully synchronous, an immature female gametocyte might be classified as a male gametocyte due to fewer ribosomes being present (which could result in a less purple appearance), or an immature male might appear like a female gametocyte due to its acicular shape. Only approximately 57-66% of stage V parasites were sexed by light microscopy when all five characteristics were identified; however, when excluding at least one of the characteristics, approximately 92-96% of the

parasites could be sexed. This study suggests that not all five characteristics are needed to be identified to establish sex ratios. Most frequently the characters such as females are larger than males and the shape of the end of the cells were not distinctive enough to make a conclusive decision, however colour of the gametocytes and the size of the location of nuclear material were the most reliable and distinctive characters to distinguish between male and female gametocytes.

IFA revealed that expression of Alpha-tubulin II was observed from stage I onwards, increasing during gametocytogenesis, as previously reported (Fennell *et al.*, 2008). However, many gametocytes did not react with Alpha-tubulin II at stage I and II, which may have been due to a combination of factors. It is likely that expression levels were too low for detection and frequently asexual 'contamination' within the cultures is observed until day 5. These asexual stages were most likely picked up with DAPI, but not by Alpha-tubulin II antibodies.

When observing stage II and III gametocytes, high expression levels of Alpha-tubulin II were seen at the 'straight' side of the developing cell. This coincides with the large amounts of sub-pellicular microtubules present at these locations observed by electron microscopy (Sinden, 1982). In this study it cannot be excluded that Alpha-tubulin II cross reacted with Alpha-tubulin I, as they are 94.5% similar at the amino acid level and both are observed in the sexual stages (Holloway, 1990). However, one study suggested that no cross reaction occurred between Alpha-tubulin II and Alpha-tubulin I during SDS page (Fennell *et al.*, 2008) and Alpha-tubulin II antibodies used in this study were obtained from the same source (MR4). Since Alpha-tubulin II is far more abundant in male than in female gametocytes (Khan *et al.*, 2005; Kooij *et al.*, 2005; Fennell *et al.*, 2008), it is likely that the antibodies used here did not cross react, as lower expression levels were observed in the female compared to the male gametocytes.

During stage V, Alpha-tubulin II expression was observed to be more homogenously distributed within the cell, compared to the earlier stages, but expression levels were seen to be highest towards the tips of the cell. Sinden, (1982) suggested that sub-pellicular microtubules disappeared once the pellicular membrane is fully formed in mature gametocytes and it is the sub-pellicular microtubules that are responsible for organizing the assembly of the pellicular membranes (Sinden, 1982). Observation of the expression of Alpha-tubulin II supports Sinden's findings, as in mature stages no aggregations of Alpha-tubulin II were observed at the circumference of the cell. This loss of the sub-pellicular

microtubules allows the gametocyte to lose its acicular shape and forms into a more rounded shape, believed to aid in sequestration (Nacher, 2004).

Microtubules possess the ability to rapidly polymerize and depolymerise to change their location and function; therefore it is likely that the Alpha-tubulin II proteins reassemble into different types of microtubules. Interestingly, no morphological study has previously mentioned that a greater number of microtubules are present in male gametocytes, compared to female gametocytes. For example, Sinden (1982) did not report the presence of more microtubules in males than female gametocytes in his electron microscopy studies. It is possible that male gametocytes contain more Alpha and Beta tubulin throughout development, as the mature male gametocytes will require large amounts of axonemal microtubules formed during the activation process of the gametocyte. Yet it is possible that male gametocytes, which will undergo several rounds of cell division, contain higher levels of spindle associated microtubules.

Pfg377 was observed to be expressed from stage III onwards, as previously reported (Alano *et al.*, 1995b; Severini *et al.*, 1999; Silvestrini *et al.*, 2000a). This study supports that Pfg377 is strictly female specific, even though osmiophilic bodies have been reported to occur in male gametocytes (Sinden, 1982). The observations of Pfg377 accumulating at the circumference of the activated gametocyte and being released after gametocyte activation supports the theory that osmiophilic bodies aid in the emergence of the macrogamete (de Koning-Ward *et al.*, 2008). Also, similar granules have been described in asexual stages, reported to be released after merozoite invasion (Culvenor *et al.*, 1991), most likely serving similar functions of Pfg377 in females. However, it is also possible that Pfg377 serves more than one function, as Pfg377 could be also released to attract nearby male gametes by chemotaxis, for example. A mechanism like this could increase the chances of fertilization, or even increase the chances of outbreeding if the male gametes could recognize Pfg377 from different genotypes. It was observed here that flagella were directed towards nearby gametes, suggesting that male gametes might be able to sense the presence of female gametes. It may even be possible that Pfg377 polymorphic regions, may aid in the recognition of certain genotypes, thereby facilitating the chances of outbreeding. It would be of great interest to further investigate whether chemotaxis could play a role in transmission of malaria parasites and use this information for studying transmission blocking strategies.

Another possible function of Pfg377 is that it could serve as a 'smoke screen', after it is released from the RBC, to deter antibodies from binding to the exflagellating male gametes, for example. This could also explain the polymorphic region of Pfg377, as this would aid the gametocyte to change the target of its smoke screen. An investigation evaluating if Pfg377 antibodies are found in natural infections would shed some light on this question.

Interestingly, it was also observed that the sex ratio decreased further after gametocytes were activated. This may have been due to an artefact of slide preparation, e.g. exflagellating males were washed off. Otherwise it may be possible that male gametocytes are activated faster than female gametocytes and that male gametes were not noted, due to their small size, it is also possible that male gametocytes are more fragile and are destroyed during the activation process.

However, no study has observed the life span of *P. falciparum* male and female gametocytes *in vitro*. Since male gametocytes undergo three rounds of mitotic division to produce eight gametes, compared to producing one female gamete, it is possible that several gametes produced are actually non-functional. It was also observed using IFA that some flagella did not contain any gametes, it is likely that during the exflagellation process, some flagella fail to draw out the male gamete.

A lower fecundity of males could also explain the observation that increased infectivity occurs when the sex ratio is more male biased at low gametocyte densities (Mitri *et al.*, 2009), as more male gametocytes are needed to ensure fertilization; but once a threshold is reached, enough males are present to fertilize all the female gametocytes.

Competition among male gametes to fertilize a female gamete is also likely to exist, as timing is crucial: every male gamete is in a race to be the first one to encounter a female gamete to ensure fertilization. Male gametes may, therefore, be under selection pressure to be activated more rapidly to increase the chances of finding a mate.

This study has shown a novel method that allows quantifying sex ratios using IFA with one sex specific protein, Pfg377 and one non-sex specific protein Alpha-tubulin II. Slides prepared from natural infections from The Gambia did not allow successful sex ratio quantification, indicating that slides must be prepared carefully to allow for the sexing of gametocytes. An additional novel

method is therefore needed to facilitate sex ratio quantification from large field samples and to better understand the factors that may shape gametocyte sex ratios.

6 SEX RATIO ANALYSIS USING QUANTITATIVE REAL-TIME PCR

6.1 INTRODUCTION

6.1.1 Sexual stage and sex specific proteins for the development of a qRT-PCR assay.

In order to establish sex ratios using a qRT-PCR assay, mRNA levels of sexual stage and specific proteins need to be quantified. Since the sex specific dyneins investigated in Chapter 4 proved unsuitable for this work, an alternative female specific protein, Pfg377 (PFL2405c), was used. This protein has previously been shown to allow accurate detection of gametocytes, of up to one gametocyte per microlitre by RT-PCR (Menegon *et al.*, 2000). Pfg377 is only expressed in gametocytes from stage III onwards and contains conserved (Region 1) and highly polymorphic regions (Region 3) (Menegon *et al.*, 2000). The conserved region would serve as a good target for gametocyte quantification using qRT-PCR whilst the polymorphic region can allow the identification of polyclonal infections using RT-PCR (Menegon *et al.*, 2000).

In *P. falciparum*, Pfs16 has been used to quantify all sexually committed parasites (Schneider *et al.*, 2004), as Pfs16 is localized in the parasitophorous vacuole which forms in gametocytes from stage I onwards (Bruce *et al.*, 1994). Conversely Pfs25 is only expressed on the surface of the zygote and ookinete and has therefore been used to quantify transcripts of mature gametocytes only (Schneider *et al.*, 2004; Mlambo *et al.*, 2008; Marangi *et al.*, 2009; Oesterholt *et al.*, 2009). Pfs16 (PFD0310w) and Pfs25 (PF_100303) have both shown to allow quantification of the total number of sexual parasites present within an infection (Schneider *et al.*, 2004) and will serve here to quantify the total numbers of gametocytes within a sample for the analysis of sex ratios.

Alpha-tubulin II mRNA is transcribed at low levels in asexual parasites and female gametocytes (Kooij *et al.*, 2005; Fennell *et al.*, 2008), which was also confirmed using IFA in this study and Alpha-tubulin II can therefore not be used as a sex specific target for the qRT-PCR assay. However, gametocyte sex ratios can still be obtained by comparing the number of female gametocytes (Pfg377) to the total numbers of gametocytes present (Pfs16 or Pfs25). In addition, observing Pfg377 and Alpha-tubulin II transcription levels during gametocytogenesis will allow comparison of the expression levels previously observed using IFA.

6.1.2 qRT-PCR: Relative quantification

There are two methods to analyze qRT-PCR data: absolute quantification determines the copy number of the transcript of interest, whereas relative quantification describes the difference between transcription levels of a target and a reference gene (Livak *et al.*, 2001). The relative quantification method is the preferred method when no absolute numbers are needed and when RNA amounts are limited, as it is the case with gametocyte RNA. Samples collected from natural infections are likely to contain low gametocyte densities and thus RNA and blood densities obtained from patients are likely to vary. Relative quantitation of sexual stage and sex specific transcripts of gametocyte cDNA preparations will therefore be evaluated as a potential tool for estimates of sex ratios using qRT-PCR. Quantification methods were adapted from Price *et al.*, 2004, discussed in section 6.3.7.

6.2 AIMS AND OBJECTIVE

1. Observe transcription levels of sexual stage and sex specific proteins during gametocytogenesis of cultured parasites using RT-PCR.

2. Measure relative expression levels of sex specific and sexual stage proteins during gametocytogenesis of cultured parasites in samples of known sex ratios using qRT-PCR.

3. Measure relative expression levels of sex specific and sexual stage proteins using qRT-PCR from natural infections.

4. Calibrate the relative expression values of sexual stage and sex specific proteins with the sex ratios previously observed using IFA and light microscopy.

6.3 MATERIALS AND METHODS

See chapter 3, for general material and methods.

6.3.1 Design of primers and probes of Pfs16, Pfs25, Pfg377 and Alpha-tubulin II

Primers for Pfs16 and Pfs25 were designed after Schneider *et al.*, 2004 and primers for Pfg377 were specifically designed to target Region 3, previously described by Menegon *et al.*, (2000). The Alpha-tubulin II sequence was retrieved from PlasmoDB and primers were designed using http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. Probes were designed by eye, with reference to the appropriate clustal alignment. All primers and probes were obtained through MWG-Biotech (Germany), see table 6.1.

Gene	**Pfs16 (PFD0310w)**
Target Stage	I-V
Forward (SA07)	**5'TTCTTCGCTTTTGCAAACCT'3**
Reverse (SA08)	3' AAAGGCATTTTGTCAGCAGAA'5
Probe (Pfs16)	**ROX-GCCCGCTGGAAAAGGATC-BHQ1**
Size cDNA	191bp
Size DNA	**191bp**
Gene	**Pfs25 (PF10_0303)**
Target Stage	I-V
Target Stage	**V**
Forward (SA11)	5' TAAATAAACCATGTGGAGATTTTT'3
Reverse (SA12)	**3' CAAGTTACATTCTTACATTCATTTGG'5**
Probe (Pfs25)	CY5-CCCGTTTCATACGCTTGTAA-BHQ1
Size cDNA	**164 bp**
Size DNA	164 bp
Gene	**Pfg377 (PFL2405c)**
Target Stage	III-V
Forward (SA09)	**5' CCCCATTTCCTCCTAAAGTACC'3**
Reverse (SA10)	3' CTGGTTCTGCTTCTGGTTCC'5
Probe (Pfg377)	**JOE-CGAACCAGAAATGGAAC-BHQ1**
Size cDNA	191 bp
Size DNA	**191 bp**
Gene	**Alpha-tubulin II (PFD1050w)**
Target Stage	I-V
Forward (SA05)	**5' TGAACATGGAATTCAACCGG'3**
Reverse (SA06)	3' CGTCAACGACGGTGGGTTC'5
Probe (PfA)	**FAM-CAAGTCGTTGCTGGTGG-BHQ1**
Size cDNA	152 bp
Size DNA	**272 bp**

Table 6.1: List of primers and probes. The primers were used for RT-PCR and qRT-PCR, whereas the probes were only used for the qRT-PCR assay. Stages at which transcription has been observed, sequence of the forward and reverse primers and the probe sequence are shown. Predicted size of the PCR product is also shown from cDNA and DNA.

6.3.2 RT-PCR targeting Pfs16, Pfs25, Pfg377 and Alpha-tubulin II

cDNA was prepared from parasites harvested on day 1, 3, 5, 7 and 11. The master mix for the RT-PCR was made with the following for one 25µl reaction: 2.5µl of 10 x NH_4 buffer (Bioline), 0.5µl of 50mM $MgCl_2$ (Bioline), 0.5µl of 2.0µM of 5µM dNTPs (Promega), 1µl of 5µM of the chosen forward and reverse primers, 0.5µl of 50 units/µl of Taq (Bioline) and 3-5µl of cDNA. Nuclease free water (Promega) was used to fill the reaction to a volume of 25µl. Superscript positive and superscript negative samples were used in each PCR reaction to determine whether DNA contamination was present in the original RNA. Positive controls, containing 3D7 DNA and negative controls containing nuclease free water (Promega) were also used in each run. PCR cycling conditions are shown in the appendix, section 8.3. cDNA and DNA samples were loaded with 1 x blue/orange loading dye (Promega), sizes were determined by using 10µl of a ladder with 100bp intervals (Hyperladder IV; Promega). Bands were analyzed with ultraviolet light after running a 1.2% (0.5 x TBE) agarose gel (Sigma-Aldrich), containing 2.5µl of ethidium bromide (10mg/l) at 100 V for 1 to 1.5 hours.

6.3.3 qRT-PCR targeting Pfs16, Pfs25, Pfg377 and Alpha-tubulin II

A multiplex approach was devised in which four sets of primers and four probes were used in one reaction. A 25µl master mix was prepared, including 2.5µl of 10 x NH_4 buffer (Bioline), 05µl of 50mM $MgCl_2$ (Bioline), 2µl of 5µM dNTPs (Promega), 2µl of each primer at 5µM (SA05, SA06, SA07, SA08, SA09, SA10, SA11 and SA12), 0.5µl of each 5µM probe (Pfs16, Pfs25, Pfg377 and Alpha-tubulin II), 0.5µl of 50 units/µl of *Taq* (Bioline), and 2-5µl of cDNA. Samples were run in triplicate for accurate quantification; superscript negative samples served as a control to allow detection of DNA contamination of the cDNA. Positive controls, using DNA and negative controls using nuclease free water (Promega) were also used in each run. Samples were run on the Rotorgene 3000 (Corbett). Cycle conditions were 95°C for 6 minutes, and 40 cycles at 96°C for 15 seconds, 49°C for 60 seconds and 70°C for 15 seconds. Conditions were optimized for the amount of *Taq* polymerase used, forward and reverse primer concentration, probe concentration, $MgCl_2$ and various annealing temperatures.

6.3.4 qRT-PCR: Gametocyte quantification

When applying the comparative Ct method, it is assumed that the amplification efficiency (AE) is consistent within a range of cDNA concentrations (Livak *et al.*, 2001). Therefore a serial dilution of cDNA was needed to establish a standard curve to validate the amplification of the reference and target genes. This allows observing the amplification efficiency over different cDNA concentrations. Stage V MACs® purified gametocytes were used for the serial dilution and were quantified with a standard haemocytometer (C-Chip, Neubauer, Germany). Superscript positive samples, positive controls and negative controls were run in triplicates, whereas only one superscript negative sample was used to ensure no DNA contamination was present in the cDNA. 2µl of cDNA per reaction were used and the AE was tested over a 2-fold dilution to a concentration of 700 to 11 gametocytes per microlitre.

6.3.5 Efficiency of the primers and probes

The ΔΔCt method assumes the percentile AE is 100%, or 1, (doubling of the PCR products per cycle) and the relative expression is calculated as 2^ (-ΔΔCt) to account for the exponential properties of the PCR (Livak *et al.*, 2001; Price *et al.*, 2004). Therefore the AE of all probes and primers were first set equal to 100% (or 1), followed by lowering the AE to < 100%, as the validation test (see section 6.3.6) was not always passed when the AE was set equal to 100 %. The efficiency was calculated based on the equation: E=10^ (-1/slope), the slope of the standard curve, which was established by plotting a graph of the serial dilution against the Ct values. When the slope was equal to -3.32, it indicated a 100% AE, and the AE should always be > 90% (0.9) for replicable experiments (Ginzinger, 2002). The efficiency was tested for the primers Pfs16, Pfs25, Pfg377 and Alpha-tubulin II.

6.3.6 The validation experiment

A validation experiment allows observing if the AE of the reference and target gene remain constant at different concentrations. This is done by observing the absolute value of the slope. The slope of the ΔCt versus the log concentration of cDNA should be +/- 0.1 to pass this test (Applied Biosystems User Bulletin No.2). This test ensures that the amplification of the primers and therefore the fluorescent signal of the probe remain constant at different cDNA concentrations. The validation test was tested with a 100% AE. The validation was tested with Pfg377

versus Pfs16, Pfg377 versus Pfs25, Pfs16 versus Pfs25, Alpha-tubulin II versus Pfs16 and Alpha-tubulin II versus Pfs25.

6.3.7 Quantification of the relative expression

The data in this study were analyzed using the comparative Ct method, where it is assumed that the target and reference genes have the same efficiency within a range of cDNA concentrations. This comparative method, where the change of expression, the ΔΔCt (Delta Delta Ct) is quantified was used here, see figure 6.1. The derivation of the 2^(-ΔΔCt) method equation is described in the Applied Biosystems Bulletin No. 2. Pfs16 and Pfs25 were used here as alternative endogenous controls, whereas Pfg377 and Alpha-tubulin II were used as reference genes. Relative expression of Pfg377 versus Pfs16 or Pfs25 gave relative expression values of female gametocytes versus total number of gametocytes, which were then compared to the sex ratios that had previously been established by light microscopy and IFA. Alpha-tubulin II was still included, even though it was not sex specific, as it may be useful in later studies to quantify sexual and asexual parasites.

The first step of quantifying the ΔΔCt method involved assigning the gDNA samples to a relative expression value of 1, to serve as a calibrator. This was done by subtracting the mean ΔCt values of the DNA samples from each other and raising this value to the power of two, to obtain a relative expression value of one. For the cDNA samples, the mean ΔΔCt value is measured by subtracting the average ΔCt of the target gene from the average ΔCt of the gDNA sample to obtain a relative expression value of the reference and target gene. cDNA from stage III-V and activated gametocytes was analyzed here. Relative expression calculations were performed in an Excel spreadsheet with the relevant formulae provided by Ric Price. See the attached CD for observing the relative quantifications calculated with Excel.

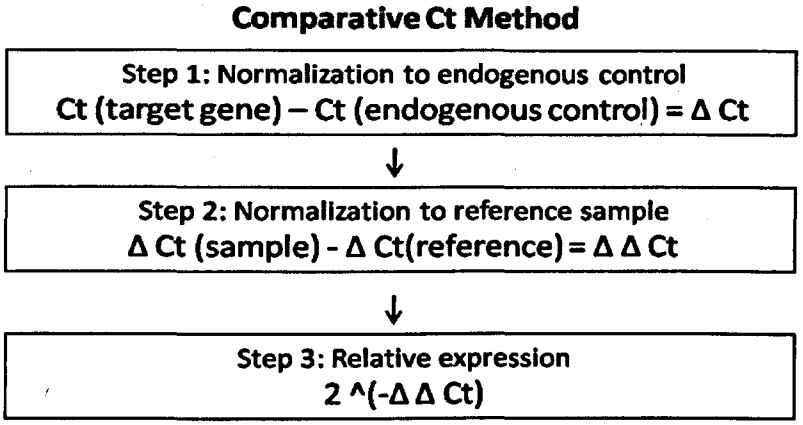


Figure 6.1: Overview of the Delta Delta Ct method. Representation of the three steps needed to calculate the relative expression using the comparative method (ΔΔCt) method, adjusted from the Applied Biosystems, User Bulleting No.2

Data were obtained by the manual selection of a threshold value corresponding to early exponential amplification, with discrimination between superscript positive and superscript negative samples. Superscript positive samples were mostly run in triplicate, in the majority of experiments, with at least one superscript negative control. When the Ct values were > than 35, superscript negative samples revealed amplification, or the spread of Ct was > 2 samples were excluded, unless otherwise noted.

6.3.8 Analysis of samples from natural infections

Samples were collected in The Gambia in 2001 (Sutherland *et al.*, 2005), either at the day of admission, day seven, or day fourteen after treatment. 25µl of the patient's blood, was stored in 1.5ml Tri-Reagent, the blood was thoroughly mixed, incubated at room temperature for 10 minutes and stored in Tri-Reagent at -70°C (except for the transportation to the UK, where samples were kept on dry ice). RNA was extracted as previously described (see section 3.3) with the exception that the RNA was finally eluted in 10µl (instead of 30µl) of nuclease free water (Promega). This was to increase the RNA concentration due to the potentially lowered quality of samples caused by the extended storage periods and non optimal freezing conditions during the transportation process. Amounts of cDNA varied, depending on the quality (very low transcription levels), approximately 6µl - 10µl of cDNA were used in each sample.

6.4 RESULTS

6.4.1 RT-PCR of Pfs16, Pfs25, Pfg377 and Alpha-tubulin II

The patterns of transcription for four sexual-stage specific genes during gametocytogenesis were detected by conventional RT-PCR and were therefore qualitative rather than quantitative, are shown in figure 6.3, the corresponding parasites per stage are shown in table 4.4. Analysis of RT-PCR products by gel electrophoresis revealed single products of the expected size. Pfs16 transcripts were detected throughout gametocyte development, whereas transcripts of the gamete antigen Pfs25 were seen between the day 7 and day 11 samples of gametocyte preparations. Alpha-tubulin II and the female-specific protein Pfg377 transcripts were first detected on day 1 and day 2 respectively, but were noticeably more abundant in gametocyte preparations after day 6. All four genes were transcribed between day 7 and 11 of gametocyte preparations, which best represent the mature sexual stages detectable in peripheral blood of *P. falciparum* infected patients.

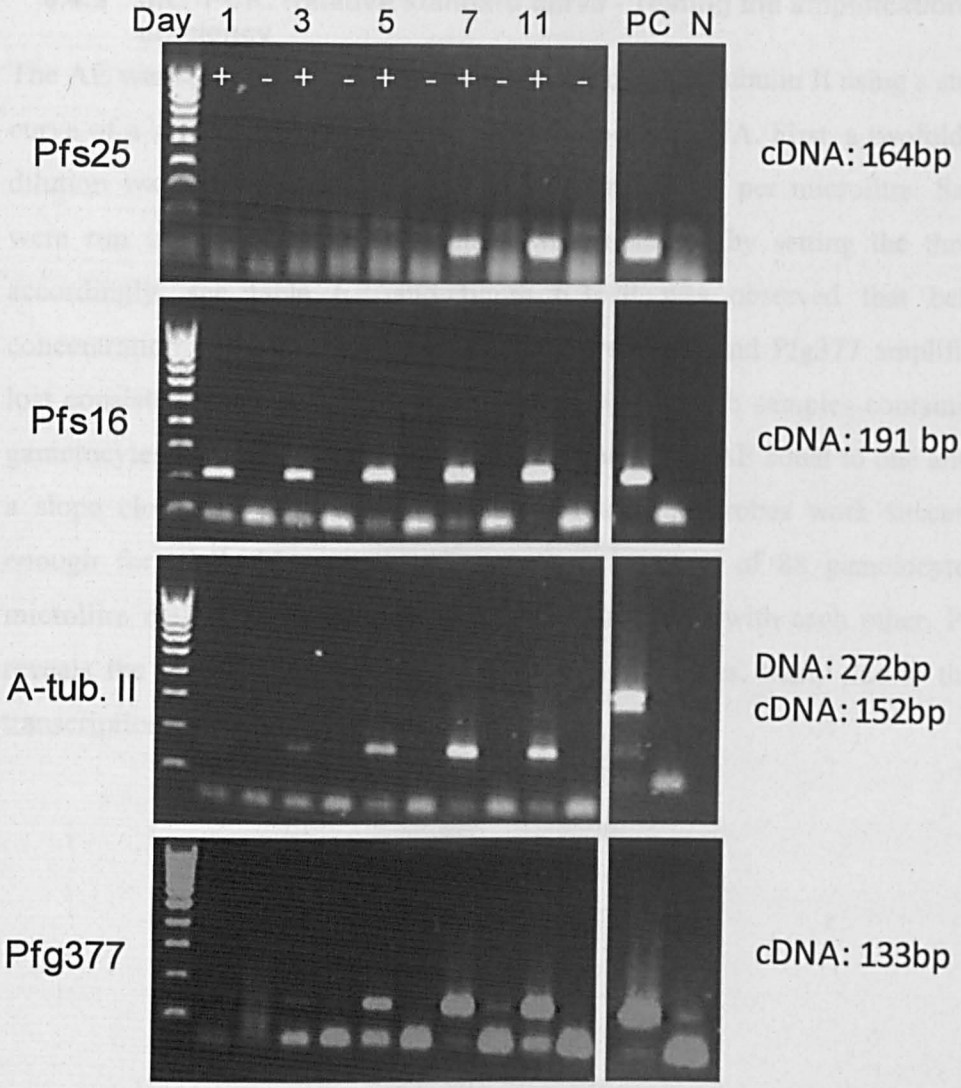


Figure 6.2: RT-PCR of four sexual stage and sex specific proteins during gametocytogenesis. Transcripts of Pfs25, Pfs16, Alpha-tubulin II (A-tub.II) and Pfg377 were amplified from stage I-V. Gel electrophoresis of amplified products; + and – refer to presence or absence of reverse-transcriptase in the cDNA reaction prior to amplification. PC is noted as positive control and N is noted as the negative control. See table 4.4 for % gametocytes observed per stage. It should be noted that the cultures were not 100% synchronous.

6.4.2 qRT-PCR: Relative standard curve - Testing the amplification efficiency

The AE was tested for Pfs16, Pfs25, Pfg377 and Alpha-tubulin II using a standard curve of a serial dilution using stage V gametocyte cDNA. First, a twofold serial dilution was made; containing 700 to 11 gametocytes per microlitre. Samples were run in triplicates and Ct values were obtained by setting the threshold accordingly, see table 6.2 and figure 6.3. It was observed that below a concentration of 88 gametocytes per microlitre Pfs25 and Pfg377 amplification lost consistency. No reliable detection was possible with samples containing 11 gametocytes, or lower. However, all probes reached an AE equal to one and with a slope close to -3.32, suggesting that primers and probes work successfully enough for replicable experiments at a concentration of 88 gametocytes per microlitre or higher. When comparing all four probes with each other, Pfg377 reveals the lowest and the largest variation in Ct values, likely due to the low transcription levels of this gene.

		Pfs16	Pfs25	Pfg377	A.tub.II
Number of gametocytes	Threshold AE	0.00095 1	0.00374 1	0.00371 1	0.02024 1
1400	1	13.66	15.88	19.89	18.96
1400	1	13.17	17.17	21.71	19.03
1400	1	12.73	17.4	22.2	18.86
700	0.5	14.24	18.88	22.65	20.32
700	0.5	15.12	18.96	23.6	20.59
700	0.5	14.55	18.4	23.18	19.95
350	0.25	15.6	17.53	23.8	20.74
350	0.25	14.31	19.92	25.37	21.45
350	0.25	14.47	19.38	22.06	20.69
175	0.125	16.86	20.12	25.81	22.05
175	0.125	16.11	20.4	19.17	23.07
175	0.125	17.79	20.61	24.78	22.78
88	0.0625	17.15	20.01	28.11	22.9
88	0.0625	16.04	20.41	23.21	22.76
88	0.0625	17.29	22.26	23.22	23.92
44	0.03125	17.23	21.86	*	21.97
44	0.03125	18.08	21.48	26.76	23.05
44	0.03125	18.65	*	*	23.96
22	0.015623	18.89	*	29.56	24.95
22	0.015623	19.06	23.02	*	*
22	0.015623	*	*	*	*

Table 6.2: Ct values of a two-fold serial dilution when AE = 1. Samples were run in triplicates and number of gametocytes used in each serial dilution is shown on the left. Thresholds were set manually and AE was set to 1. Fields marked with an asterisk (*) were exluded, as they did not yield sufficient amplification. This serial dilution was done with stage V gametocyte cDNA.

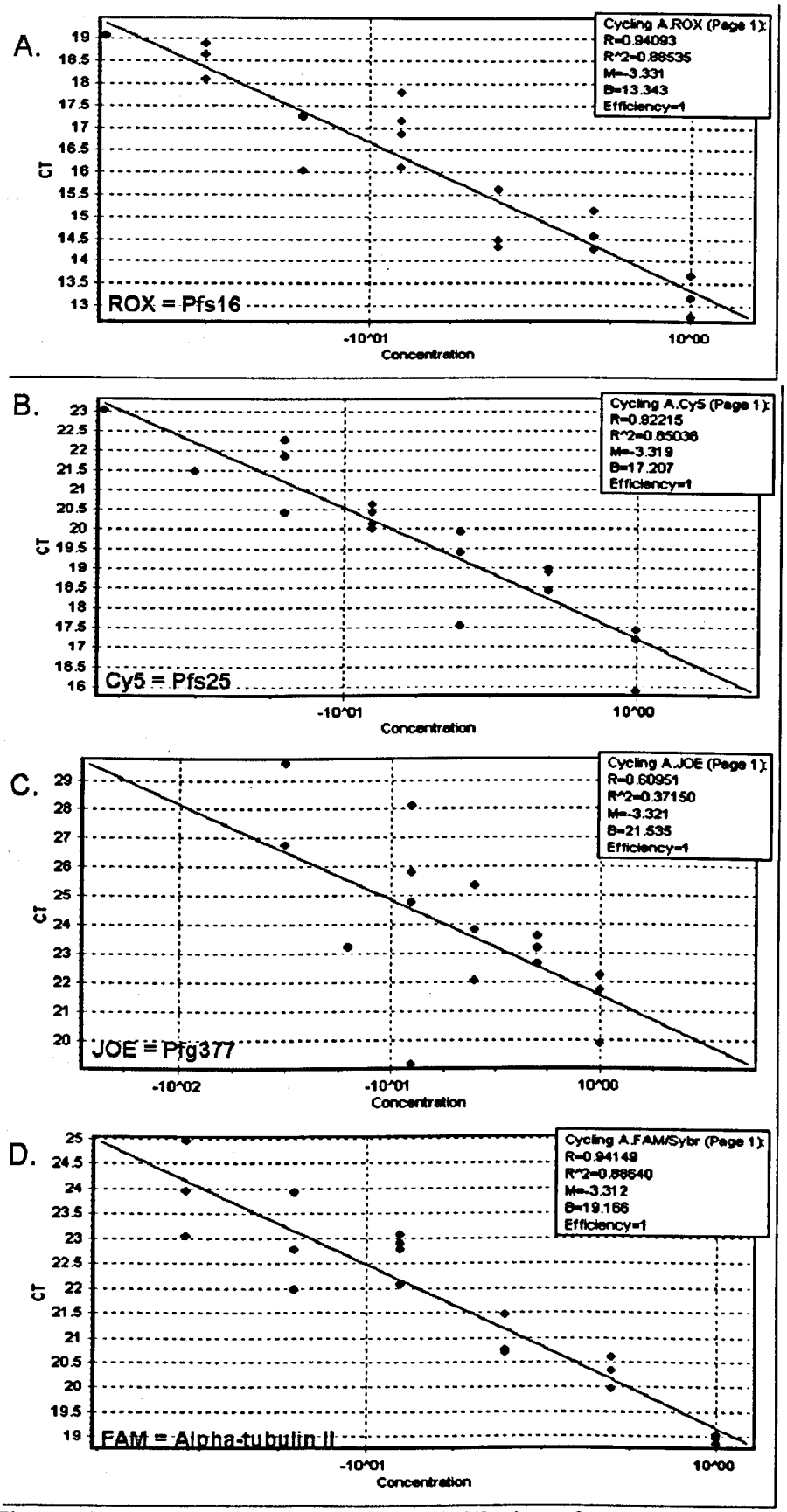


Figure 6.3: Standard curve of a serial dilution of stage V gametocyte cDNA when the AE = 1. A.) Pfs16, B.) Pfs25, C.) Pfg377 and D.) Alpha-tubulin II are shown over a two fold serial dilution.

6.4.3 Validation test, where AE = 1

For the validation experiment two requirements need to be fulfilled: firstly the efficiencies of the target and reference gene must be approximately equal and that the slope of the ΔCt versus the (log) cDNA concentration must be +/- 0.1. This ensures an equal amplification efficiency at different cDNA concentrations. The validation was tested for Pfg377 versus Pfs16, Pfg377 versus Pfs25, Pfs16 versus Pfs25, Alpha-tubulin II versus Pfs16 and Alpha-tubulin II versus Pfs25. Alpha-tubulin II versus Pfs16 had a slope of -0.07 and was therefore the only pair that passed the validation test when the AE = 1, see figure 6.4. Large variations bewteen the ΔCt values can be seen when observing the standard error. Pfs16 versus Pfs25 showed the most consistent ΔCt values over the serial dultution.

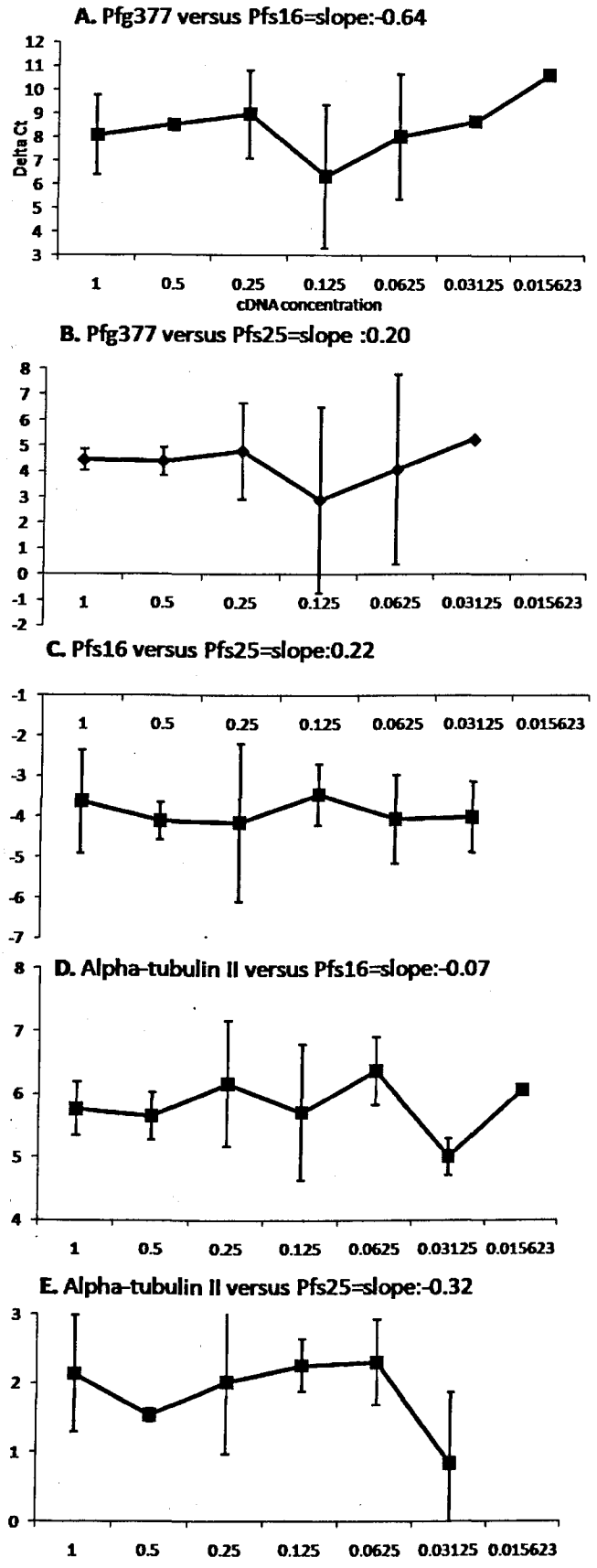


Figure 6.4: Validation test when AE=1. Plot of the log cDNA concentration of the serial dilution versus the Δ Ct (Delta Ct), showing the best fit line of A.) Pfg377 versus Pfs16, B.) Pfg377 versus Pfs25, C.) Pfs16 versus Pfs25, D.) Alpha-tubulin II-Pfs16 (Alpha-tubulin II and E.) Alpha-tubulin II versus Pfs25.

6.4.4 Interpretation of the validation test

The validation test was used to observe that the amplification of the reference of the target gene remained constant at different cDNA concentrations. In this study large variations of the ΔCts were observed, yet target and reference genes of Alpha-tubulin II versus Pfs25 passed the validation. However, lowering the AE (minimum of 90% AE) allows all reference and tareget genes to pass the validation test (data not shown). Also, it should be noted that a slope can still be zero, even when large fluctuations of Ct values exist, as the fluctuations can cancel each other out. Overall the validation test can be easily manipulated and may be passed when lowering the AE accordingly.

6.4.5 Analysis of the relative expression

Relative expression was quantified from stage III (day 5), stage IV (day 9), stage V (day11), activated gametocytes (AG), asynchronous culture (AS) and samples collected from patients in the field (GA). Asynchronous cultures consisted mainly of asexual parasites, stage IV and stage V gametocytes. Gambian samples were chosen from day 0 of admission and consisted of asexual parasites and gametocytes.

In stage III gametocytes, Pfg377 mRNA was detected to be expressed at lower levels than Pfs16 and Pfs25 mRNA, see figure 6.7 A). Alpha-tubulin II transcripts were also found to be less abundant than Pfs16 transcripts. On the contrary Alpha-tubulin II had transcripts at higher levels than Pfs25 and the same were found for Pfs16 in comparison to Pfs25 and when comparing it to Pfs25 versus Pfg377. This showed that Pfs25 and Pfg377 contain low mRNA levels during this early stage of gametocytogenesis. Samples (a) and (b) were taken from different cultures and revealed to have very similar relative expression levels of all proteins investigated.

In stage IV gametocytes, Pfg377 mRNA was observed to be expressed at lower levels than Pfs16 and Pfs25 mRNA in all three culture samples (a), (b), and (c), see figure 6.7 B). On the contrary Pfs25 was observed in much higher abundance than Pfg377. Alpha-tubulin II transcripts were also observed to be less abundant than Pfs16 and Pfs25 transcripts, except for one analysis of sample (b), where Alpha-tubulin II was observed to be slightly higher than Pfs16.

Transcription levels of Pfs16 in comparison to Pfs25 were observed to be relatively similar and in sample (a), however Pfs16 was more abundant than in sample (b). This showed that Pfs25 has far more abundant mRNA transcript levels at stage IV compared to Pfg377 and Alpha-tubulin II.

In stage V gametocytes, Pfg377mRNA was detected to be expressed at lower levels than Pfs16 and Pfs25 in all three samples (a) and (b), see figure 6.7 C). In comparison, Pfs25 was found in much higher abundance than Pfg377. Alpha-tubulin II transcripts were also found to be less abundant than Pfs16 and Pfs25 transcripts, as observed previously. Transcription levels of Pfs16 in comparison to Pfs25 were observed to be relatively similar, as found in stage IV, yet in sample (a) Pfs16 was found to be more abundant than in sample (b). This showed that Pfs25 transcription levels are increasing over gametocyte maturity and that Pfs25 contains higher levels of mRNA in comparison to Pfg377 and Alpha-tubulin II.

In activated gametocytes, Pfg377 mRNA was observed to be expressed at lower levels than Pfs16 and Pfs25 in all three samples (a, b, and c), whereas Pfs25 was found in much higher abundance than Pfg377, see figure 6.7 D.). Alpha-tubulin II transcripts were also found to be less abundant than Pfs16 and Pfs25 transcripts, even more though when it was observed at stage V. However, transcription levels of Pfs16 in comparison to Pfs25 were found to be far less abundant, which was not observed at earlier stages of gametocytogenesis. This showed that Pfs25 transcription levels are increasing once gametocytes are activated and the higher levels of Pfs25 mRNA were found once gametocytes were activated.

An asynchronous culture consisting of large numbers of asexual parasites, stage IV and mainly stage V gametocytes (courtesy of Chiara Andolina), see figure 6.7 E.). These samples may represent closely what one would expect to find in patients, as asexual parasites are mixed with gametocytes. In the samples taken from the asynchronous cultures Pfg377 mRNA was found to be expressed at lower levels than Pfs16 and Pfs25 in all three samples (a) and (b). On the contrary Pfs25 was found in much higher abundance than Pfg377. Alpha-tubulin II transcripts were also found to be less abundant than Pfs16 and Pfs25 transcripts, similar to what was found in stage IV and V gametocytes.

However, transcription levels of Pfs16 in relative comparison to Pfs25 were found to be less abundant in sample (a) and (b), yet one sample of (a) and one sample of (b) revealed that Pfs16 was slightly more abundant than Pfs25,

suggesting that in these asynchronous cultures transcription levels of Pfs16 and Pfs25 were similar. By observing the transcript level of an asynchronous culture it can be suggested that this culture must have mainly contained mature gametocytes (no slides were available of this asynchronous culture).

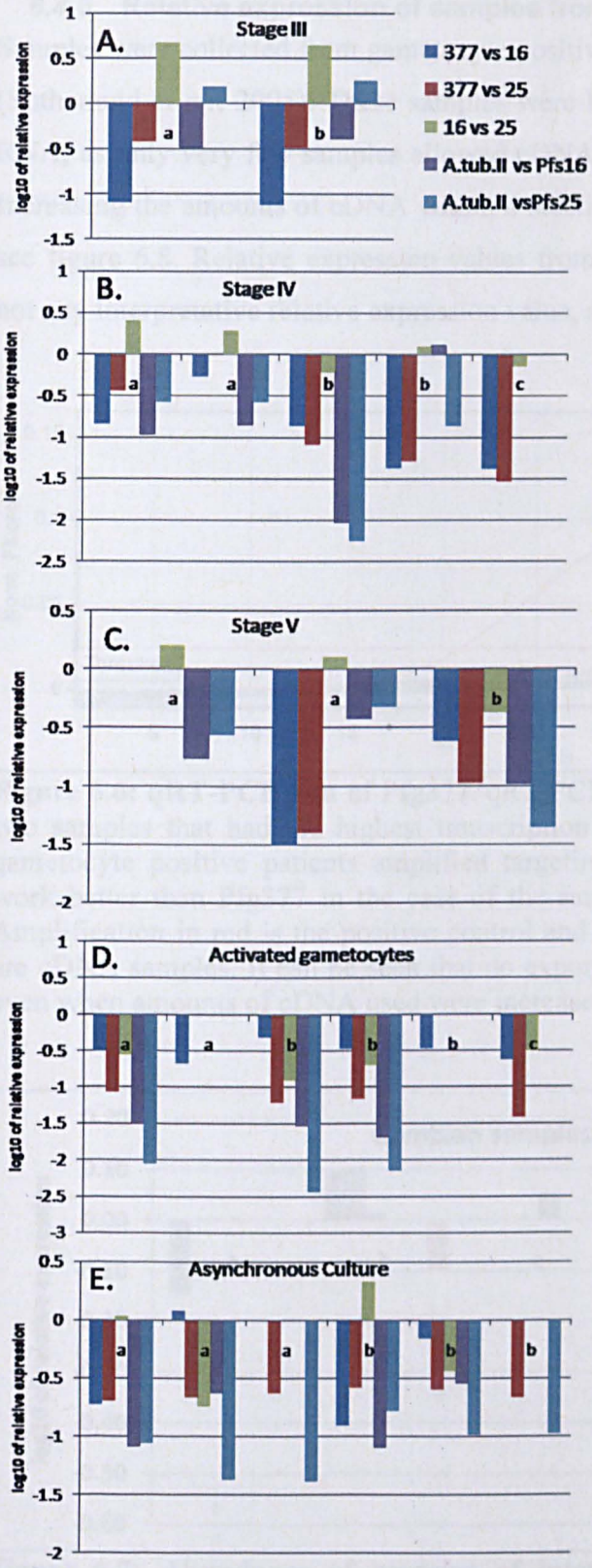


Figure 6.5: Relative expression levels of genes of interest during gametocytogenesis. Log 10 of the relative expression values of A.) stage III, B.) stage IV, C.) stage V, D.) activated gametocytes, and E.) an asynchronous gametocyte culture. Relative expression values were measaured of Pfg377 versus Pfs16 (337 vs 16), Pfg377 versus Pfs25 (377 vs 25), Pfs25 versus Pfg377 (25 vs 377), Pfs16 verus Pfs25 (16 vs 25), Alpha-tubulin II versus Pfs16 (A.tub.II vs 16), and Alpha-tubulin II versus Pfs25 (A.tub. II vs 16).

6.4.6 Relative expression of samples from natural infections

Samples were collected from gametocyte positive patients in The Gambia in 2001 (Sutherland *et al.*, 2005). These samples were likely to have contained degraded RNA, as only very few samples allowed cDNA amplification at very low levels. Increasing the amounts of cDNA within a reaction did not increase amplification, see figure 6.8. Relative expression values from four different samples did yield not any interpretative relative expression value, see figure 6.9.

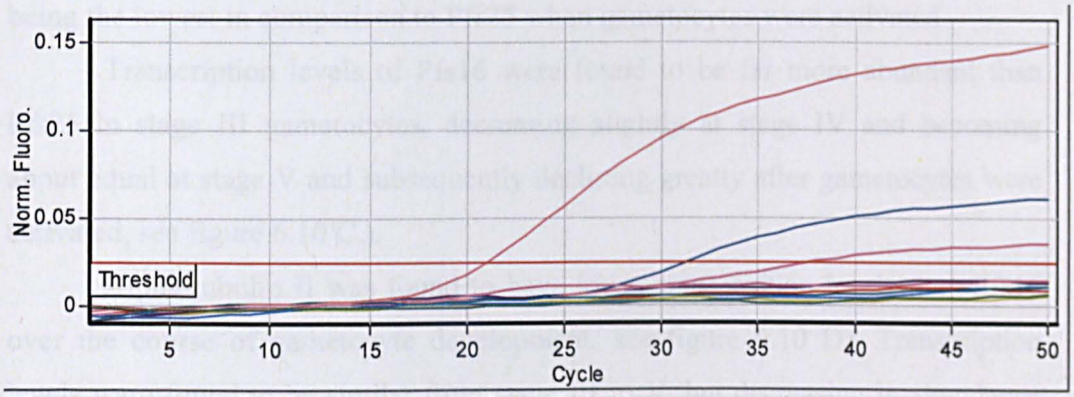


Figure 6.6: qRT-PCR run of Pfg377. qRT-PCR representation of a run with the two samples that had the highest transcription levels, collected from Gambian gametocyte positive patients amplified targeting Pfg377 (other probes did not work better than Pfg377 in the case of the samples collected in The Gambia). Amplification in red is the positive control and the blue and pink amplifications are cDNA samples. It can be seen that no exponential amplification is occurring, even when amounts of cDNA used were increased to 10µl per reaction.

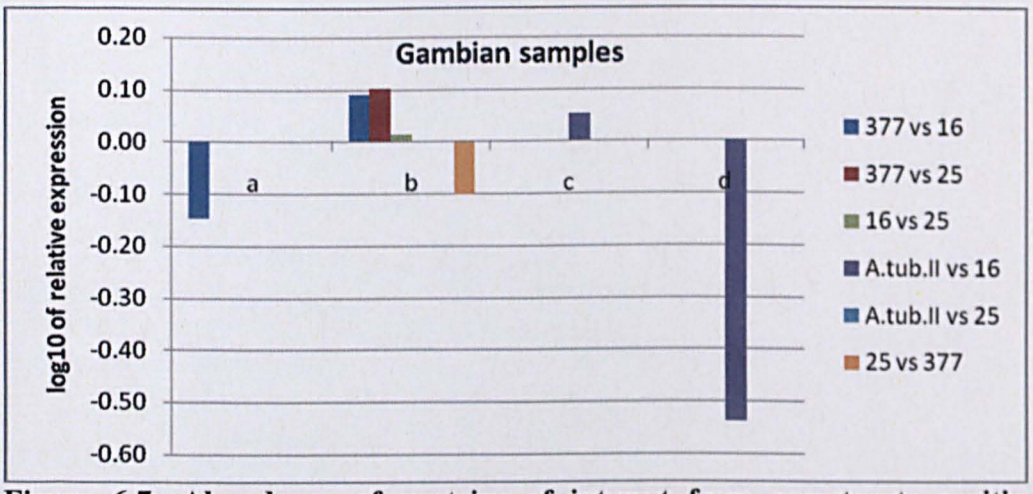


Figure 6.7: Abundance of proteins of interest from gametocyte positive patients. Log 10 of the relative abundance of Pfg377 versus Pfs16 (377 vs. 16), Pfg377 versus Pfs25 (377 vs. 25), Pfs25 versus Pfg377 (25 vs. 377), Pfs16 versus Pfs25 (16 vs. 25), Alpha-tubulin II versus Pfs16 (A.tub.II vs16) and Alpha-tubulin II versus Pfs25 (A.tub.II vs. 25.); a), b), c) and d) represent analysis performed on four separate samples collected from gametocyte positive patients in The Gambia in 2001.

6.4.7 Summary: relative expression during gametocytogenesis

It was found that Pfg377 has lower transcript numbers than Pfs16 throughout the course of gametocyte development, see figure 6.10 A.). However, Pfg377 transcripts were found to increase in abundance in comparison to Pfs16 during gametocyte development and increased after gametocytes were activated.

Pfg377 was also found to have lower transcription levels than Pfs25 over the course of gametocyte development, see figure 6.10 B.). In comparison to Pfs25, Pfg377 transcripts decrease in abundance over gametocyte development, being the lowest in comparison to Pfs25 when gametocytes were activated.

Transcription levels of Pfs16 were found to be far more abundant than Pfs25 in stage III gametocytes, decreasing slightly at stage IV and becoming about equal at stage V and subsequently declining greatly after gametocytes were activated, see figure 6.10 C.).

Alpha-tubulin II was found to have lower transcription levels than Pfs16 over the course of gametocyte development, see figure 6.10 D). Transcription levels were found to be similar from stage III to V, but decreasing in abundance once gametocytes were activated

At stage III Alpha-tubulin II was found to have more abundant transcription levels than Pfs25, see figure 6.10 E.). However, expression levels decrease and Alpha-tubulin II is less abundant at stage IV and V compared to Pfs25, decreasing even further once gametocytes were activated.

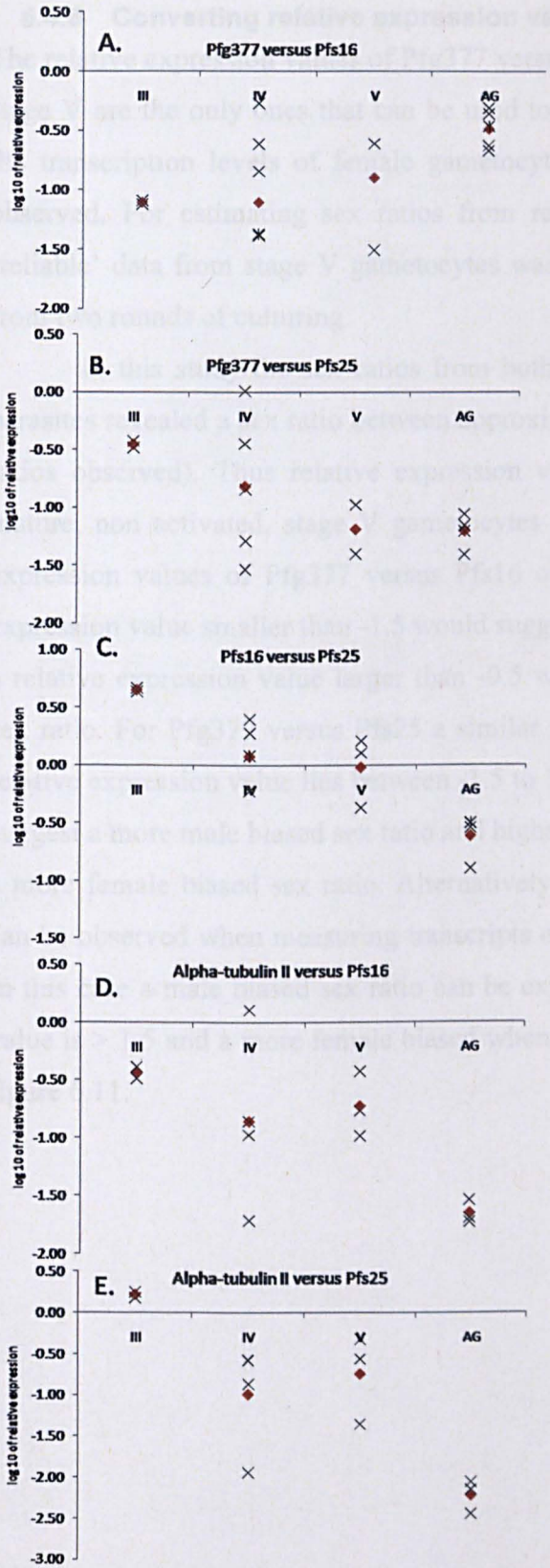
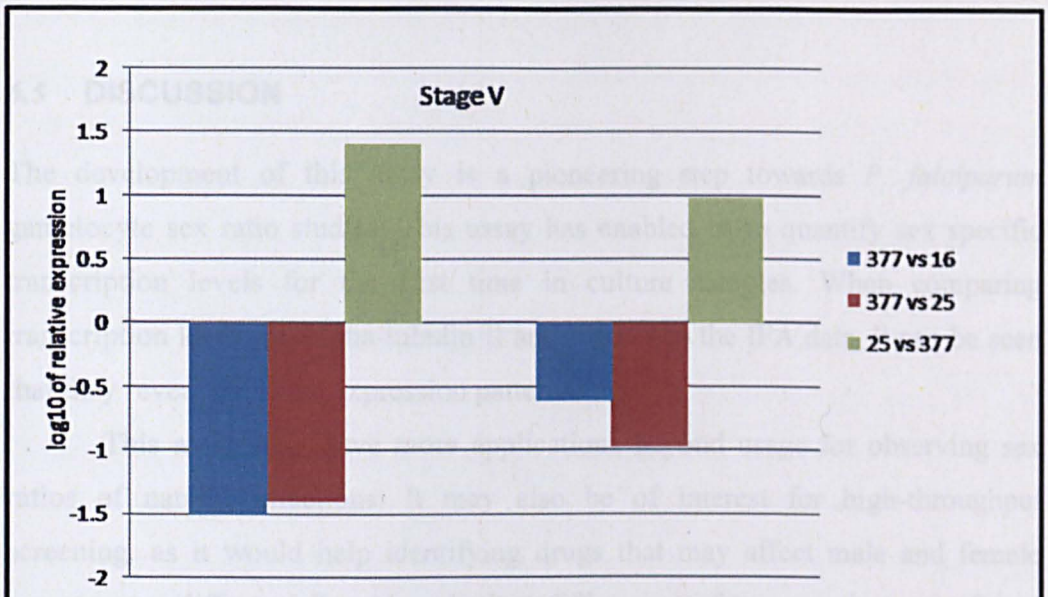


Figure 6.8: Relative expression during gametocytogenesis. Log10 of the relative expression of A.) Pfg377 versus Pfs16, B.) Pfg377 versus Pfs25. C.) Pfs25 versus Pfg377, D.) Pfs16 versus Pfs25, E.) Alpha-tubulin II versus Pfs16, F.) Alpha-tubulin II versus Pfs25. The mean relative expression for each stage is shown in red.

6.4.8 Converting relative expression values into gametocyte sex ratios

The relative expression values of Pfg377 versus Pfs16 and Pfg377 versus Pfs25 of stage V are the only ones that can be used to estimate sex ratios, as these reflect the transcription levels of female gametocytes to total number of gametocytes observed. For estimating sex ratios from relative expression values, the most 'reliable' data from stage V gametocytes was chosen here, which were obtained from two rounds of culturing.

In this study the sex ratios from both light microscopy and IFA of 3D7 parasites revealed a sex ratio between approximately 0.11 - 0.14 (including all sex ratios observed). Thus relative expression values to estimate the sex ratio for mature, non activated, stage V gametocytes should theoretically reflect relative expression values of Pfg377 versus Pfs16 of -1.5 to -0.5. However, a relative expression value smaller than -1.5 would suggest a more male biased sex ratio and a relative expression value larger than -0.5 would suggest a more female biased sex ratio. For Pfg377 versus Pfs25 a similar sex ratio can be expected when the relative expression value lies between -1.5 to 1.0, whereas a lower sex ratio would suggest a more male biased sex ratio and higher transcription levels would suggest a more female biased sex ratio. Alternatively positive relative expression values can be observed when measuring transcripts of Pfs25 versus Pfg377 for example. In this case a male biased sex ratio can be expected when the relative expression value is > 1.5 and a more female biased when the relative expression is < 1.0, see figure 6.11.



Log 10 Relative expression	Predicted SR ≥ 0.11	Observed SR = 0.11 - 0.14	Predicted SR ≤ 0.14
Pfg377 versus Pfs16	≤ -1.5	-1.5 to -0.5	≥ -0.5
Pfg377 versus Pfs25	≤ -1.5	-1.5 to 1.0	≥ 1.0
Pfs25 versus Pfg377	≥ 1.5	1.0 to 1.5	≤ 1.5

Figure 6.9: Relative expression 'calibrator'. Log 10 of the relative expression from stage V gametocytes of Pfg377 versus Pfs16 (377 vs 16), Pfg377 versus Pfs25 (377 vs 25) and Pfs25 versus Pfg377 (25 vs 377). Sex ratios that were observed and that are predicted are noted. Observed values were taken from light microscpoy and IFA, whereas the predicted sex ratio was predicted from the relatice expression values.

6.5 DISCUSSION

The development of this assay is a pioneering step towards *P. falciparum* gametocyte sex ratio studies. This assay has enabled us to quantify sex specific transcription levels for the first time in culture samples. When comparing transcription levels of Alpha-tubulin II and Pfg377 to the IFA data, it can be seen that they reveal the same expression patterns.

This assay may have more applications beyond usage for observing sex ratios of natural infections. It may also be of interest for high-throughput screening, as it would help identifying drugs that may affect male and female gametocytes differentially. Also, distinct differences of transcription were found between stage V and activated gametocytes, therefore this assay may be of interest to be used in transmission studies, as it may be able to help revealing optimal maturity of gametocyte cultures, thereby increasing the chances of successful transmission in laboratory studies. Also, qRT-PCR assays may help understanding biological functions of proteins by observing transcription levels through gametocyte development. The use of this assay may be less costly and time consuming than obtaining the appropriate antibodies for IFA studies for example. The benefit of using the relative quantification method is that it compensates for variation within experiments. For example, different amplification efficiencies of probes and primers, or different amounts of cDNA of samples are accounted for. This is done by setting the relative expression value of the DNA control to 1, which calibrates for variation.

The relative expression levels that were quantified here provided quantitative data that can be calibrated to the sex ratios previously observed using light microscopy and IFA, as the relative expression values of Pfg377 versus Pfs16 and Pfg377 versus Pfs25 reflect the transcription levels of the female gametocyte to the total number of gametocytes targeted. Relative expression values for sex ratio analysis were measured from stage V gametocytes from culture and should reflect the same transcription levels of gametocytes found in the peripheral blood.

Pfs16 has been observed to be transcribed following the invasion of the RBC (Dechering *et al.*, 1997), therefore mRNA from asexual parasites may be amplified when using Pfs16 as the reference gene, thereby influencing the

observed sex ratio. Whether Pfs 16 transcription levels are amplified in sexually determined parasites before sequestration occurs needs to be further investigated. This could be investigated by observing whether Pfs16 is transcribed in infected patients that have not yet developed gametocytes and do not show transcription levels for sequestered gametocytes, which can be tested by targeting Pfs25 by qRT-PCR, for example.

One problem with the development of this assay was the frequent presence of DNA contamination from the malaria parasites and the difficulty of consistently obtaining high quality cDNA. The qRT-PCR assay revealed even the slightest DNA contamination, which was not detected by RT-PCR. It was therefore important to always include superscript negative samples in the analysis, to ensure only cDNA was amplified. Using more costly procedures to purify RNA may decrease chances of DNA contamination, but this option may not always be applicable for large field studies. One option would be to explore a more efficient method for the production of cDNA. Low quality cDNA could have been caused by the addition of inhibitory compound during the RNA extraction, for example, as leftovers from reagents used for RNA extraction may lead to inaccurate quantification of mRNA (Radstrom *et al.*, 2004). Also, different mRNAs degrade at different rates and optimizing RNA storage may improve availability of homogenous qualities of mRNA (Nolan *et al.*, 2006).

This qRT-PCR assay was not very sensitive, as amplification was lost consistently below 44 gametocytes per microlitre. However, Pfs16 and Alpha-tubulin II were sensitive enough to allow amplification of 11 parasites per microlitre. Gametocyte samples collected here were concentrated using MACs® purification, thereby eliminating sources of human RNA, such as leukocytes. Yet samples taken from naturally infected individuals would contain higher levels of RNA, from white blood cells for example, which may allow increasing the carryover of RNA and yielding a higher sensitivity, even though parasite densities would be much lower.

Low transcription levels of Pfg377 might cause problems for field samples with low gametocyte densities. Here we used random primers for the cDNA production, which may have amplified certain mRNAs more efficiently than others (Bustin *et al.*, 2004) and could have affected Pfg377 transcription levels. This problem could be solved by targeting a different part of the gene, or purify parasites from natural infections using the purification process Nycodenz®. This

process is more suitable to purify small volumes of blood, as parasites are separated based on a density layer and are less likely to get lost, compared to using the MACS magnet, which requires to be washed with large volumes of media.

Pfg377 was the only sex specific protein used here and was observed to have low transcription levels, which may cause problems when quantifying gametocytes from natural infections, as they frequently occur at low densities. Exploring additional female probes, such as Pfs47 (van Schaijk *et al.*, 2006), for example, may serve as a control and might have stronger transcription levels which will ensure successful amplification at low gametocytaemia. Even though Alpha-tubulin II could not be used for obtaining relative expression ratios for determining sex ratios, it was still included in this analysis to provide a comparator for expression levels of sexual stage proteins which may provide information of asexual densities found within natural infections for future studies.

There are several sex specific proteins described in *P. berghei* that have orthologs in *P. falciparum*, that should be further explored and could prove to have high transcription levels, which would be ideal for the analysis of relative expression levels. There are a large number of male specific proteins associated with axoneme and flagella and two female specific and six male specific kinase/phosphatise proteins (Khan *et al.*, 2005) that should be further investigated in *P. falciparum*.

Comparing the relative expression values of Pfg377 versus Pfs16 revealed that transcription levels increased during gametocyte development. Pfg377 is associated with osmiophilic bodies, which are needed for the emergence of the female gamete, therefore one would predict that transcription increases during the later stages of gametocyte development, as it was observed when comparing Pfg377 to Pfs25; Pfg377 decreases, as Pfs25 transcription levels were very low at stage III but increased considerably from stage IV onwards.

Pfs25 has been associated with the zygote and ookinete (Dechering *et al.*, 1999) and therefore only has high transcription levels during the later stages of development, as was observed here. In contrast, Alpha-tubulin II had higher transcription levels during the early stages of development, which decreased during the later stages of gametocyte development in comparison to Pfs16 and Pfs25. When comparing relative overall expression values at different gametocyte stages, it becomes evident that little variation exists at stage III and activated

gametocytes. These observations confirm the validity of the assay, as they reveal transcription levels as previously reported (Bruce *et al.*, 1994; Alano *et al.*, 1995a; Severini *et al.*, 1999; Schneider *et al.*, 2004; Kooij *et al.*, 2006; de Koning-Ward *et al.*, 2008; Fennell *et al.*, 2008). However, it should also be noted that gametocyte cultures were not always 100% synchronous, therefore it is difficult to tell at which exact stage transcription levels are observed. The relative expression analysis from female parasites (Pfg377) to total parasites (Pfs16 or Pfs25) to establish reliable sex ratios requires the analysis of more samples. It is also necessary to evaluate whether early stage V gametocytes have different transcription levels to late stage V gametocytes, as this may cause some variation in the relative expression data.

Overall, this assay has opened new doors for quantifying transcription levels of malaria parasites. This assay was developed for estimating sex ratios from natural infections, but this assay may have more applications to obtain a better understanding of the gametocyte biology *in vitro* and *in vivo*.

7 FINAL DISCUSSION AND FUTURE WORK

7.1 SUMMARY OF RESULTS

7.1.1 Chapter 4: Investigation of DHCs

Sex specific DHCs were investigated here for the first time in *P. falciparum*. It was found that the DHCs targeted were transcribed from stage I onwards, which has never been reported from a sex specific protein in *P. falciparum* and may serve as a novel marker for sexual differentiation, which should be further studied in regard to the maturity of the gametocyte culture. The DHCs targeted here did not serve as ideal tools for sex ratio quantification. However, many more DHCs exist and have never been investigated and should be further explored in malaria parasites. DHCs may also be of great interest for molecular studies and as novel drug and vaccine targets and should be further investigated.

7.1.2 Chapter 5: Sex ratio analysis by microscopy

MACS® purification of gametocytes was used here for the first time to quantify gametocyte sex ratios using light microscopy and IFA, which allowed to sex gametocytes at much larger densities than previously reported. IFA analysis permitted for the first time observing expression of sex specific proteins during gametocytogenesis in large densities of gametocytes and also allowed establishing gametocyte sex ratios from stage IV gametocytes onwards for the first time, which was not based on the subjective identification of characters. Sex ratio analysis by IFA may be of interest for other studies investigating the morphology of male and female gametocytes during gametocytogenesis. It was also found that the sex ratio increased further once gametocytes were activated. Establishing sex ratios using light microscopy and IFA allowed to measure sex ratios with the help of two different methods, which allowed to later calibrate the qRT-PCR relative expression data.

7.1.3 Chapter 6: Sex ratio analysis by qRT-PCR

The qRT-PCR assay used here is the first assay developed that allows establishing relative quantitative data that can be used to estimate sex ratios of *P. falciparum*, by calibrating these against the sex ratios established both by light microscopy and IFA. This assay also allowed determining the maturity of the culture, by

comparing the relative expression values of the sexual stage and sex specific genes. This may be of further interest for optimizing the age of the culture for transmission studies in the laboratory. This assay now needs to be optimized for the further use of peripheral blood samples collected from natural infections.

7.2 GENERAL DISCUSSION AND FUTURE WORK

Recent advancements in malaria molecular biology, such as the sequencing of multiple *Plasmodium* genomes, are enabling us to improve our understanding of the parasite and its complicated interactions with both human and mosquito hosts. Despite all our advances, many important biological questions about this parasite remain to be answered. To this date only four sex specific proteins of *P. falciparum* have been studied and further investigations of the gametocyte proteome are needed to gain more insight into the biology of the sexual stages and to identify novel methods to block transmission of the parasites to the mosquito.

In chapter 4 sex specific dyneins were investigated for the first time in *P. falciparum.* The DHCs studied here were found to be the earliest transcribed sex specific proteins – the first ever reported. Analysis of the amino acid sequence of the DHCs investigated here suggested that they belong to the cytoplasmic dyneins and are likely to play a role in the rearrangements of chromosomes. Male specific dyneins investigated might be involved in the three rounds of mitosis; and the female specific dyneins might be involved in preparation for meiosis, which both occur at the onset of gametogenesis. It is important to identify and understand the function of proteins, such as dyneins, as they may lead to the discovery of novel targets for microtubule inhibitors which could be used as potential novel antimalarials (Kappes *et al.*, 2007). These microtubule inhibitors could prevent the polymerisation of the microtubules, which could interfere with the development of male or female gametocytes and block transmission. For example, sex specific DHCs microtubule inhibitors might prevent fertilization from occurring, by blocking the proper alignment of chromosomes. Dyneins have already been of interest for vaccine development for other parasites, such as schistosomes (Githui *et al.*, 2009) and leishmaniasis (Stober et al., 2006) and should be further investigated in malaria parasites as potential novel transmission blocking targets.

In Chapter 5 a novel combination of gametocyte purification (Saeed *et al.*, 2008) and greatly improved immune fluorescent staining IFA method was

described for the investigation of sexual stage and sex-specific protein expression during gametocytogenesis. This method allowed not only the sexing of gametocytes in a non-subjective way, but also permitted the observation of morphological characters from high gametocyte densities, at the same time. However, as mentioned earlier, few details of sex specific proteins are known which limits the advancement of IFA studies. If a greater selection of sex-specific proteins were available, this could lead onto a wider range of potential IFA targets. This can only be achieved with an improved understanding of the proteome.

Such proteomic analysis for *P. falciparum* could be done in the same way as it has been done previously with *P. berghei* (Khan *et al.*, 2005), for example. The first step would require separating the sexes leading to the identification of sex specific proteins. Following the strategies of Khan *et al.*, 2005, this would include transfecting male and female gametocytes with a sex specific fluorescent protein, and then using fluorescence activated cell sorting to separate the sexes. This would be followed by mass spectrometry for the identification of individual proteins. Once all sex specific proteins have been identified, the optimal proteins to target male and female gametocytes for sex ratio analysis using qRT-PCR could be potentially selected.

To increase sensitivity of the qRT-PCR assay presented in Chapter 6, the selected proteins should have relatively high and constant transcription levels. Ideally these proteins would be transcribed in mature gametocytes only and are not present in sexually determined ring stage parasites, as might be the case with Pfs16 (Dechering *et al.*, 1999; McRobert *et al.*, 2004). It would also be essential to follow transcription levels as gametocytes mature and observe changes in transcription levels to obtain non-fluctuating relative expression values. The protein sequence targeted should also be checked for homology to other *Plasmodium* species and the human host, to prevent non-specific amplification when sequences are very similar to each other.

It would be also of interest to use sex specific proteins that are transcribed only before sequestration, as this would allow one to compare the gametocyte sex ratio before and after sequestration. This may elucidate how gametocytes are affected by the immune system and at what rate they may be destroyed. Dyneins investigated in this study had low transcription levels, but Khan *et al.*, (2005)

reported a number of different male specific dyneins that may be of interest for further investigation.

The detection of male and female gametocytes at submicroscopic levels is important to obtain precise and non-subjective data on the gametocyte sex ratios. Only with novel accurate detection methods will it be possible to evaluate the effects of drug treatment on the parasites, transmission dynamics and transmission blocking strategies. This assay needs to be optimized to allow for obtaining precise sex ratio data, which could be done by establishing a serial mixture of gametocytes with a sex ratio that is all male biased to a sex ratio that is all female biased. Such a serial mixture of different sex ratios could be also done after male and female gametocytes were successfully separated by FACS analysis. Using a serial dilution of different gametocyte sex ratios would allow for the setting of the exact relative expression values for each sex ratio.

An inherent problem remains when studying sex ratios in natural gametocyte infections. This is due to the number of variables related to gametocyte dynamics which are not currently considered when studying sex ratios. Our limited knowledge of these influences could potentially adjust the interpretation of such data. For example, the longevity of male and female gametocytes has not been studied extensively in *P. falciparum* and whether any differences in their survival rates exist is still unknown. If the life span of one sex differs, this may affect sex ratios when observing it over time. Several recent studies have suggested that sex ratios may be affected due to the different half-lives of male and female gametocytes in the presence of drugs (Sowunmi *et al.*, 2009).

If females have a life span that differs from that of males, one could expect to observe a different sex ratio at different time points in natural infections, see figure 7.0. Several studies have found that the gametocyte sex ratio becomes more male biased over time both *in vitro* and *in vivo* (Paul *et al.*, 1999; Reece, 2003; Reece *et al.*, 2008), which could be due to the possibility that more males need to be produced to ensure fertilization. This observation has been hypothesized to occur because of immune responses against the sexual stages, which requires that more males must be produced to increase the chances of successful transmission (Paul *et al.*, 2002a). It is possible that males, for example, do not survive as long as females when circulating in the blood and that the differences in longevity among clones may also exist, which would affect the sex ratio. In this study, it

was observed that male gametocytes activate faster than females *in vitro*. This phenomenon may not occur in natural hosts as such, but it still reveals biological differences exist between male and female gametocyte biology that may affect the sex ratio *in vivo*.

Differential timing of male and female gametocyte development may be beneficial to the parasite to avoid self-fertilization. Dichogamy, the separation in time of gender expression has frequently been reported in hermaphroditic plants, where the female gamete matures before the male gamete (protogyny), or the male gamete before the female gamete (protoandry) (Bertin *et al.*, 1993). It is possible that similar mechanisms are present in malaria parasites to increase the chances of outbreeding. A study is needed that determines the exact half-lives of male and female gametocytes. Preferably this should be done with different clones, as differences are likely to exist among different genotypes. Following this, it would be necessary to investigate how certain antimalarial drugs affect the half-lives of male and female gametocytes.

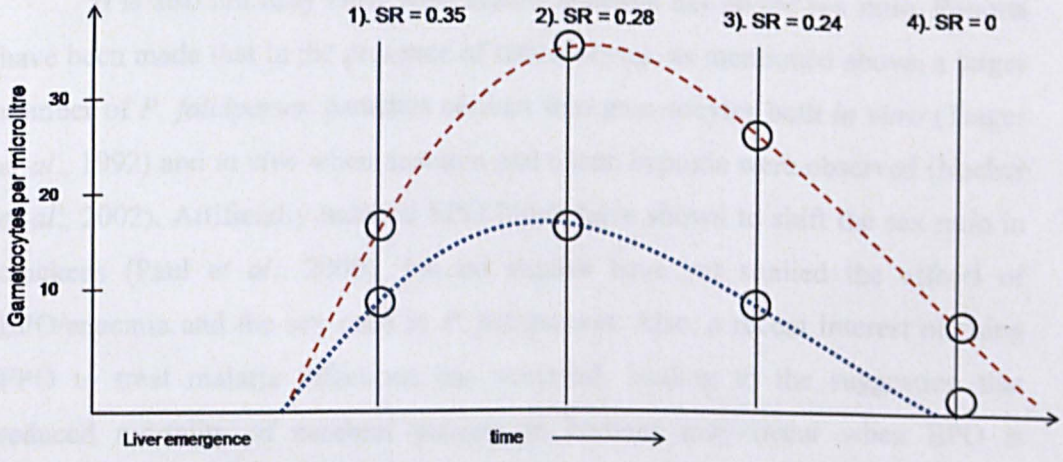


Figure 7.0 : Hypothetical life span of male and female gametocytes. Female gametocytes (red line) may have a longer life span than male gametocytes (blue line). If differences in their life span exist than one can expect to find a change of the sex ratio at different time points (1-4).

In natural infections, the genetic heterogeneity of the parasite and host are likely to influence gametocytes differentially; therefore it would be beneficial to evaluate such factors in the laboratory under controlled conditions. For future studies, this qRt-PCR sex ratio assay could be first used in the laboratory to evaluate certain factors that may be involved in shaping the sex ratio. In natural infections, host factors may influence gametocyte sex ratios and it is therefore useful to study certain aspects (host nutrition, other parasitic infections and

anaemia for example) under controlled conditions. The sex ratio assay developed here could also be used to compare relative expression levels observed in natural infections, to evaluate the optimal maturity of gametocytes for laboratory transmission studies.

Host factors, such as haemoglobinopathies may also influence gametocyte sex ratios and should be considered. For example, it is possible that sickle cell disease affects gametocytogenesis and/or the gametocyte sex ratios, by affecting one sex more than the other. Sickle cell cause an increase of reticulocytes, which may increase commitment of sexual stage parasites, as it has been shown in the laboratory (Trager *et al.*, 1992). Disorders such as sickle cell and alpha thalassemia are frequently found in malaria endemic regions and due to their prominence in certain areas, it is possible that malaria parasites may have adapted their reproductive strategies accordingly, potentially including the sex ratio. It is possible that one sex may be more affected than the other by such blood disorders; it may therefore require large longitudinal cohort studies to evaluate whether hosts factors are present that may affect the results of the study.

It is also not fully clear what effects anaemia has on the sex ratio. Reports have been made that in the presence of reticulocytes, as mentioned above, a larger number of *P. falciparum* parasites convert into gametocytes both *in vitro* (Trager *et al.*, 1992) and *in vivo* when anaemia and tissue hypoxia were observed (Nacher *et al.*, 2002). Artificially induced EPO levels have shown to shift the sex ratio in chickens (Paul *et al.*, 2000), but no studies have yet studied the effects of EPO/anaemia and the sex ratio in *P. falciparum.* Also, a recent interest of using EPO to treat malaria infections has occurred, leading to the suggestion that reduced mortality of cerebral malaria in humans may occur when EPO is administered to the patient (Casals-Pascual *et al.*, 2009). Investigating the effects of host factors such as anaemia/EPO levels is therefore not only essential for sex ratio studies, but will give us clues how EPO may affect the parasite and host interactions.

Another important aspect that needs to be investigated when studying gametocyte sex ratios is whether host immune responses affect gametocytes differentially. Gametocyte surface antigens (GSA) have only been identified recently (Saeed *et al.*, 2008), but the question of whether plasma antibodies may affect male and female gametocytes differentially is still unanswered. This could be solved by using plasma from gametocyte positive patients in the laboratory and

evaluating its affect on one sex compared to other. Studying gametocytes in the laboratory may not extrapolate to what one could expect to find in natural infections. However, it may reveal some information on the biology of the sexual stages that allow for improved interpretation of data from natural infections.

People infected with malaria are likely to carry more than one parasite clone; both in areas with low and high transmission intensities (Babiker, 1998; Paul *et al.*, 1998). When evaluating sex ratios from natural infections, the sex ratio may be calculated from polyclonal infections, which are likely to vary among each other. Therefore, when observing sex ratios over time; different sex ratios can be expected to be different at different time points. It is likely that variation amongst genotypes in the relation to gametocyte density, timing, half-life and sex ratio exist. In addition, gametocyte clones of one genotype are likely to overlap and clones are likely to vary from one another in their longevity. Therefore when observing sex ratios over time from drug trials, for example, final conclusions need to be drawn carefully, as gametocytes may be a mixture of clones originating from different genotypes, see figure 7.7.1.

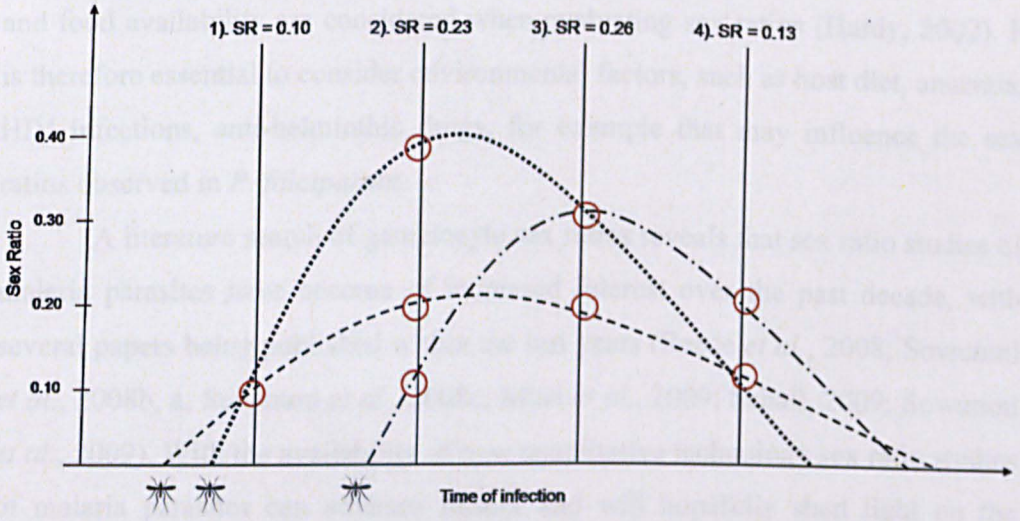


Figure 7.7.1: Hypothetical sex ratio of polyclonal infections over time. Representing a hypothetical infection with three inoculations (dotted lines) at different time points (see mosquito). Sex ratio of each clone was measured at four different time points and combined from all clones. Representing that sex ratios, especially in high transmission areas, consists from more than one clone and due to different inoculation times and intrinsic differences of the sex ratio, should be expected to change over time.

Once the qRT-PCR assay has been optimized and factors that could influence the sex ratio have been studied in the laboratory, longitudinal field studies of gametocyte sex ratios from natural infections would be needed. This study would include asymptomatic gametocyte carriers and patients who are treated with antimalarial and other drugs that may be administered to people living in malaria endemic regions (such as antibiotics or treatment for diseases such as HIV). Sex ratios should be evaluated in correlation to longevity, effects of drug treatment (if applicable), EPO levels and anaemia, immune responses, host factors and whether the sex ratio is measured from a polyclonal or monoclonal infection (see figure 7.7.2).

Host and parasite factors should always be considered when studying gametocyte sex ratios in natural infections, as sex ratios may be effected by a variety of factors. Sex ratio studies of other taxa commonly include external factors in their studies that may influence the sex ratios. For example in mammals, social rank, food availability, and stress may influence sex ratios, whereas in arthropods for example, temperature, population densities, bacterial infections, and food availability are considered when evaluating sex ratios (Hardy, 2002). It is therefore essential to consider environmental factors, such as host diet, anaemia, HIV infections, anti-helminthic drugs, for example that may influence the sex ratios observed in *P. falciparum*.

A literature search of gametocyte sex ratios reveals that sex ratio studies of malaria parasites have become of increased interest over the past decade, with several papers being published within the last years (Reece *et al.*, 2008; Sowunmi *et al.*, 2008b, a; Sowunmi *et al.*, 2008c; Mitri *et al.*, 2009; Schall, 2009; Sowunmi *et al.*, 2009). With the availability of new quantitative technology sex ratio studies of malaria parasites can advance further and will hopefully shed light on the biology of sexual reproduction and on transmission blocking strategies, aiming to reduce the burden of malaria.

Optimization of the qRt-PCR assay:

1. Proteomic analysis of male and female gametocytes:
2. Identify novel sex specific proteins
3. Make serial mixtures with different sex ratios and calibrate the assay precisely

Use the qRT-PCR assay to investigate gametocyte biology:

1. Determine the half-life of male and female gametocytes
2. Investigate the effects antimalarials have on male and female gametocytes
3. Investigate how EPO affects sex determination
4. Study how plasma from infected patients affect mature male and female gametocytes

Use the qRT-PCR assay to observe sex ratios from natural infections:

1. Large longitudinal sex ratio field studies should consider:
   -Mono/polyclonal infarctions?
   -Half-lifes of male and female gametocytes?
   -EPO/anaemia?
   -Sex specific antibodies?
   -Genetic host factors?

Figure 7.7.2: Overview of the future work for *P. falciparum* sex ratio studies. Overview of the future work for sex ratio studies of *P. falciparum*. Ideally, the future work would involve three steps; step one would allow the optimization of the qRT-PCR assay in the laboratory, the second step would require to study factors that may influence the male and female gametocyte longevity in the laboratory, the third step would allow using the assay to observe natural infections considering all factors that may influence sex ratios.

8 APPENDICES

8.1 Buffers, solutions and media

ICM (Incomplete Media)
RPMI 1640 (with 25mM HEPES and L-Glutamine), Gibco

CM (Complete Media)
RPMI 1640 (with 25mM HEPES and L-Glutamine), Gibco
0.005% hypoxanthine
10% human AB+ pooled heat-inactivated serum (National blood service)
40mg/L gentamyacin

Cryopreservative
3% D-sorbitol
0.65% NaCl
28% Glycerol

Synchronization Solution
RPMI 1640 (with 25mM HEPES and L-Glutamine), Gibco
15 % D-sorbitol RPMI

Giemsa Buffer
0.1% Di-sodium hydrogen orthophosphate
0.07% Potassium dihydrogen orthophosphate
Adjust to a pH of 7.4

Giemsa Stain
Giemsa solution (BDH) was diluted 1:9 with Giemsa buffer

TBE
89mM Trizma base
89mM Boric acid
2mM EDTA

Thawing solution
(1) RPMI 1640 (with 25mM HEPES and L-Glutamine), Gibco
12 % NaCl
(2) 1.6 % NaCl
RPMI 1640 (with 25mM HEPES and L-Glutamine), Gibco
(3) 0.9 % NaCl + 0.2 % D- glucose
RPMI 1640 (with 25mM HEPES and L-Glutamine), Gibco

8.2 RT-PCR: cycling conditions

Primers: SA01& SA02
1. 94°C for 5 minutes
2. 94°C for 15 seconds
3. 50°C for 30 seconds
4. 70°C for 30 seconds
Cycle steps 2. to 4. were repeated 44 times

Primers: SA03 & SA04
1. 94°C for 3 minutes
2. 94°C for 30 seconds
3. 50°C for 1 minute
4. 68°C for 2 minutes
Cycle steps 2. to 4. were repeated 39 times

Primers: SA05-SA06
1. 95°C for 6 minutes
2. 95°C for 5 seconds
3. 58°C for 1 minute
4. 70°C for 30 seconds
Cycle steps 2. to 4. were repeated 39 times

Primers: SA07-SA08
1. 94°C for 6 minutes
2. 94°C for 5 seconds
3. 53°C for 1 minute
4. 70°C for 30 seconds
Cycle steps 2. to 4. were repeated 39 times

Primers: SA09-SA10
1. 96°C for 6 minutes
2. 96°C for 5 seconds
3. 59°C for 1 minute
4. 70°C for 30 seconds
Cycle steps 2. to 4. were repeated 39 times

Primers: SA11-SA12
1. 94°C for 6 minutes
2. 94°C for 5 seconds
3. 54°C for 1 minute
4. 70°C for 30 seconds
Cycle steps 2. to 4. were repeated 39 times

8.3 qRT-PCR: sex ratio assay supplementary data

cDNA dilution	Mean Delta Ct	Standard error
1	8.08	1.67
0.5	8.51	0.11
0.25	8.95	1.85
0.125	6.33	3.00
0.0625	8.02	2.62
0.03125	8.68	
0.015623	10.67	
slope	-0.64	

Table 1: (Figure 6.4 A): ΔCt values of Pfg377 versus Pfs16, when AE =1. cDNA concentration and the mean ΔCt of Pfg377 versus Pfs16, indicating the standard error of the mean and the slope of the best fit line. Samples were run in triplicates.

cDNA dilution	Mean Delta Ct	Standard error
1	4.45	0.40
0.5	4.40	0.55
0.25	4.80	1.88
0.125	2.88	3.64
0.0625	4.08	3.71
0.03125	5.28	
0.015623		
slope	0.20	

Table 2: (Figure 6.4 B): ΔCt values of Pfg377 versus Pfs25, when AE =1. cDNA concentration and the mean ΔCt of Pfg377 versus Pfs25, indicating the standard error of the mean and the slope of the best fit line.

cDNA dilution	Mean Delta Ct	Standard error
1	-3.63	1.27
0.5	-4.11	0.46
0.25	-4.15	1.95
0.125	-3.46	0.75
0.0625	-4.07	1.09
0.03125	-4.02	0.87
0.015623		
slope	0.22	

Table 3: (Figure 6.4 C): ΔCt values of Pfs16 versus Pfs25, when AE =1. cDNA concentration and the mean ΔCt of Pfs16 versus Pfs25, indicating the standard error of the mean and the slope of the best fit line.

cDNA dilution	Mean Delta Ct	Standard error
1	5.76	0.42
0.5	5.65	0.37
0.25	6.17	1.00
0.125	5.71	1.08
0.0625	6.37	0.54
0.03125	5.01	0.29
0.015623	6.06	
slope	-0.07	

Table 4: (Figure 6.4 D): ΔCt values of Alpha-tubulin II versus Pfs16, when AE =1. cDNA concentration and the mean ΔCt of Alpha-tubulin II versus Pfs16, indicating the standard error of the mean and the slope of the best fit line.

cDNA dilution	Mean Delta Ct	Standard error
1	2.13	0.84
0.5	1.54	0.10
0.25	2.02	1.04
0.125	2.26	0.38
0.0625	2.30	0.62
0.03125	0.84	1.03
0.015623		
slope	0.32	

Table 5: (Figure 6.4 E): ΔCt values of Alpha-tubulin II versus Pfs25, when AE =1. cDNA concentration and the mean ΔCt of Alpha-tubulin II versus Pfs25, indicating the standard error of the mean and the slope of the best fit line.

Stage	Sample	377 vs. 16	377 vs. 25	16 vs. 25	A.tub.II vs. 16	A.tub.II vs. 25	25 vs. 377
III	a	-1.09	-0.42	0.67	-0.49	0.18	0.42
III	b	-1.12	-0.48	0.64	-0.39	0.25	0.48
IV	a	-0.84	-0.45	0.40	-0.98	-0.58	0.45
IV	a	-0.27	0.01	0.28	-0.85	-0.58	-0.01
IV	b	-0.62	-0.84	-0.22	-1.72	-1.94	0.84
IV	b	-1.37	-1.29	0.09	0.11	-0.87	1.29
IV	c	-1.39	-1.53	-0.14	*	*	1.51
V	a	*	*	0.21	-0.77	-0.56	*
V	a	-1.51	-1.40	0.11	-0.43	-0.32	1.40
V	b	-0.61	-0.98	-0.37	-0.99	-1.35	0.98
AG	a	-0.36	-1.06	-0.55	-1.73	-2.05	1.06
AG	a	-0.67	*	*	*	*	*
AG	b	-0.31	-1.21	-0.90	-1.54	-2.44	1.21
AG	b	-0.46	-1.16	-0.51	-1.69	-2.15	1.13
AG	c	-0.62	-1.41	-0.50	*	*	1.41
AS	a	-0.73	-0.70	0.03	-1.09	-1.06	0.70
AS	a	0.07	-0.67	-0.74	-0.63	-1.37	0.67
AS	a	*	-0.63	*	*	-1.39	0.63
AS	b	-0.69	-0.59	0.08	-0.89	-0.78	0.59
AS	b	-0.16	-0.60	-0.44	-0.55	-0.98	0.60
AS	b	*	-0.67	*	*	-0.98	0.67
GA	a	-0.15	*	*	*	*	*
GA	b	0.09	0.10	0.01	*	*	-0.10
GA	c	*	*	*	0.05	*	*
GA	d	*	*	*	-0.54	*	*

Table 8. (Figure 6.7): Relative expression during gametocytogenesis. Log10 of relative expression of Pfg377 versus Pfs16 (377 vs. Pfs16), Pfg377 versus Pfs25 (377 vs. 25), Pfs16 versus Pfs25 (16 vs. 25), Alpha-tubulin II versus Pfs16 (A.tub.II vs. P), Alpha-tubulin II versus Pfs25 (A.tub.II vs. 25), Pfs25 versus Pfg377 (25 vs. 377). Stages III-V, activated gametocytes (AG), asynchronous culture (AS), and Gambian samples (GA). Fields marked with an asterisk (*) did not yield amplification or too poor amplification for analysis. Fields with a bold border had a spread Ct ≥ 2 and the samples were removed due to the large differences of the Ct values.

Stage	III	IV	V	AG
Mean	**-1.11**	**-1.11**	**-0.90**	**-0.48**
	-1.09	-0.84	-1.51	-0.36
	-1.12	-0.27	-0.61	-0.67
	*	-0.62	*	-0.31
	*	-1.37	*	-0.46
	*	-1.39	*	-0.62

Table 9. (Figure 6.10A): Relative expression of Pfg377 versus Pfs16 over gametocytogenesis. Log 10 of the mean relative expression values and the individual expression values of Pfg377 versus Pfs16 over gametocytogenesis. Fields containing an asterisk did not yield any results.

Stage	III	IV	V	AG
Mean	**-0.45**	**-0.82**	**-1.19**	**-1.21**
	-0.42	-0.45	-1.40	-1.06
	-0.48	0.01	-0.98	-1.21
	*	-0.84	*	-1.16
	*	-1.29	*	-1.41
	*	-1.53	*	

Table 10. (Figure 6.10B): Relative expression of Pfg377 versus Pfs25 over gametocytogenesis. Log 10 of the mean of the relative expression values and the individual expression values of Pfg377 versus Pfs25 over gametocytogenesis.

Stage	III	IV	V	AG
Mean	**-0.44**	**-0.86**	**-0.73**	**-1.65**
	-0.49	-0.98	-0.77	-1.73
	-0.39	-0.85	-0.43	-1.54
	*	-1.72	-0.99	-1.69
	*	0.11	*	*

Table 13. (Figure 6.9D): Relative expression of Alpha-tubulin II versus Pfs16 over gametocytogenesis. Log 10 of the mean of the relative expression values and the individual expression values of Alpha-tubulin II versus Pfs16 over gametocytogenesis. Fields containing an asterisk did not yield any results.

Stage	III	IV	V	AG
Mean	**0.22**	**-0.99**	**-0.75**	**-2.21**
	0.18	-0.58	-0.56	-2.05
	0.25	-0.58	-0.32	-2.44
	*	-1.94	-1.35	-2.15
	*	-0.87	*	*

Table 14. (Figure 6.10E): Relative expression of Alpha-tubulin II versus Pfs25 over gametocytogenesis. Log 10 of the mean of the relative expression values and the individual expression values of Alpha-tubulin II versus Pfs25 over gametocytogenesis. Fields containing an asterisk did not yield any results.

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