CHAPTER 3

APPLICATION OF A 3D SCAFFOLD: A SPOROZOITE-HEPATOCYTE MODEL

3.1. INTRODUCTION

3.1.1. Malaria

The liver constitutes the largest organ in the human body and is responsible for performing critical tasks such as plasma protein synthesis, hormone and bile production, glycogen storage, metabolism of xenobiotics, decomposition of erythrocytes and lipid digestion. The liver supports almost every organ in the body and is vital for our survival; however, this organ is also involved in many diseases, of which malaria ranks amongst one of the most important and widespread. An accurate estimation of the extent of morbidity and mortality caused by malaria is difficult but ~216 million cases and 655 000 deaths were reported for 2010 (131).

Malaria is caused by the protozoan parasite belonging to the genus *Plasmodium*. Five species account for almost all human infections (*P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi)*. *P falciparum* causes the majority of infections in Africa and is responsible for most of the instances of severe disease and mortality (132). The parasite's life cycle, as illustrated in Figure 3.1, begins when the parasite is transmitted as a liver-infective sporozoite to the mammalian host through the bite of an infected female *Anopheles* mosquito. An average of 15-123 sporozoites are injected under the skin of the host and enter the blood circulation system where they migrate to the liver. There they infect hepatocytes and begin to develop into merozoites (133, 134). Tens of thousands of merozoites develop per invading sporozoite over a period of days to ~2 weeks (depending on the species) and are released into the blood stream where they rapidly adhere to and invade erythrocytes. Further infectious merozoites are subsequently generated through asexual replication inside erythrocytes, and this intra-erythrocytic cycle is also where malaria-associated pathology becomes detectable (135).

The malaria parasite's life cycle also comprises a phase of sexual reproduction, which takes place in the mosquito vector. The switch to sexual development starts in the human host, with the formation of male and female gamete precursors (gametocytes), and is a prerequisite for transmission of disease to the mosquito vector (136). Once ingested by the female mosquito the

male and female gametes form zygotes, which develop into oocysts. From these oocysts sporozoites emerge and travel to the salivary gland of the mosquito ready to be injected into the next host. Sporozoites are generated in multinucleated oocysts located between the epithelium and the basal lamina of the mosquito midgut. When a mature oocyst bursts, sporozoites are released into the mosquito's haemolymph; when the sporozoites reach the mosquito salivary glands they attach to and invade gland cells. Sporozoites transit through the cytoplasm of the gland cells and exit into the secretary cavity where they await the next blood meal for transmission to the host (137).

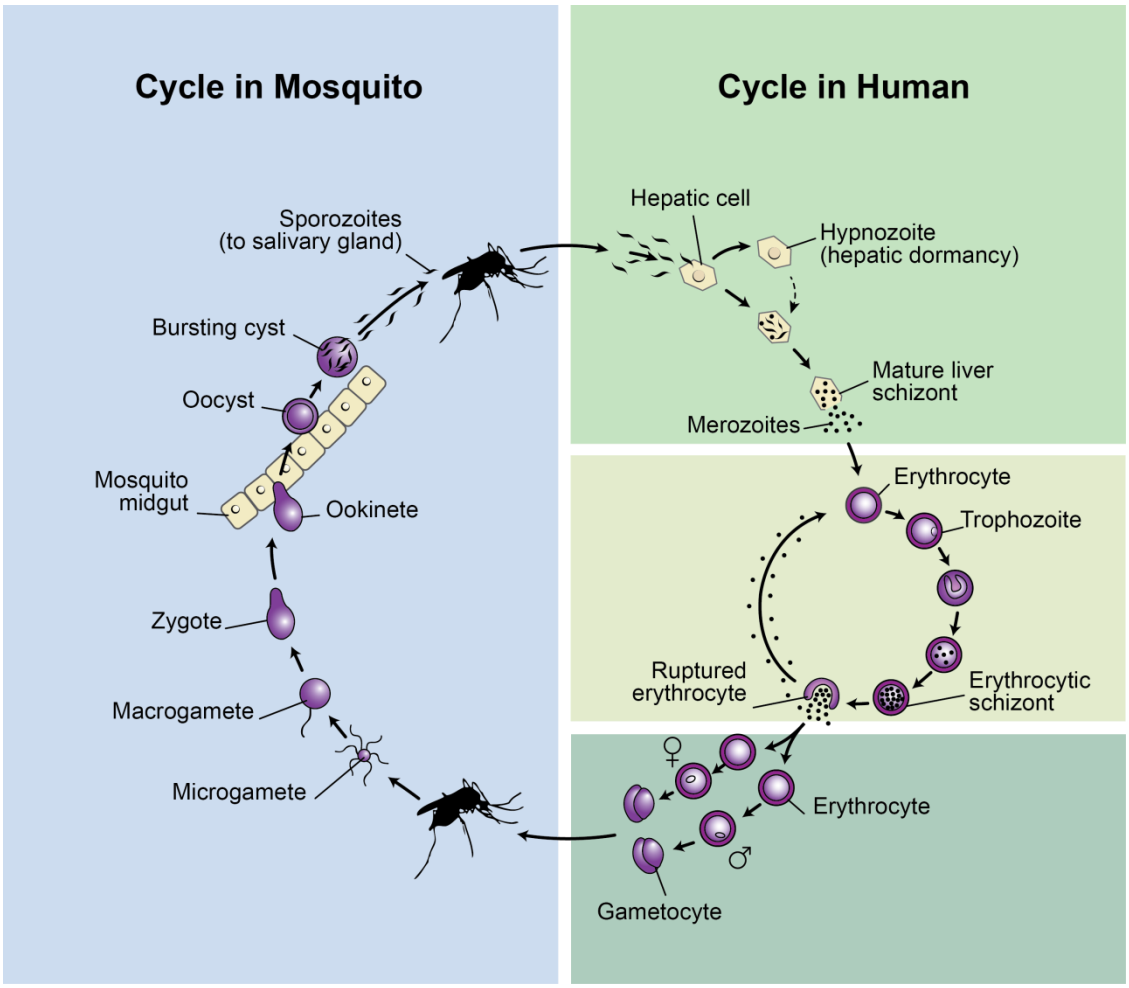

Figure 3.1. The life cycle of *P. falciparum* parasites in the human host and the *Anopheles* mosquito vector. Malaria develops in two phases: an exo-erythrocytic and an intra-erythrocytic phase. When an infected mosquito feeds on its host, sporozoites in the mosquito's saliva enter the bloodstream and migrate to the liver multiplying asexually and asymptomatically forming thousands of merozoites over a period of 5 - 16 days which, following rupture of their host cells, escape into the blood and infect erythrocytes. This is the beginning of the erythrocytic stage of the life cycle. Within the erythrocytes, the parasites multiply further. A merozoite infects an erythrocyte and matures into a schizont, which releases more merozoites into the circulatory system to invade fresh erythrocytes approximately every 48 h. Several such amplification cycles occur and it is at this stage that the clinical signs of malaria are observed. Some merozoites are triggered to form the sexual stage parasites (gametocytes), which are ingested by a female mosquito when taking a blood meal. The male and female gametes form zygotes, which develop into oocysts. After 8 - 15 days these oocysts sporozoites emerge and travel to the salivary gland of the mosquito ready to be injected into the next host. Adapted from (138).

3.1.2. Sporozoite hepatocyte invasion

The proposed journey of the sporozoite to the hepatocyte and subsequent liver stage development starts when the infectious sporozoite is deposited into the skin by the mosquito vector and subsequently enters the bloodstream through a capillary endothelial cell. Some sporozoites may also enter draining lymph nodes and can partially develop within the lymphoid endothelium. After being transported by the bloodstream through the body, the sporozoites eventually reach the liver. In the liver sinusoids, the blood flow is very slow and sporozoites are able to adhere to the liver endothelium via circumsporozoite protein recognition of heparan sulphate proteoglycans (HSPGs) protruding into the sinusoidal lumen through endothelial fenestrations (139). Following adhesion, the sporozoites glide along the endothelium looking for a point of entry to the space of Disse and the underlying hepatocytes (140). The sporozoite then traverses a number of hepatocytes before invading a hepatocyte with the formation of a parasitophorous vacuole membrane. Although contentious, transgenic mice (homozygous op/op known to have few Kupffer cells) (141) and intra-vital microscopy using infected live mice with red or green fluorescent *P. berghei* sporozoites (142) have pointed to Kupffer cells (resident macrophages in the liver) as the main gateway into the liver. In addition, it has been shown that sporozoites can transmigrate Kupffer cells and macrophages *in vitro* (142, 143).

At least two cell-invasive motilities have been documented for malaria parasites: cell-infection (137) and cell-traversal motility (144). Cell-infection motility is accompanied by vacuole formation and is followed by parasite development into exo-erythrocytic (EE) forms, whereas cell-traversal motility involves plasma-membrane disruption and is followed by migration through the cytoplasm and eventual escape from the cell. In their 2002 paper, Mota *et al.* revealed that cell traversal motility can be identified by a conventional cell-wound assay and they proposed that this motility is necessary for sporozoites to be activated for hepatocyte infection (145). Once inside the hepatocyte the sporozoites develop into EE forms, undergo schizogony and produce thousands of erythrocyte-infectious merozoites that enter the sinusoid packaged in extrusomes / merosomes (in order to avoid immune detection) and are subsequently released in the bloodstream (146, 147).

3.1.3. *In vitro* culturing of hepatocytes for malaria sporozoite invasion

In recent years, the malaria parasite has become ever-more resistant to the current line of available drugs and in certain regions is increasingly resistant to the last line of defence, artemisinin (148). Most available antimalarials are designed to target the pathogenic blood

stages in humans and to address the constant threat of drug resistance (149). Thus there is a renewed interest in new methods to tackle malaria, in particular the development of solutions that combat EE development including liver-stage infection as well as gametocyte formation. Despite being asymptomatic, the liver stage of *Plasmodium* infection is both immunologically active (150) and one where a rich array of host–parasite interactions takes place (146, 151, 152). In endemic areas relapse of vivax malaria is a major cause of malaria in young children, and an important source of malaria transmission. Relapse also occurs in *P. ovale* infections (153). This recurrence arises from persistent liver stages of the parasite (hypnozoites) (153). Molecules that efficiently target the parasite stages in the liver would offer protection from the development of the blood stages and resultant malaria symptoms. As only approximately 100 sporozoites may be introduced by a bite, there are also likely to be many orders of magnitude fewer parasites at this stage than in an active blood stage infection, reducing the possibility of resistance arising (154). New compounds targeting this stage of the disease are therefore highly desirable; at the same time the liver stage represents the most suitable opportunity for developing long-term vaccine solutions.

Much of what researchers know today about the *Plasmodium* EE stages is as a result of research done using *Plasmodium* spp. that infects rodents, specifically*, P. berghei* and *P. yoelii*. These two species present differences in infectivity, which depend not only on the species but also on the clone and the genetic background of the rodent host (133). *In vitro* systems have been developed to study the EE stages of these malaria parasites in various human and mouse cell-lines including HepG2, Huh-1/2, Huh-7, HSS-102, HC04, WI38 and HeLa cell-lines grown in 2D (155). The sporozoites of human and primate malaria parasites will readily infect primary cells but these cells cannot be grown continuously in culture and are thus not ideal for experimental purposes. However, Sattabongkot *et al*. (113) developed the hepatocyte cell-line, HC04, that could support the complete development of the EE stage parasites from *P. falciparum* and *P. vivax* but infectivity was low; ~0.066% and 0.041% for *P. falciparum* and *P. vivax* infections, respectively. As a direct result of the low levels of infectivity, down-stream applications to study the parasite including global strategies like genomic and proteomic investigations are less feasible. Similarly, effective testing of antimalarial drugs on *P. falciparum*-infected hepatocytes remains a major challenge, primarily due to inconsistent and unreliable *in vitro* culturing systems developed for sporozoite invasion into hepatocytes. Thus, a new, reliable *in vitro* model that more easily permits researchers to study the *P. falciparum*-liver interaction is needed to expedite a more relevant understanding of malaria liver infection.

Chapter 3: An application of a 3D scaffold: A sporozoite-hepatocyte model

With the knowledge that the infectious sporozoites traverse several hepatocytes prior to cell infection, it was hypothesised that the newly developed 3D system described in Chapter 2 may provide a suitable basis for developing an improved host-pathogen model of malaria infection. Thus, the major focus of this chapter was to establish if a greater level of *in vitro P. falciparum* sporozoite invasion could be achieved in hepatocytes that have been cultured on 3D non-woven scaffolds compared to conventional 2D systems. Although *P. berghei* sporozoites have previously been produced *in vitro* in low numbers (156), an effective *in vitro* system for the generation of sporozoites poses as a major hurdle to this field of malaria drug discovery (155), especially for *P. falciparum*. Thus, the maintenance of mosquito colonies infected with malaria parasites is a pre-requisite for liver stage research. To date, this has not been achieved for *P. falciparum* in South Africa, limiting our research to the asexual stages of these parasites. The first objective of this chapter was, therefore, to establish methods to infect an *Anopheles* mosquito colony with *P. falciparum* gametocytes, to allow sexual replication in the mosquitoes and obtain a reliable source of *P. falciparum* sporozoites for subsequent use in the 3D hepatocyte cell culture model.

3.2. MATERIALS AND METHODS

3.2.1. *In vitro* cultivation of asexual *P. falciparum* cultures

Continuous asexual cultures of *P. falciparum* parasite strains 3D7, 7G8 and NF54 (MRA-1000 ATCC, MR4, ATCC® Manassas, Virginia) were maintained *in vitro* according to a modified Trager and Jensen method which supports intracellular parasite development (157). The erythrocyte and serum preparation, culture thawing and general maintenance of asexual stage parasites are described below.

Type A+ or O+ blood was collected in EDTA vacuum tubes, transferred to centrifuge tubes using aseptic technique and centrifuged at 2500*g* for 5 min at room temperature. The plasma and buffy coat were aspirated and an equal volume of wash medium [RPMI with L-glutamine and HEPES (Lonza Walkersville) supplemented with 0.4% (w/v) D-glucose (Sigma-Aldrich Chemie), 88 mg/L hypoxanthine (Sigma-Aldrich Chemie) and 48 mg gentamycin (Sigma-Aldrich Chemie)], was added. The erythrocytes were re-suspended and centrifuged at 2500*g* for 5 min, after which the supernatant was aspirated. The procedure was repeated 3 to 4 times to reduce the presence of leukocytes. Washed erythrocytes were re-suspended in an equal volume of wash medium and stored at 4°C. The wash medium was aspirated and replaced twice a week to preserve the erythrocytes.

Human serum (male, type A+, negative for HIV, hepatitis and syphilis, aspirin, antibiotic and drug free) was purchased from the Interstate Blood Bank (Tennessee, USA). The units of serum were filter sterilised through both a 47 mm type A/D glass fibre filter (PAL Corporation, Ann Arbor, Michigan, USA) and a 47 mm 0.22 PES filter (PAL Corporation). The units of serum were pooled (a minimum of four donors per pool) and aliquots were frozen at -20°C. The human serum was not heat inactivated on the advice of Megan Dowler from the Walter Reed Army Institute of Research (personal communication). Ethical approval for the use of donor blood and serum was conferred by the University of Pretoria and CSIR research ethics committees.

Glycerol-frozen aliquots of asexual *P. falciparum* 3D7, 7G8 and NF54 parasites were removed from storage at -80°C and quickly thawed in a water bath at 37°C. The thawed aliquot was transferred to a centrifuge tube under sterile conditions in a laminar flow cabinet. The osmotic potential of the thawed stocks were gradually reduced by the drop-wise addition of 200 µL 12% (w/v) NaCl solution, followed by pipette-mixing for ~10 – 20 s and then the drop-wise addition of 1.8 mL 1.6% (w/v) NaCl, again followed by pipette-mixing for ~10 – 20 s. The parasites were

collected by centrifugation at 2500*g* for 5 min at room temperature. The supernatant was aspirated and 10 mL culture medium [wash medium with 0.5% (m/v) AlbuMAX® II purified lipid-rich bovine serum albumin (Gibco®) for the 3D7 and 7G8 parasites, and RPMI with L-Glutamine and HEPES (Lonza Walkersville) with 88 mg/L hypoxanthine (Sigma-Aldrich Chemie) and filter sterilised human serum (A+ male, Interstate Blood Bank) for the NF54 parasites], preheated to 37°C, was added. This was followed by 500 µL freshly collected, washed, type A+, packed erythrocytes to establish a ~5% haematocrit culture. The erythrocytes were suspended and transferred to 75 cm^2 culture flasks (T75, Nunc) and gassed for 30 s with a special gas mixture containing 90% nitrogen, 5% oxygen and 5% carbon dioxide (Air Products, Johannesburg, South Africa). The flasks were sealed air-tight and incubated at 37°C.

Parasite growth was monitored daily by visual microscopic inspection of thin smears which were fixed with methanol (MeOH) and stained with a 10% (v/v) Giemsa stock solution (Merck) in phosphate buffered saline (PBS, pH 7.4, Lonza Walkersville). Parasitaemia was calculated by counting the equivalent of 1000 erythrocytes using the Miller technique for reticulocyte counting (158) at 1000x magnification with an Olympus BX41 microscope. This counting technique requires an evenly distributed erythrocyte smear across the microscope field and makes use of an ocular micrometer disk. It consists of two squares whose area has a ratio of 1:9; the small square occupies one corner of the large square. The principle involved in the use of the Miller ocular is the counting of the scarce element (parasites) in the large square and the estimation of the incidence of the common element (erythrocytes) from counts made in the small square only. This is performed on 10 randomly adjacent fields until a total of 100 erythrocytes were counted in the small squares. By extrapolation, the equivalent of 1000 erythrocytes were inspected for parasites and the percentage parasitaemia was calculated as follows:

$$\% \text{ Parasitemia} = \frac{100 \text{ X parasites in large squares}}{\text{Erythrocytes in small squares X } 9}$$

Parasitaemia was generally maintained between 3-5%. Culture medium was replaced daily and the parasites were gassed with the special gas mixture for 40 s before incubation at 37°C. Once thawed, the cultures were not grown for longer than 3 months to prevent genetic alteration. Ring-stage parasite-infected pellets (5-10% parasitaemia) were cryopreserved with equal volume of freezing media (20% glycerol/wash medium) on a regular basis and stocks were preserved at -80°C.

3.2.2. *In vitro* cultivation of sexual stage *P. falciparum* parasites (gametocyte cultivation)

Formal training in gametocyte culturing, mosquito feeding, mid-gut and salivary gland dissections was received at the Armed Forces Research Institute for Medical Sciences (AFRIMS) in Bangkok under the supervision of Dr Jetsumon Sattabongkot.

A method for the development of *in vitro P. falciparum* parasites' sexual stages (gametocytes) using strains 3D7, 7G8 and NF54 was subsequently developed using a combination of protocols by Carter *et al.* (159), the candle jar method from the AFRIMS (Dr Sattabongkot; personal communication) as well as from Megan Dowler (Walter Reed Army Institute of Research, WRAIR; personal communication). The methodologies were compared in order to obtain cultures with consistently high numbers of gametocytes and are described below.

3.2.2.1. Candle jar method

The candle jar method is used routinely at AFRIMS and Walter Reed Army Institute of Research with good success. A 5 mL asexual parasite culture grown in a T25 flask with a parasitaemia of between 3-5% was transferred aseptically to a 50 mL tube to which 1.8 mL fresh A+ erythrocytes was added for a 6% starting haematocrit in 23.2 mL culture media (no antibiotics, 10% human serum added). This was mixed well and 5 mL was added to each well of a 6-well tissue culture plate. The plate was then placed into a candle jar and the candle lit. The lid of the candle jar was secured with a very small gap until the candle burned out, thereafter, the lid was closed; vacuum grease was used around the lip of the lid to ensure a tight seal. Media changes were performed daily and a slide warmer set to 37°C was set up in the hood to prevent excessive cooling of the gametocyte cultures whilst outside of the incubator.

The gametocyte cultures were sub-cultured routinely on a Monday/Thursday cycle using the youngest culture in the series. One well of the six was used to start a new culture as described above. Each culture set started out in 6-well plates, the contents of the wells (25 mL) were then pooled and transferred to a T75 flask around day 10/11 or when candle jar space was not available for the newest subculture. Proper use of the candle jar results in the gas phase being 2-3% CO_2 and 14-17% O_2 compared to the 5% CO_2, 5% O_2 mixture used for gassing flasks. Only 3D7 and 7G8 parasite strains were available at the time of this experiment.

3.2.2.2. Flask method

The method for sexual stage culturing in a culture flask was based on the protocol developed by Carter *et al.* (159). An unsynchronised, asexual *P. falciparum* parasite stock culture at a parasitaemia above 4% was diluted to a starting parasitaemia of 0.5-0.7% at a 6% haematocrit. The diluted culture was made up to 15 mL using complete media (no antibiotics, 10% (/v/) human serum) in a T75 tissue culture flask and gassed with the special gas mixture (5% O_2, 5% CO_2 in N_2) for 40 s. Media changes were performed daily with pre-warmed media and a slide warmer set to 37°C was again used to prevent excessive cooling of the cultures whilst outside the incubator. After 4-5 days, when the asexual stage parasitaemia reached 5-10%, thin smears were made, fixed with MeOH and stained with a 10% (v/v) Giemsa stock solution (Merck) in phosphate buffered saline (PBS, pH 7.4, Lonza Walkersville) in order to examine the cultures for changes in morphology associated with stress, such as slightly triangular ring forms. When these signs were observed, the haematocrit was reduced to approximately 3.5% by increasing the amount of fresh media added from 15 mL to 25 mL. The cultures were maintained with daily media changes of 25 mL until mature gametocytes (stage V) were observed.

The gametocyte cultures initiated in flasks were not sub-cultured. New gametocyte cultures were started from the stock flask of asexual parasites. This method was tested using both 3D7 and 7G8 parasite strains as well as NF54 (MRA-1000 ATCC, MR4) parasites.

3.2.2.3. Monitoring of exflagellation

Gametocyte cultures were inspected for gametogenesis (exflagellation) of the male gametocyte (microgamete) by monitoring exflagellation microscopically from day 13 onwards. Terminally differentiated malarial gametocytes remain in a developmentally arrested state until they are taken up by the mosquito (160). The gametocytes then undergo gametogenesis in the mosquito mid-gut within minutes after ingestion of the infected blood meal. *In vitro* gametocyte cultures are assessed prior to mosquito feeding for the presence of exflagellation, an indication of parasite maturity and potential infectivity.

Exflagellation slides were made during the daily media change after removing the media and making a thin smear. Before fresh media was added, the contents of the flask were gently mixed and a small drop of culture material placed on a clean slide and covered with a petroleum jelly-ringed cover slip. The cover slip was gently tapped into contact with the drop. Exflagellation was best observed when the density of the drop allowed the cells to be closely packed together

in a single layer rather than crowded in overlapping layers. Exflagellation was checked after 15 min at 21°C at 200x magnification. In order to observe the exflagellating parasite one must rest one's eyes on the visible field and look for vibrating/jiggling movement and small areas where cells have been cleared to determine whether an exflagellating gametocyte is responsible. A magnification higher than 200x is not recommended as Brownian motion is then strongly visible and may obscure the motions caused by exflagellation. Rating exflagellation is very subjective but was rated using the scale currently used at Walter Reed: 1+: poor exflagellation (1-2 every 5 fields); 2+: some exflagellation (4-10 every 5 fields); 3+: good exflagellation (3-5 per field); 4+: excellent exflagellation (10-15 per field).

3.2.3. Mosquito rearing

The mosquitoes used in this study were kindly reared by Professor Lizette Koekemoer and staff at the Vector Control Reference Unit/Malaria Entomology Research Unit at the National Institute for Communicable Disease (NICD, Sandringham, Johannesburg). The mosquitoes fed are listed in Table 3.1.

Table 3.1. Mosquitoes fed for malaria infection studies. The mosquitoes were reared at the Vector Control Reference Unit/Malaria Entomology Research Unit at the National Institute for Communicable Disease in Johannesburg, South Africa.

Species	Colony name	Country of origin
Anopheles gambiae	SUA	Liberia
An. gambiae	TONGS	Ivory Coast
An. funestus	FUMOZ	Mozambique

3.2.4. Mosquito feeding and dissections

3.2.4.1. Mosquito preparation

Approximately 150 female *An. gambiae* and *An. funestus* mosquitoes (4-7 and 14-21 days post emergence (dpe), respectively) were collected from the insectary for feeding. *An. funestus* mosquitoes were used at 14-21 dpe as this was the age that the staff at the NICD were previously able to successfully infect these mosquitoes with *P. berghei*, a rodent malaria. The mosquitoes were placed in the Richard Hunt Insectary (a secure laboratory, NICD, Johannesburg, South Africa) at 26°C and 80% humidity and kept in a 16/8 h light/dark cycle. The mosquitoes food source (sugar pads: cotton wool soaked in a 10% sugar solution) were

removed 24 h prior to feeding the mosquitoes in order to starve the mosquitoes so that they would subsequently feed well on the blood.

Mosquito membrane feeders (Hemotek system, Discovery Workshops, UK) were set at 38°C and baudruche transparent membrane (supplied with the membrane feeder) was stretched over the feeder and secured using an O-ring. The gametocyte cultures produced in section 3.2.2.2 were used. For the purposes of mosquito feeding, the asexual parasites were not selectively killed once gametocytes began to form, thus, a mixed gametocyte population existed (stages I-V) containing a small percentage of asexual parasites. The sexual cultures consisted of predominantly mature gametocytes (stages IV-V) from days 14 through 17. Gametocyte cultures (approximately 2% gametocytaemia) were spun down for 5 min at 2500*g* in a centrifuge pre-warmed to 37°C and the pellet (approximately 500 µL) gently re-suspended in an equal volume fresh O+ erythrocytes and 2.5-3 mL warm human serum (from section 3.2.1.). This feed mixture was placed into the feeders; as a general rule, gametocytes from 1 flask filled one feeder, that is a 500 µL culture pellet with fresh blood and serum. The feeders with the blood were place on the netting covering the cartons of mosquitoes and the feeders and cartons were covered with a damp cloth. Blowing in the cartons as soon as the mosquitoes started feeding was performed at regular intervals every 5-10 min to increase the numbers of mosquitoes finding the feeder. The feed was completed when the feed mix was drained or crusty/dried or when visual inspection showed the mosquitoes were replete; this could be as soon as 15-20 min. Unfed mosquitoes were separated from the fed ones and the fed mosquitoes' sugar pads were replaced.

3.2.4.2. Mosquito mid-gut dissections and sporozoite isolation from salivary glands

Oocysts are visible in an infected mosquitoes' mid-gut from 7 days post-infected blood meal, but are easiest to see 9-10 days after the feed (Dr J Sattabongkot, personal communication). The mid-guts of 10 mosquitoes per fed colony were checked each day for oocysts 7-10 days after feeding. The mosquitoes were taken out of the carton and immobilised by placing them in the freezer for 2 min. They were then placed in a Petri dish and kept on ice so that they did not revive. Each mosquito was placed in a drop of sterile PBS and using a stereo-microscope, the dissection needles were placed firmly, but gently enough so as not to squash the mosquito, onto the thorax and the other needle was placed on the bottom segments of the mosquito's abdomen. The needle on the abdomen was gently pulled downward until the mid-gut was visible (Figure 3.2). The mid-guts were stained with 0.1% mercurochrome for several minutes to

stain the oocysts before placing a cover slip over them. They were viewed at 100x magnification (using bright field microscopy).

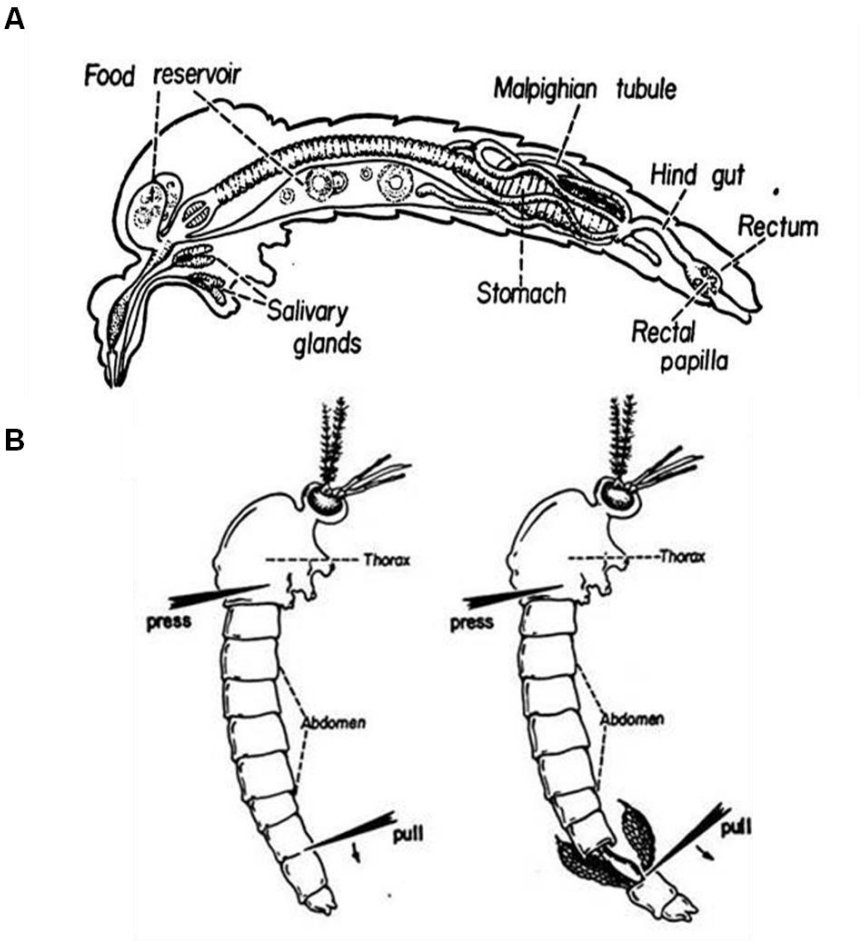

Figure 3.2. Mosquito mid-gut dissections. **A.** Diagrammatic side view of the female mosquito showing internal organs (ovaries omitted) (161). **B.** The position of the needles on the thorax and lower segment of the mosquito to dissect out the mid-gut (161).

Lying under the alimentary canal in the forward part of the thorax are the salivary glands. There are two sets of these, each having three lobes with a common duct which joins the duct from the other set a short distance before they enter the base of the hypopharynx (162). Each of these lobes is made up of a layer of secreting cells which produces the saliva that is injected into the wound as soon as the insect pierces the skin of the victim; it is in these glands that one finds the sporozoites. Sporozoites should be visible 16-22 days post feeding. Mosquitoes were dissected (20 individual mosquitoes per colony) every second day from days 16-22 to microscopically detect sporozoites. Mosquito salivary gland dissections were performed as described by Strome *et al.* (163) using a stereo microscope. Each knocked mosquito was placed in a drop of PBS and the dissection needles placed on the head and thorax as illustrated in Figure 3.3. The head of the mosquito was gently removed from the thorax to expose the

glands, which were then severed from the head. After recovering the gland a cover slip was placed on top of the gland to rupture it and the sporozoites viewed with phase contrast at 200x magnification.

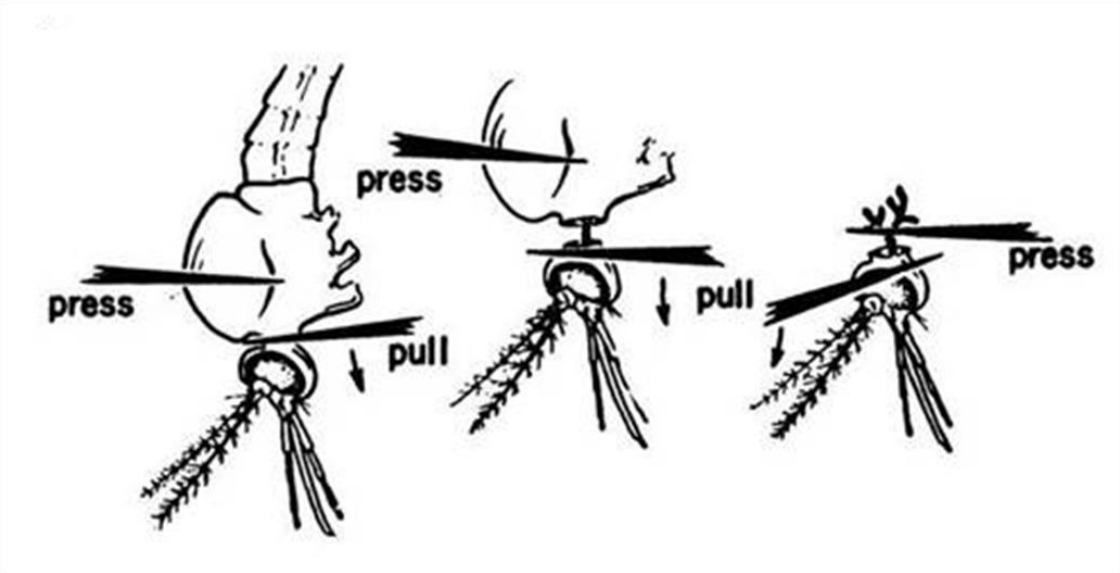

Figure 3.3. Mosquito salivary gland dissections. The position of the dissection needles on the thorax and head of the mosquito and removal of the salivary glands (161).

3.2.5. Populating 3D scaffolds and 2D wells with hepatocytes

The methodology applied was the same as in Chapter 2 section 2.2.3. Briefly, scaffolds (PP-*g*-PNIPAAM-B, Table 2.2) of size 5x5x3 mm were sterilised in 70% ethanol for 1 h, rinsed three times in 1x PBS and allowed to dry in a laminar hood. The scaffolds were soaked overnight at 37°C in the cell culture media specific for the cell-line used, to reduce any surface tension effects as well as to ascertain whether the scaffolds had been effectively sterilised prior to seeding the non-woven scaffolds with the hepatocytes (114). The HC04 hepatocytes were cultured in 1:1 DMEM:Hams F12 media (Lonza) with 2 mM L-glutamine and supplemented with 10% (v/v) FCS, 100 g/mL penicillin and 10 µg/mL streptomycin (Sigma-Aldrich Chemie). Hepatocytes growing in culture flasks were trypsinised and re-suspended to a final concentration of 2 x 10^6 cells/mL. A 200 µL aliquot of this cell suspension was gently dripped onto each of the non-woven scaffolds in a 96-well plate, resulting in a final concentration of 4 x 10^5 cells seeded into each scaffold.

The scaffolds were incubated at 37°C for 2 hpi to allow the cells to attach to the scaffolds. Thereafter, the scaffolds were removed from the 96-well tissue culture plate using sterile tweezers and placed into the well of a 6-well tissue culture plate with 3 mL media and cultured

under standard conditions. Scaffolds containing the HC04 hepatocytes were maintained in culture for a period of 14-16 dpi with media changes performed every 48 hpi.

Two days before infecting the hepatocytes with the sporozoites (see below), 2 x 10^4 HC04 hepatocytes were seeded into the wells of a 96-well plate and maintained under standard conditions. These served as the 2D control; three wells per time point were seeded as well as three wells to be used as the uninfected control. On the day of infection, the hepatocytes were approximately 60-70 % confluent (155).

3.2.6. Seeding sporozoites into 2D hepatocytes and 3D scaffolds

Prior to seeding the 2D hepatocytes and 3D scaffolds with the sporozoites, the scaffolds were aseptically removed from the media in the 6-well plate and each placed into individual wells of an empty 6-well plate; this was done so that when the sporozoites were seeded into the scaffold they were not able to float off into the surrounding media. The media was aspirated out of the wells of the 96-well plate where the 2D cells were growing.

Cryopreserved infectious *P. falciparum* NF54 sporozoites were obtained from Sanaria Inc (Rockville, MD, USA) as six vials of non-attenuated sporozoites in a liquid-nitrogen dry shipper with a temperature monitoring device so as to maintain the viability of the cryopreserved sporozoites. The vials contained 195 542 ± 8 151 sporozoites, each with sporozoite membrane integrity of 88.6% ± 1.35%, as determined by Sanaria Inc. A 6 day hepatocyte potency assay resulted in 22 parasites expressing PfMSP-1 per well, also as determined by Sanaria Inc. The sporozoites were shipped in a temperature monitored container ensuring the cold chain was not broken at any point.

Upon their arrival, the vials were transferred to a liquid nitrogen tank and stored in the liquid nitrogen vapour phase. The sporozoites (20 µL, ±195 000 sporozoites per vial) were thawed for 30 s in a circulating water bath set to 37°C. They were resuspended in 180 µL invasion media [1:1 DMEM:Hams F12 media (Lonza) with 2 mM L-glutamine and supplemented with 10% (v/v) human serum (Interstate Blood Bank), 200 g/mL penicillin and 20 µg/mL streptomycin (Sigma-Aldrich Chemie)] as stipulated in the protocol supplied with the parasites. The sporozoites were gently mixed and 40 µL (approximately 4 x 10^4 sporozoites) was added to each 3D scaffold (n=3) and 2D well (n=3; an approximate 1:2 ratio of sporozoite to hepatocytes). The sporozoites were allowed to invade for 6 h under standard incubation conditions following which media was gently added to the 3D scaffold and fresh media replaced the 40 µL in the 2D wells. 2D and 3D

scaffolds were harvested on days 3 and 7 post invasion; 2D samples were trypsinised and pelleted via centrifugation at 500*g* for 5 min, the supernatant was aspirated and the pellet was washed with an equal volume of PBS. All the samples were stored in liquid nitrogen to retain the stability of the samples.

3.2.7. gDNA isolation

Frozen 2D and 3D samples from section 3.2.6 were removed from liquid nitrogen storage and completely defrosted. gDNA was isolated with the DNeasy® Blood and Tissue Kit (Qiagen) according to the kit instructions. Proteinase K (20 µL) and 40 µL RNAse A (10 mg/mL, Fermentas Life Sciences, Ontario, Canada) were added to the samples and incubated at room temperature for 2 min. Following this, 200 µL Buffer AL was added to each sample. Buffer AL contains chaotropic salts and detergents, the chaotropic salts allows DNA to bind to the silica matrix (164) in the DNeasy spin column. The samples were vortexed and incubated at 56°C to lyse the cells. The samples were then placed into QIAShredder columns and centrifuged at 16 000*g* for 3 mins to ensure that the hepatocytes and sporozoites were fully lysed as well as to separate the cells from the 3D scaffolds. Ethanol (200 µL of 100%) was added to the samples and mixed well. Thereafter the samples were pipetted into a DNeasy spin column and centrifuged at 6 000*g* for 1 min. The DNA-bound membrane was then washed with 500 µL buffer AW1 followed by 500 µL buffer AW2. The samples were spun for 3 min at 16 000*g* to ensure that any residual ethanol was removed from the membrane. They were then placed in a clean 1.5 mL centrifuge tube and the gDNA eluted by gently pipetting 100 µL ddH_20 onto each of the membranes. The samples were incubated at room temperature for 1 min and thereafter spun at 16 000*g* for 1 min to elute the DNA.

The concentration of the gDNA was determined by measuring the absorbance at 260 nm by UV spectrophotometry with a NanoDrop-1000. For double stranded DNA one absorbency unit equals 50 ng/µL (165). Purity from protein contamination was estimated from the 260 nm/280 nm ratio which should be between 1.7 and 1.9 (165) as proteins have a maximum absorbance at 280 nm.

3.2.8. TaqMan® assay

The principle of the TaqMan® assay is based on a fluorogenic probe (TaqMan® probe), complementary to the target sequence being added to the PCR reaction mixture. This probe is an oligonucleotide with a reporter dye (fluorophore; FAM) attached to the 5' end and a quencher

dye (TAMRA) attached to the 3' end. While the probe is not hydrolysed, the quencher and fluorophore remain in close proximity to each other. During the PCR reaction the probe anneals in the selected specific region between the forward and reverse primers; the polymerase then carries out the extension of the primer and replicates the template to which the TaqMan® probe has bound. The 5' exonuclease activity of the polymerase cleaves the probe, releasing the fluorophore away from the close vicinity of the quencher. The fluorescence of the fluorophore increases and is detected by the light cycler.

Three controls were included in the TaqMan® assay. gDNA was extracted from asexual parasite cultures (trophozoites) with a parasitaemia of 5%, 2.5%, 1%, 0.1% and 0.01% using the same methodology described in sections 3.2.7. gDNA was also extracted from ± 3 $x10^4$ sporozoites to be used as a positive control as well as from uninfected hepatocytes growing in the 3D non-woven scaffolds and in 96-well tissue culture plates as described in section 3.2.6.

Primers to detect the 18s rRNA gene in *P. falciparum* were supplied by Dr Jetsumon Sattabongkot, (AFRIMS, Bangkok, Thailand); they were designed by Professor Osamu Kaneko (Institute of Tropical medicine (NEKKEN), Nagasaki University, Nagasaki, Japan). The primer and probe sequences are as follows: forward primer: 5'- GTG TGT CTA ACA CAA GGA AGT -3'; reverse primer: 5'- ACA ATT CAT CAT ATC TTT CAA TCG GTA-3' and probe: 5'- FAM TTG TAC ACA CCG CCC GTC GCT C TAMRA-3'.

Primers to detect the β-actin gene of HC04 hepatocytes, which serves as the control gene for data normalisation, were from the paper of Tanaka *et al.* (166). Their sequences are as follows: forward primer: 5'-CAG TGT GAC ATG GTG CAT CT-3'; reverse primer: 5'- GTG AGG ATC TTC ATG AGG TAG TCA-3' and TaqMan® probe: 5'-FAM-ACG TTG CTA TCC AGG CTG TGC T- TAMRA-3'. The β-actin hepatocyte gene was used to correct for the differing number of hepatocytes within the 2D and 3D culture systems to generate a relative ratio of EEF's per hepatocyte in the 3D vs the 2D system. The primers and probes were synthesised by Integrated DNA Technologies (Leuven, Belgium).

Each 20 µL reaction contained 1 x TaqMan® Universal Master Mix II (Applied Biosystems), 200 nM of both the forward and reverse primers, 50 nM probe, 10 µg gDNA template and the balance up to 20 µL, nuclease-free water. The thermal cycles were as follows: 95°C for 10 min followed by 55 cycles of denaturing at 95°C for 15 s and extension at 60°C for 1 min. Three biological replications were run and each with technical triplicates.

The presence of the *P. falciparum* 18s rRNA gene was determined as well as the hepatocyte β-actin gene in each of the samples in order to generate a ratio to correct for the differing number of hepatocytes in the 2D and 3D system. The ratio generated from the 3D system was then compared relative to the 2D ratio to gauge which system permitted greater sporozoite invasion per hepatocyte.

3.3. RESULTS

3.3.1. Gametocyte production and exflagellation

Previously described methods for the production of *P. falciparum* gametocytes were compared to determine optimal conditions for gametocyte production for subsequent use in mosquito feeding to obtain sporozoites for hepatocyte infection studies. Several conditions could induce *P. falciparum* parasites to differentiate from asexual forms to their sexual gametocytes, and both the candle jar and flask methods described here depend on high parasite numbers and reduced haematocrit resulting in parasite stress to induce gametocytogenesis (Dr Sattabongkot and colleagues at AFRIMS, Megan Dowler at the Walter Reed Army Institute of Research; personal communications, (159, 167)). Indicators of parasite stress include triangular ring stages as well as hazy or faint looking trophozoites and schizonts (159).

Comparison of the candle jar method to the flask method indicated that, for the majority of *P. falciparum* strains used, the flask method was superior in its ability to produce gametocytes (Figure 3.4). The candle jar method proved to be cumbersome and impractical given incubator space as a limiting factor. Additionally, this culturing method resulted in the formation of many spiculated cells (possible erythrocyte artefacts) as well as pyknotic, dead parasites showing signs of degradation including dark and lumpy parasite staining (168). Degenerate stage IV and V gametocytes appear as dark, lumpy, constricted or broken parasites with pigments often dispersed in irregular clumps (168) such as the gametocytes observed in Figure 3.4 (red box). Comparatively, gametocytes could be routinely produced in T75 culture flasks (flask method, (159)) for 7G8 and NF54 *P. falciparum* strains. For this method, the overall morphology of both sexual and asexual parasites in the culture was consistent with normal / healthy parasites and very few pyknotic, dead parasites were observed (Figure 3.4). Moreover, the erythrocytes themselves were much better adapted to this culturing method compared to the high number of spiculated erythrocytes observed with the candle jar method.

In addition to testing the various gametocyte production methods, several strains of *P. falciparum* parasites were evaluated for their ability to produce gametocytes. The 3D7 *P. falciparum* parasite strain continually failed to produce gametocytes even under different experimental methods used (Figure 3.4). However, qualitative morphological monitoring indicated that *P. falciparum* NF54 parasites proved superior to 7G8 parasites in their ability to generate gametocytes of various developmental stages (Figure 3.4).

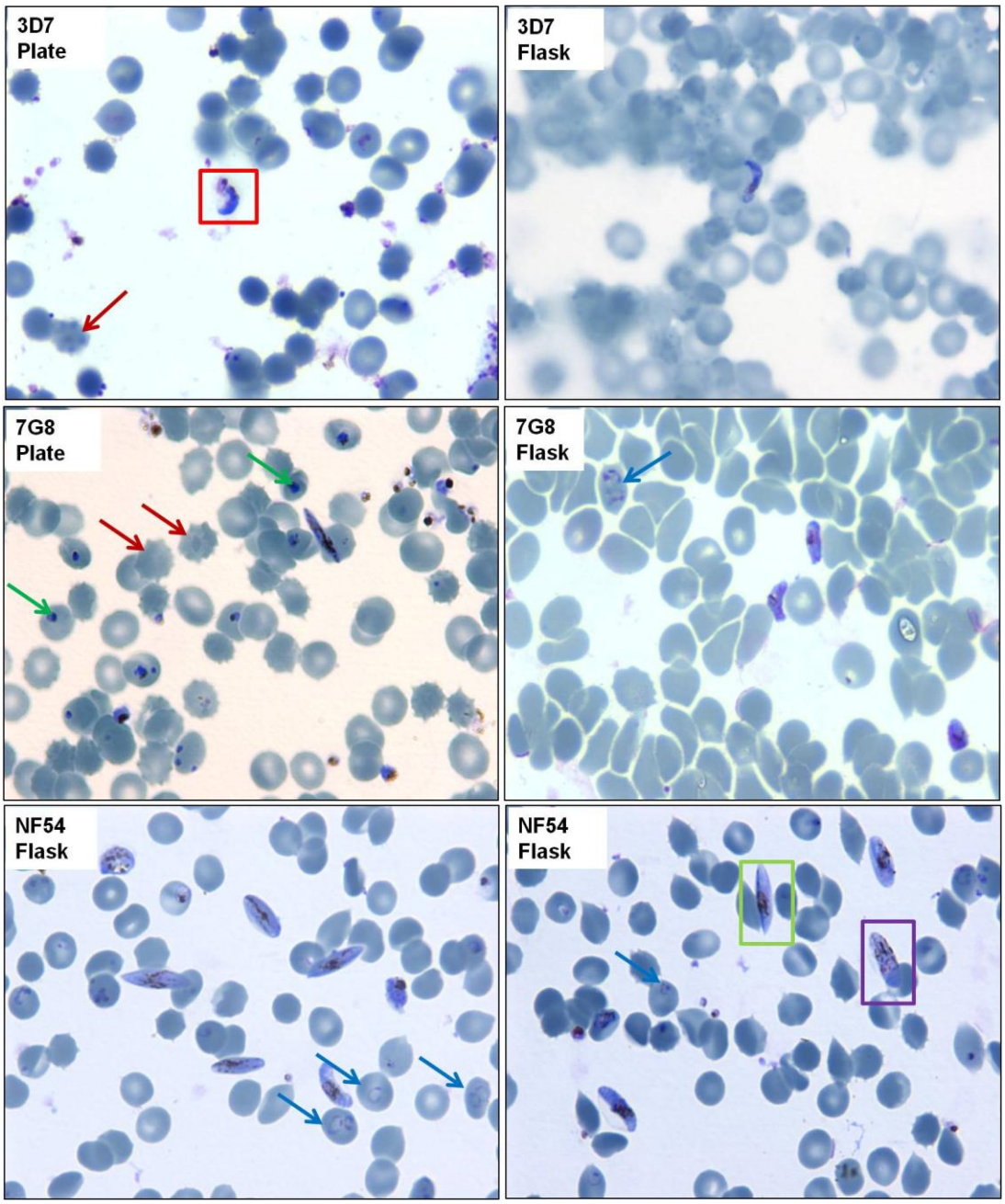

Figure 3.4. A qualitative comparison of the different gametocyte producing methods using 3 parasite strains after 10 days in culture. Culturing *P. falciparum* 3D7 and 7G8 parasites in 6-well plates in the candle jar did not result in good gametocyte production; many spiculated erythrocytes were observed as indicated by the red arrows and the green arrows indicate dead parasites. The 3D7 parasite as outlined in the red box appears dark and lumpy, an indication of degeneration (168). Using the flask method to initiate gametocytes in 3D7 and 7G8 parasites fewer dead parasites were observed but gametocyte production remained suboptimal as very few gametocytes were observed. Ten days after initiating gametocyte production using the flask method with *P. falciparum* NF54 parasites, good gametocyte production was observed as judged by the abundance of gametocytes observed across the microscope slide. The cultures consisted of predominantly stage IV and V parasites as indicated by the green and purple box respectively, though it was not uncommon to see earlier stages. Ten days after gametocyte initiation asexual parasites were still observed as indicated by the blue arrows. All parasites were stained with Giemsa stain and observed at 1000x magnification using an Olympus BX41 light microscope.

Quantitative analysis of various observed parameters of the gametocyte production methods was subsequently performed. The parameters measured include the number of spiculated cells, asexual parasites, dead/pyknotic parasites, parasites outside erythrocytes, degenerate gametocytes and healthy gametocytes (Figure 3.5). Three represented microscope fields were counted per parasite strain and culturing method and the variables are expressed as a percentage of the total erythrocytes per field. Based on these results, it is evident that the candle jar method results in extreme stress for both the parasites as well as erythrocytes in the culture. High levels (~2-fold and 3.5-fold increase for the 3D7 and 7G8 parasites, respectively) of spiculated cells were observed when culturing with the candle jar method vs. using the flask method. Additionally, dead asexual parasites and parasites outside erythrocytes were observed at higher numbers in the candle jar method compared to the flask method. From Figure 3.5, it is also clear that the flask method was the only method able to produce ~7 ± 3% gametocytaemia.

Comparison of strain-specific differences in gametocyte production again iterated that *P. falciparum* NF54 parasites are the most reliable gametocyte producing strain. Producing gametocytes from *P. falciparum* 7G8 parasites (flask method) still resulted in 8 ± 2% spiculated cells in addition to 3 ± 2% asexual parasitaemia still present in the culture 10 days after induction of gametocyte production. This indicates that these parasites are unable to maximally convert asexual parasites to gametocytes. In contrast, for the *P. falciparum* NF54 parasites, very few (4 ± 2%) spiculated cells, pyknotic or degenerate parasites were observed, indicating that overall this parasite strain was adequately adapted to the culture conditions for gametocyte production. Additionally, this was the only strain able to produce ~7 ± 3% gametocytaemia; 9.5-fold more than for the 7G8 parasites.

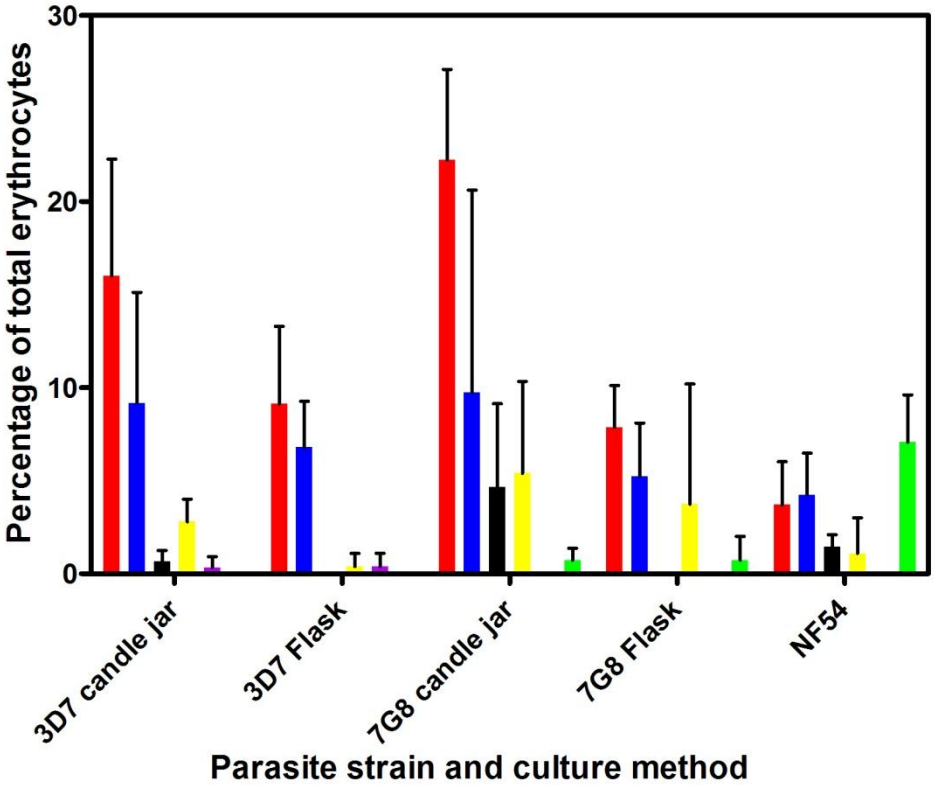

Figure 3.5. A comparison of culture health and gametocyte production between the candle jar and flask method and the three parasite strains. Various observed parameters in each of the culture methods (flask/plate) and for each of the different parasite strains (3D7, 7G8 and NF54) after 10 days in culture were measured. NF54 parasites were only cultured using the flask method. The number of spiculated erythrocytes (red bars), asexual parasites (blue bars), dead/pyknotic parasites (black bars), parasites outside the erythrocytes (yellow bars), degenerate gametocytes (purple bars) and healthy gametocytes (green bars) were counted. Data are from one representative experiment for each strain and culture method tested and are represented as the average of the percentage of the total number of erythrocytes from three adjacent microscopes field ± standard deviations.

After identifying the flask method and *P. falciparum* NF54 strains as the optimal combination for the production of gametocytes, the stage-specificity, production of male and female gametocytes and kinetics of gametocytogenesis could be evaluated. All five previously described stages of gametocyte maturity during gametocytogenesis of NF54 strain *P. falciparum* parasites could be detected (Figure 3.6 A). Stage descriptions are as described by Carter and Miller (168). Stage I gametocytes are the earliest stage at which gametocytes may be distinguished from a trophozoite; they are small rounded parasites one third to half the diameter of an erythrocyte, the cytoplasm is clear or light staining and is never vacuolated or amoeboid. Stage II gametocytes are identified by one side of the parasites becoming extended giving the parasite an overall half-moon shape; one side is smoothly rounded and the other side straight, and the two ends are sharply pointed. At this stage the parasite occupies up to half the erythrocyte; the erythrocyte is not distorted; pigment formation is almost complete and the granules tend to spread out along the axis of elongation (Figure 3.6). Stage III gametocytes are

identified when the pointed ends of stage II begin to become bluntly rounded and if still visible the erythrocyte becomes markedly distorted along the axis of the steadily elongating parasite (Figure 3.6). Stage IV gametocytes continue to grow along the direction of its axis and the two ends become pointed once again; pigment granules remain widely spread over the length of the parasite which is not bent or curved. Stage V gametocytes represent the point at which the gametocytes reach full morphological maturity and are crescent shaped (Figure 3.6).

In addition to the ability to successfully produce all stages of gametocytes, both male and female mature gametocytes were seen in the cultures from days 10 - 16 in fully mature stage V gametocyte cultures (Figure 3.6 B). At this stage, male and female gametocytes can be differentiated from one another. The mature macrogametocyte (female) is characterised by blue staining cytoplasm and is more elongated than the microgametocyte (male). The chromatin and pigment granules of the females are closely aggregated at the centre of the parasite; both chromatin and pigment granules are more spread out in the male (168) (Figure 3.6). Based on this morphological evaluation, it is therefore clear that an optimised protocol was established for gametocyte production with *P. falciparum* NF54 parasites, resulting in mature male and female gametocytes necessary for mosquito feeding studies.

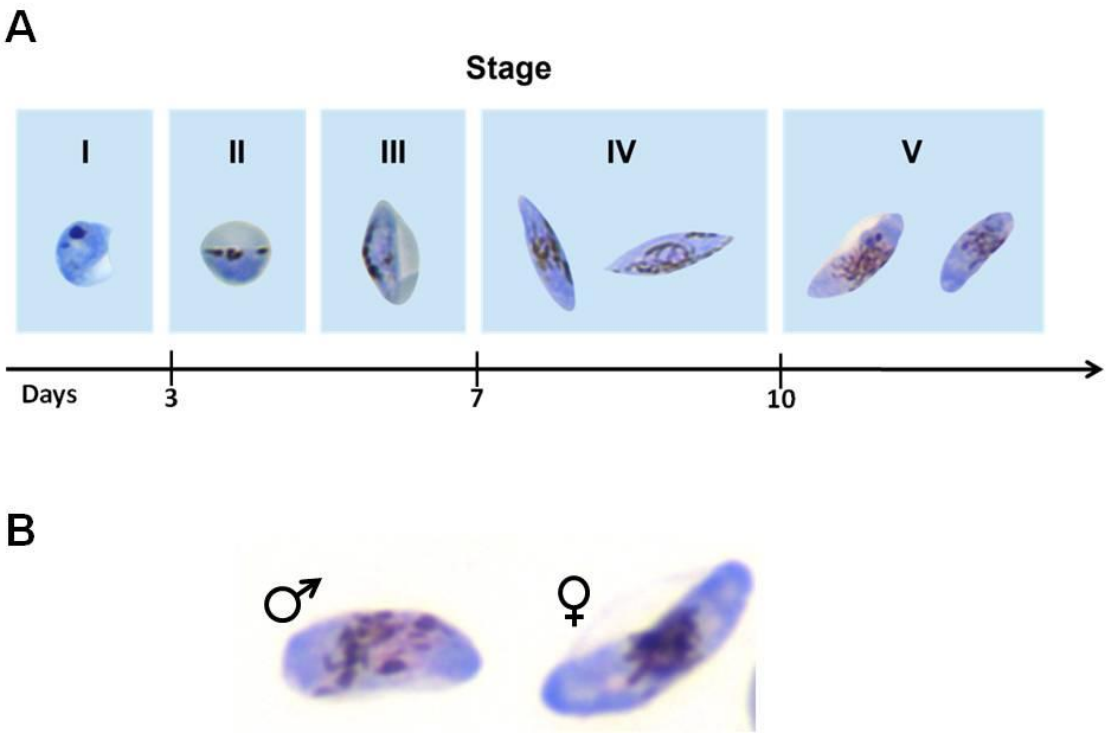

Figure 3.6. Gametocyte staging and sexing. **A.** Identification of Giemsa stained *P. falciparum* NF54 gametocyte stages I-V visualised at 1000x magnification using a light microscope (Olympus BX41). Stage II and III parasites were visualised after 3 days in culture stage IV were present after 7 days in culture and mature gametocytes were prevalent after 10 days in culture. **B.** The mature macrogametocyte (female) is characterised by blue staining cytoplasm and is more elongated than the microgametocyte (male). The chromatin and pigment granules of the females are closely aggregated at the centre of the parasite; both chromatin and pigment granules while tending to centralise are more spread out in the male (168).

In addition to the morphological evaluation of gametocyte production, the kinetics of gametocyte production could be monitored as a measure of successful gametocyte production, which should correlate to a reduction in the number of asexual forms of the parasite (169). Under optimal conditions, the flask method resulted in the production of *P. falciparum* NF54 sexual stage parasites which could be first detected on day 3 after gametocyte induction based on microscopic detection of stage I and II parasites (Figure 3.5). At this stage, the culture was still predominated with asexual forms of the parasite (parasitaemia ~8%) as assessed microscopically and these asexual forms decreased around day 8 when gametocyte production increased (Figure 3.7). The production of sexual stage parasites steadily increased until gametocytaemia reached a peak of 2-6% of predominantly stage IV-V on day 15 under optimal conditions (Figure 3.7). The highest gametocytaemia observed at 6% was correlated to a rapid increase in parasitaemia to 8% on day 3, peaking at ~12% on day 5 followed by a decrease to 2% by day 7 (Figure 3.7A). Comparatively, a parasitaemia of 8% reached by day 9 may not be sufficient to trigger gametocyte formation and resulted in only a 2% gametocytaemia (Figure 3.7B). Few or no asexuals were observed from day 13 onwards (Figure 3.7). This was,

however, not a general phenomenon for each gametocyte culture and in some instances asexual parasites were observed up to day 16 (data not shown).

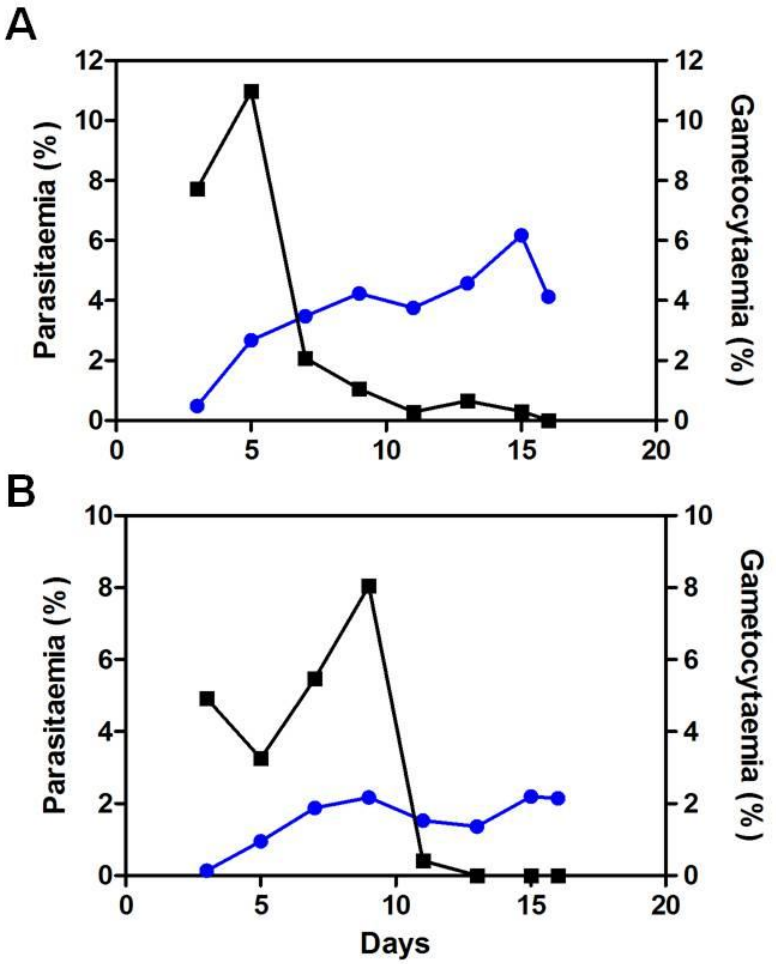

Figure 3.7. *P. falciparum* gametocytogenesis**.** Two independent gametocyte cultures were initiated using a mixed (asynchronous) population of parasites at 0.6% parasitaemia. The process of gametocytogenesis is depicted graphically in a high yielding culture (maximum gametocytaemia of 6%) (A) and a lower yielding culture (maximum gametocytaemia of 2%) (B). The number of asexual parasites (black line, solid squares) increased followed by a rapid decrease leading to a parasitaemia (asexual) of zero by day 16. Sexual forms were first detected on day 3 in both cultures (blue line, solid circle).

A certain proportion of the ring forms that appear in culture develop into gametocytes; this percentage represents the rate of conversion of asexual parasites to gametocytes (168) and is calculated using the following equation:

$$\text{Rate of conversion of asexual parasites to gametocytes} = \left[\frac{\text{No. of stage II gametocytes per 100 erythrocytes counted 48 h after ring forms}}{\text{No. of ring forms per 100 erythrocytes}} \right] \times 100$$

The two independent cultures described above (Figure 3.7) were examined on day 3, counting the number of ring-stage asexual parasites and again on day 5 for stage II gametocytes to

determine the conversion rate. The conversion rate of rings to gametocytes was 27% (2% final gametocytaemia, Figure 3.7B) and 32% (6% final parasitaemia, Figure 3.7A), respectively in the two independents cultures.

The conversion rates between the cultures do not appear to be significantly different, even though a 3-fold difference in end-point gametocytaemia is observed both cultures saw a conversion rate of ~30%. However, a correlation does appear to exist between high early parasitaemia and end-point gametocytaemia. The gametocyte cultures were initiated using an asynchronous asexual culture; the difference in end-point gametocytaemia may, therefore, be correlated to early prevalence of ring form asexual parasites. As such, the ratio of ring stage parasites to trophozoites was calculated for the first few days of the gametocyte culturing period (Table 3.2) and the two cultures were compared.

Table 3.2. The ring:trophozoite ratio. An initial high ring to trophozoite ratio was observed in culture A which rapidly declined; Culture B was observed to have an even ratio of rings to trophozoites which declined more slowly.

	Culture A	Culture B
	Ring:trophozoite ratio	
Day 3	2.3	1
Day 5	0.2	1.25
Day 7	0.083	0.59
Conversion rate	32%	27%
Final gametocytaemia	6%	2%

Culture A (Figure 3.7A) was observed to have an initial high number of rings as compared to trophozoites on day 3 of gametocyte culture (2.3 times more, Table 3.2). The number of rings re-emerging after 48 hours was substantially less as a ratio (rings:trophozoites) of 0.2 was calculated. This ratio further decreased by day 7. Culture B (Figure 3.7B) whose asexual parasitaemia increased more slowly than culture A had an even ratio of rings to trophozoites on day 3; the population of rings increase slightly 48 h later as evident by the higher observed ratio (1.25) and then as in culture A, the ring population was observed to decline.

One of the measures of functionality of *P. falciparum* gametocytes is to determine exflagellation of male gametocytes (microgametes) and this was used here to determine if the gametocytes

produced above are useful for mosquito infections. Exflagellating microgametes were observed on day 14-16 by monitoring exflagellation microscopically (Figure 3.8). Exflagellation can be identified by a small locus that rapidly vibrates/jiggles and is indicative of the presence of a viable male microgamete which, along with the female macrogamete, is necessary for oogenesis in the mosquito vector. Once good exflagellation is observed, a gametocyte culture is regarded as ready for mosquito feeding. Exflagellation rated 3+ or 4+ (as outlined in section 3.2.2.3) would be observed before a culture was deemed ready to be fed to the mosquitoes.

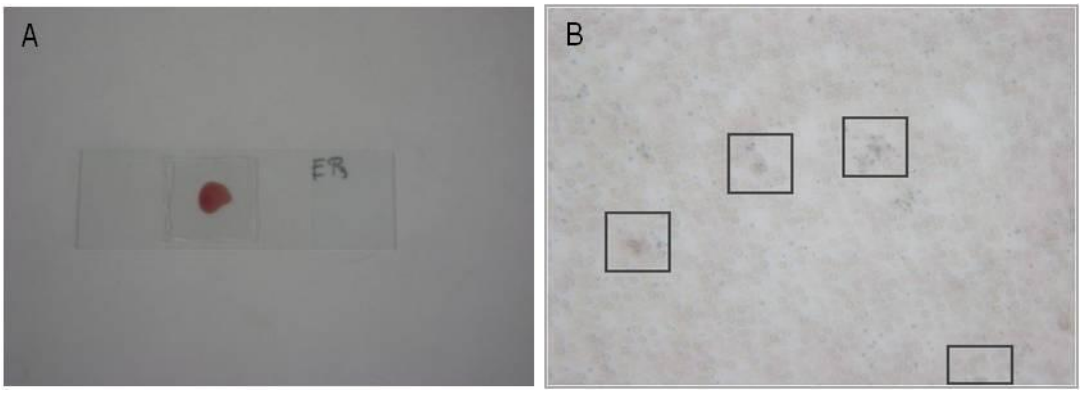

Figure 3.8. Mature microgamete exflagellation. **A.** An exflagellation slide; a drop of blood from a day 14-16 gametocyte culture is placed on a microscope slide and covered with a petroleum jelly rimmed cover slip and left to stand at room temperature for 20 min. **B.** A microscope image depicting four exflagellating microgametes in the microscope field (in grey boxes) in a blood droplet that had been at room temperature for 20 min. Visualised at 200x magnification.

3.3.2. Sporozoite invasion in 2D and 3D

3.3.2.1. Mosquito feeding and dissections

Mosquitoes were fed a parasite culture every two weeks over a period of 6 months. The mid-guts were dissected 7-10 days post feeding to determine if oocysts developed. Additionally, salivary glands were dissected 16-22 days post feeding to determine the presence of sporozoites. Although dissections were successful and were positive in detecting *P. berghei* in control systems (work conducted by Te-Chang M. Lo at the NICD), no oocysts or sporozoites could be detected after feeding *P. falciparum* NF54 gametocytes to the *An. funestus* mosquitoes as illustrated in Figure 3.9.

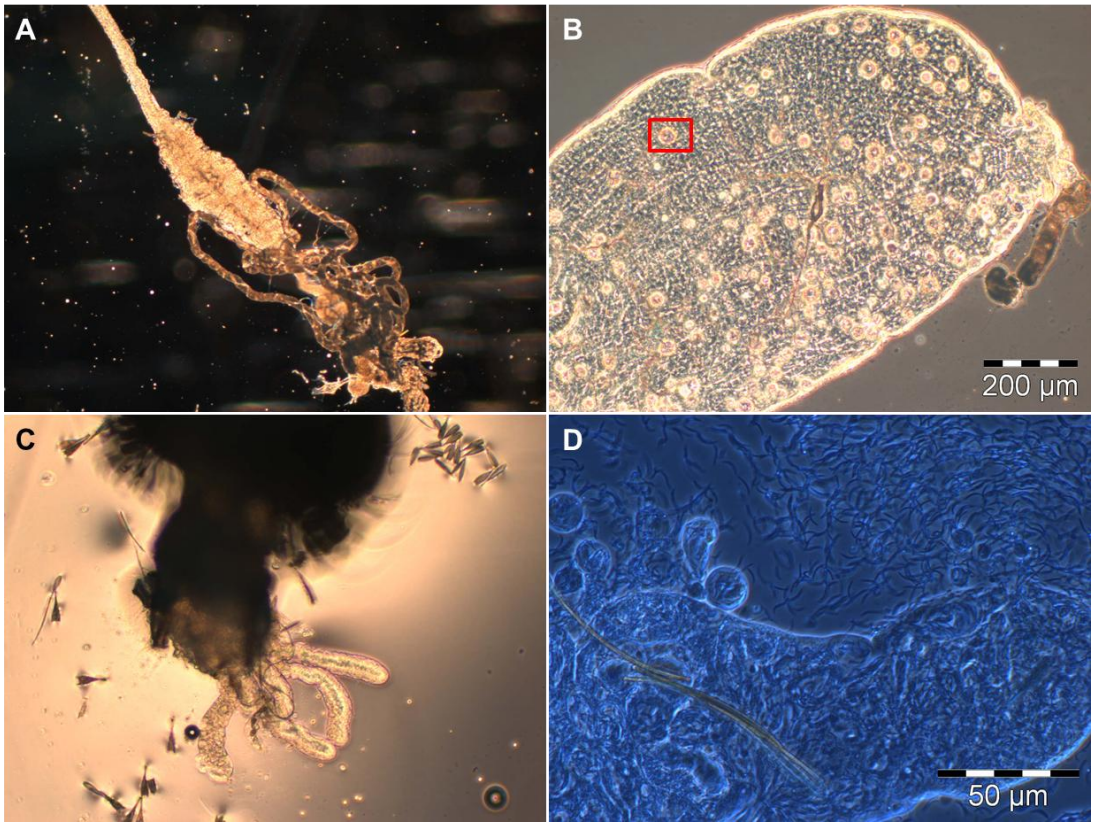

Figure 3.9. Mosquito mid-gut and salivary gland dissections. **A.** Photographed mosquito midgut and malpighian tubule at 50x magnification. **B.** FUMOZ midgut at 100x magnigfication; a *P. berghei* oocyst is outline in the red box; **C.** A bright field micrograph of uninfected mosquito salivary glands still attached the head at 50x magnification; **D**. *P. berghei* sporozoites surrounding a salivary gland. Photos B and D kindly supplied with permission by Te-Chang M. Lo, Malaria Entomology Research Unit, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, South Africa.

3.3.2.2. Sporozoite invasion of HC04 cells.

In order to enable testing of the scaffolds, *P. falciparum* NF54 sporozoites produced at Sanaria Inc. (USA) were obtained for the study. HC04 hepatocytes were maintained on PP-*g*-PNIPAAM-B scaffolds for 16 days prior to invasion with sporozoites as per section 3.2.6 (Figure 3.10 A). Another scaffold, Nylon-cont (Table 2.2. Chapter 2) was subsequently included as this scaffold has smaller pores (40 – 80 µm) and permits the hepatocytes to more densely populate the scaffold (Figure 3.6 B). This scaffold was used so that the sporozoites would be less able to navigate out of the scaffold via the pores.

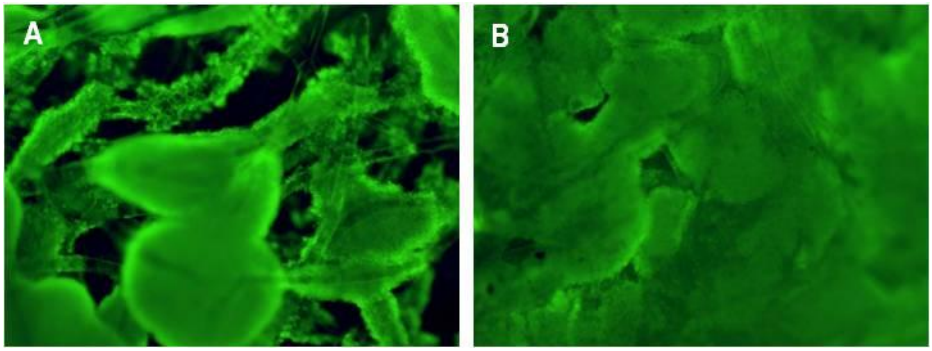

Figure 3.10. HC04 hepatocytes populating the non-woven scaffolds. **A**: PP-*g*-PNIPAAM-B and **B**: Nylon-Cont non-woven scaffolds after 16 days of growth. The cells were stained using fluorescein diacetate and visualised at 40x magnification using a standard fluorescence microscope (Olympus BX41) equipped with a 490 nm bandpass filter with a 510 nm cut-off filter for fluorescence emission.

3.3.2.3. Quantification of sporozoite invasion in HC04 cells

A qRT-PCR TaqMan® assay was performed to detect the parasites' 18S rRNA gene as an indication of sporozoite presence/invasion in HC04 cultures. The HC04 β-actin gene was used to normalise the data i.e. to correct for the differing number of hepatocytes in the 2D and 3D system. Presently the aim was not to accurately quantify parasite infection but to express invasion as a ratio of invasion in the 3D system relative to invasion in the 2D control system.

qRT-PCR was successful in detecting the *P. falciparum* parasites' 18S rRNA gene for quantification of parasites in control samples i.e. asexual parasites (5%, 2.5%, 1%, 0.1% and 0.01% parasitaemia) and purified sporozoites. Additionally, the HC04 β-actin gene was detected in uninfected hepatocytes growing in the 3D non-woven scaffolds as well as in 2D 96-well tissue culture plates. Low levels of *P. falciparum* parasites' 18S rRNA gene (very high C_T value) were detected in invaded 2D hepatocytes; average C_T values of 48.3 ± 1.3 at 3 dpi and 46.9 ± 2.7 at 7 dpi were observed (Appendix Table A1; Figure 3.11). The *P. falciparum* parasites'18S rRNA gene was however not detected in the invaded 3D hepatocytes.

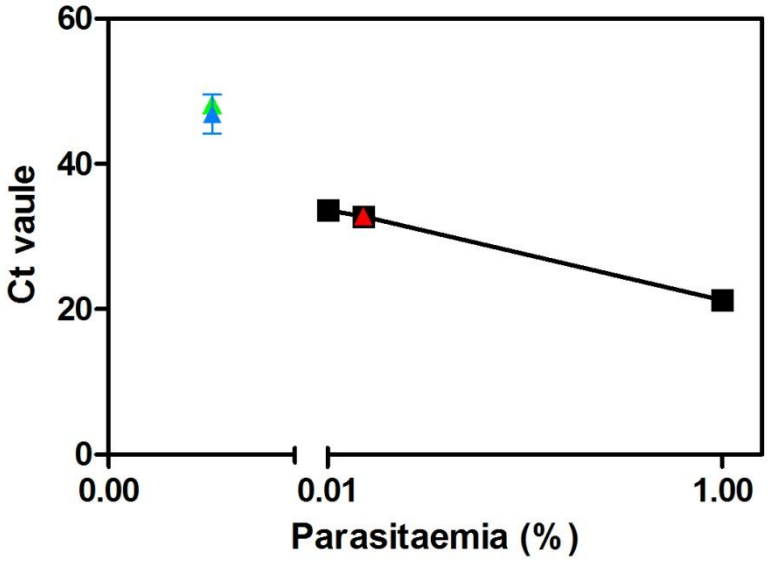

Figure 3.11. TaqMan® expression data. Asexual *P. falciparum* parasitaemia (0.01%, 0.1% and 1%) are plotted on the graph against the Ct value at which the *P. falciparum* 18s rRNA gene is first detected (black squares). The 18s rRNA gene in the positive sporozoite control (30 000 sporozoites) is plotted (red triangle); the Ct value corresponds to approximately 0.1% asexual parasitaemia. The 18s rRNA gene was detected in the 2D day 3 (blue triangle) and 7 (green triangle) samples and this parasitaemia as compared to the asexual controls falls somewhere between 0% and 0.01% parasitaemia (plotted midway for representative purposes only).

In order to determine whether the detection of the *P. falciparum* 18s rRNA gene in 2D and 3D invaded hepatocytes was being limited by the concentration of the probe or the amount of DNA being added to each reaction, the probe concentration was increased to 200 nM and the DNA concentration increased from 10 ng to 30 ng. The signal detected for each of the 2D and 3D invaded hepatocytes was not increased by the addition of increased probe concentration and additional DNA (Appendix, Table A2).

As no signal was detected in the hepatocytes growing on the 3D PP scaffolds, the experiment was repeated using the Nylon-Cont non-woven scaffold; due to the nylon having a smaller fibre diameter a non-woven mat with smaller pores is formed. It was hypothesised that if the pores were smaller, the hepatocytes would be able to more densely populate the non-woven scaffold thus allowing less of an opportunity for the sporozoites to migrate away from the hepatocytes via the porous network. The nylon non-woven scaffolds, when grafted with PNIPAAm, did not permit thermal release of hepatocytes and coupled with the denser pore network did not make this the best scaffold for work conducted in Chapter 2; it may however be better suited for sporozoite invasion compared to the PP non-woven scaffolds.

The manner in which the nylon non-woven scaffolds were seeded with sporozoites was also modified slightly. Instead of resuspending the sporozoites in 180 µL invasion media, the sporozoites (in a 20 µL pellet) were resuspended in 40 µL invasion media up to a final volume of 60 µL thus a smaller liquid volume containing the approximately 4 x 10^4 sporozoites was used to seed each of the six independent non-woven scaffolds. This was done in order that the small drop of media did not leak out of the scaffold. The protocol used to seed the 2D hepatocytes remained unchanged.

The 18s rRNA gene was detected in the 2D samples with an average C_T value of 48.98±0.7 and 48.31±1.1 at 3 and 7 dpi, respectively. The gene was again not detected in the invaded 3D hepatocytes (Appendix, Table A3) and, thus, the β-actin expression data was not used to normalise the data to correct for differing number of hepatocytes in the 2D and 3D systems.

3.4. DISCUSSION

The trigger that causes asexual parasites to form gametocytes remains unclear and there may be multiple factors that contribute to this (170). Several methods have been proposed to produce gametocytes *en masse* including the manipulation of the culture conditions and/or addition of pharmacological agents (170). Examples include the addition of lysed erythrocytes to an asexual culture (168, 171); using human serum in place of serum substitutes (172) or the addition of antimalarial drugs such as chloroquine (173). A sudden decrease of the haematocrit in fast growing ring-stage cultures causing parasite stress and subsequent conversion to large numbers of gametocytes has also been proposed (159, 174) and Ifediba and Vanderberg concluded that gametocyte production could be enhanced by reducing the haematocrit for part of the culture period (175). The sudden decrease in haematocrit and a high parasite load may reflect conditions of physiological stress in the human host that are known to correlate with increased numbers of transmission stages in blood circulation (167).

In this study two methods were tested for the generation of gametocytes. The candle jar method is used by Dr Sattabongkot and colleagues at AFRIMS and Megan Dowler at the WRAIR to generate sufficient amounts of gametocytes to feed to mosquitoes. In this study, the candle jar method was abandoned after 3D7 and 7G8 parasites failed to produce gametocytes and this method proved to have practical constraints. The second method was the flask method, which uses the reduction of the haematocrit when signs of culture stress become evident (via microscopic evaluation) to stimulate gametocytogenesis. The final culture conditions for the flask method involved starting the gametocyte culture on day 0 in a 15 mL culture volume with a 0.6% asexual parasitaemia (unsynchronised *P. falciparum* NF54 parasites) and 6% haematocrit. Using this method, a maximal gametocytaemia of approximately 2-6% was achieved using NF54 parasites. This high percentage gametocytaemia is in line with what the Parasitology Core Facility at the John Hopkins Malaria Research Institute claim to generate using the same protocol (176), however, this is higher than what has currently been described in literature.

Lelièvre *et al*. achieved a gametocytaemia (mature gametocytes stages IV-V) of approximately 1-2% using the 3D7HT-GFP parasite strain using AlbuMAXII in place of human serum, continuous addition of hypoxanthine and asexual parasite reduction using *N*-acetyl-D-glucosamine (177). Using AlbuMAXII Lelièvre *et al*. were not successful in generating gametocytes using NF54 parasites; it is, however, our experience that culturing NF54 parasites using AlbuMAXII in place of human serum is difficult which may account for their observed

results. In their 2012 paper, Roncales *et al*. compared several methods of gametocyte production with the most successful method resulting in a gametocytaemia of just under 1.5% (169). This was achieved using the *P. falciparum* NF54 parasite strain with a starting asexual parasitaemia of 0.75% and a haematocrit of 2%. During the culturing period, gametocytogenesis was stimulated by decreasing the asexual parasitaemia via sorbitol treatment and the addition of erythrocyte lysates [12]. Roncales *et al*. were not able to generate a significant number of gametocytes using the range of other methods tested, one of which was very similar to one used in this study. By starting with an asexual parasitaemia of 0.2%, reducing the haematocrit 3 days later and not chemically decreasing the asexual parasitaemia, they were only able to achieve a gametocytaemia using 3D7 parasites of 0.08 ± 0.03% and 0.19 ± 0.02% using Dd2 parasites. They did not test this method using NF54 parasites. Comparatively, in the work presented here we were able to achieve a >2% gametocytaemia when starting with a 0.6% asexual parasitaemia and a 6% haematocrit using NF54 parasites.

Culture synchronisation prior to gametocyte culture initiation should not influence the outcome of gametocytogenesis. In the protocol drafted by Carter *et al*. (178) the starting asexual parasitaemia should be 0.5% if the culture is mostly schizonts, 0.7% if the culture is mostly rings and 0.6% if the cultures comprised of a mixed population of asexual parasites. Since our cultures were not synchronised, a mixed population was observed, thus a starting parasitaemia of 0.6 % was always calculated to initiate a gametocyte culture. Mature male and female gametocytes (IV-V) were observed in culture from day 10; after days 14-16 in culture they were the predominant gametocyte stage present. Early stage gametocytes were also observed to be present in the cultures as well as a very small percentage of asexual parasites on occasion.

In contrast to the statement above, the initiating asexual culture's synchronisation status may indeed contribute to achieving higher end-point gametocytaemia. This hypothesis was further explored and upon further investigation, the gametocyte culture that reached 6% gametocytaemia (culture A) was observed to have a higher proportion of rings vs. trophozoites (approximately double) on day 3 after initiation when compared to the lower yielding gametocyte culture (culture B). From this it can be deduced that the asynchronous asexual culture used to initiate culture A was predominantly trophozoites on day 0 (assuming a 48 h life cycle). This would have resulted in the higher percentage asexual population observed earlier on in the gametocytogenesis process (Figure 3.7A). In contrast, culture B was comprised of an equal population of rings and trophozoites, thus, the asexual parasitaemia did not increase as rapidly as in culture A. After gametocyte induction, commitment occurs such that individual schizonts produce a progeny of merozoites that uniformly develop into either sexual or asexual parasites

(179). The re-emergence of rings was seen to decline more rapidly in culture A suggesting commitment to sexual stage development. This difference in end-point gametocytaemia also suggests that the trigger to form gametocytes occurs early on in the culturing protocol and for a finite period of time i.e. rings formed at later time points or after increasing the media volume due to a less rapid asexual increase are not induced to form gametocytes. As such, even though the conversion rates were ~30% for both the high and lower yielding gametocyte cultures, culture A simply had more rings at the point of gametocyte induction. Thus, a gametocyte culture initiated using a synchronised culture of asexual trophozoites may yield more gametocytes; this hypothesis remains to be validated.

Using the flask method several factors were identified to contribute towards the successful differentiation of asexual parasites into gametocytes. Firstly, *P. falciparum* asexual parasites do need to undergo a degree of stress to allow gametocyte production. The parasite cultures exceeded an asexual parasitaemia of approximately 5-10% before the amount of media added to the flasks during the daily media changes was increased. The efficacy of reducing the haematocrit to enhance gametocyte production was first documented by Ifediba and Vanderberg (175) in 1981. Sexual conversion induced *in vitro* by a sudden decrease in haematocrit concentration and a high parasite load was affirmed by Buchholz *et al.* (167). Minimising the time that the gametocyte cultures spend outside of the incubator is another factor, which aids in good gametocyte production. *In vivo*, mature gametocytes remain in a developmentally arrested state until they are taken up by the mosquito. It is within the mosquito mid-gut where the gametocytes then undergo gametogenesis within minutes after ingestion of the infected blood meal. Male gametogenesis (exflagellation) is triggered by a temperature decrease of at least 5°C and an increase in pH (160). Even though mature gametocytes are only observed from day 10 *in vitro*, to ensure that gametogenesis does not spontaneously occur during daily culture maintenance due to changes in temperature outside of the incubator, cultures were placed on a slide warmer set to 37°C; media was also thoroughly warmed prior to its addition to the culture flask (180) as a standard practice. This is a practice advocated by the researchers at AFRIMS (Bangkok), Megan Dowler (WRAIR, personal communication) and is stated in various protocols available for gametocyte culture (176, 178).

The successful generation of gametocytes in this study using NF54 *P. falciparum* parasites may be due to strain specific differences - NF54 *P. falciparum* parasites may simply be better at producing gametocytes than the other strains tested. This was certainly our experience as by using this method we were not successful in generating a significant number of gametocytes using 3D7 and 7G8 parasites. Even under the optimal conditions described above, 3D7 and

7G8 *P. falciparum* parasites failed to produce significant gametocytaemia using this method. The observation that 3D7 and 7G8 parasites do not produce gametocytes to the same extent as NF54 parasites is in agreement with Bennett *et al.* (181) who suggest that not all laboratory strains produce gametocytes to a similar extent even if they are stimulated by the same induction method. Gametocyte production can be lost in parasites that have been maintained *in vitro* for extended periods of time; *P. falciparum* parasite isolates have been shown to undergo deletions in a subtelomeric portion of chromosome 9 of about 300 kb (182). These parasites lack cyto-adherence to C32 melanoma cells and produce greatly reduced numbers of gametocytes (179). As standard practice in our laboratories, parasites are not maintained in culture for longer than 3 months, however, it is unknown to us whether our 3D7 and 7G8 parasites underwent prolonged asexual propagation, thus losing their ability to produce gametocytes, prior to us receiving them. The NF54 parasites used in this study were obtained from MR4 and were originally sourced from the lab of Megan Dowler (WRAIR) where they were used routinely to produce gametocytes for mosquito feeding.

In this study, *P. falciparum* gametocytes were generated for mosquito membrane feeding to ultimately obtain sporozoites for hepatocyte invasion studies. Culture adaptations therefore include the use of only human serum, antibiotics were omitted from the culture medium and the population of asexual parasites was not decreased via sorbitol or *N*-acetyl-D-glucosamine treatment. This was done to simplify the culturing process to allow for simple, high-throughput culturing and to generate a blood meal that would be palatable to the mosquitoes without, in any, way influencing the natural infection process we aimed to mimic. The NF54 *P. falciparum* gametocyte cultures were fed to *Anopheles spp*. mosquitoes at the NICD. However, none of the mosquitoes dissected 9-11 days post feeding had developed *P. falciparum* oocysts in their mid-guts nor were we able to obtain any sporozoites from the salivary glands of the mosquitoes at the later time points.

A number of factors may have contributed to this. Firstly, altitude may play a role as the Edenvale/ Sandringham area (where the NICD is situated) is approximately 2200 m above sea level. Altitude is an important factor in malaria transmission and it is generally stated that transmission does not normally occur above 1800 m (183). This effect is, however, usually related to lower temperatures associated with higher altitudes but temperature is a controlled parameter inside the insectary. Secondly, the mosquitoes may not be susceptible to the particular strain of malaria parasites used to infect them or the malaria parasites cultured *in vitro* may have become less infectious. Parasite genotype-vector genotype interactions are a major determinant of infection outcome (155, 184). In our study, only *An. gambiae* and *An. funestus*

were fed; other common mosquito species used for feeding that could possibly be used are *An. freeborn, An. stephensi* or and/*or An. arabiensis*. Of these *Anopheles* mosquitoes each presents with different permissiveness for malaria infection: *An. freeborni >> An. gambiae = An. arabiensis > An. stephensi* (155, 185). Finally, the presence of exflagellating microgametes was used to indicate that a gametocyte culture is functional and ready to be fed to the mosquitoes. This is, however, only an indication that viable male microgametes were present in the culture and not an indication of guaranteed infectivity. Female macrogametes, which along with the microgamete are required to form the oocysts, were observed in the *in vitro* cultures and therefore all the basic elements for successful infection were deemed to be in place. However, there is currently no measure of female macrogamete viability, which could influence oocyst formation. In the absence of detecting ookinetes or oocysts, it is currently difficult to determine if the gametocyte production protocol produced viable forms of both male and female gametes. Future feeds may make use of xanthurenic acid in the feed mix; xanthurenic acid has been identified as the putative inducer of gametogenesis in the mosquito (186). Bhattacharyya and Kumar demonstrated that by increasing the concentration of exogenous xanthurenic acid (optimum 100 mM), a gradual increase in the number of oocysts in the mid-gut of infected mosquitoes is observed (160). Alternatively, although a more challenging approach, ookinetes could be generated *in vitro* and fed to the mosquitoes (187).

P. berghei sporozoites have, however, been successfully produced at the NICD by infecting guinea pigs with the parasite and allowing the mosquito to feed on the sick animals. In this study we chose not to test our hypothesis that *Plasmodium* sporozoites may infect hepatocytes growing in 3D better than those growing in 2D by using *P. berghei* sporozoites. *P. berghei* is different to *P. falciparum* and we aimed to create a system based on the human parasite. It has previously been reported that depending on the host cell type *P. berghei* sporozoites can use several distinct pathways for invasion (188). Infection of human HepG2, HuH7 and even HeLa cell-lines by *P. berghei* does not depend on CD81 or host membrane cholesterol; invasion of hepatocytes by *P. falciparum* and *P. yoelii* depend upon these mechanisms for invasion. The existence of distinct invasion pathways may explain why *P. berghei* sporozoites are capable of infecting a wider range of host cell types *in vitro*. Therefore, optimising our 3D system using these sporozoites may not ultimately translate into a better system for *P. falciparum in vitro* studies. As a result of our inability to generate infected mosquitoes from gametocytes to produce sporozoites, cryopreserved *P. falciparum* sporozoites were purchased from Sanaria Inc. (USA) and these were used in the pilot 2D vs 3D hepatocyte invasion study.

In this study, qRT-PCR was used as the determinant of successful hepatocyte invasion of *P falciparum* sporozoites into either the 2D or 3D hepatocytes. An additional control that could be included would be to perform the TaqMan® assay on the DNA from Sanaria's 6 day hepatocyte potency assay which resulted in 22 parasites per well and correspond our C_T values to the ones obtained for these samples; this will give an insight as to whether this end-point analysis is sensitive enough for parasite quantification. The lack of plentiful sporozoites limits the testing of various new and exciting 3D culture methods and once infected mosquitoes are available to us in our own laboratories, several modifications to our 3D culturing system will be tested. An initial experiment would be to test parasite movement/penetration during the first hour in contact with the scaffold using immunofluorescence of the CSP. Once invasion has been established methods used to analyse the parasitaemia will then extend to flow cytometry to possibly assess the developmental stage the parasite is able to reach in 2D and 3D systems, qRT-PCR using RNA to assess gene copy number of the parasite *in vitro* can also be investigated. In our study, co-culture of the invaded 2D and 3D cultures with erythrocytes was also performed as outlined in (113) on 7, 14 and 21 dpi without successfully detecting any asexual parasites; this will also be conducted in future studies to assess whether the full sporozoite life cycle can be completed in the *in vitro* systems.

In vivo, hepatocytes are not directly accessible to sporozoites travelling in the bloodstream of a mammalian host; parasite passage across the sinusoidal cell layer is therefore mandatory for the onset of a malaria infection (141). The sinusoidal layer is composed of specialized fenestrated endothelia interspersed with Kupffer cells and several biochemical and physiological studies as well as various microscopic and genetic approaches have all suggested that sporozoites reach hepatocytes by passing through Kupffer cells (142, 143, 189-191). This is not the case *in vitro* where most *Plasmodium s*pecies are able to mature to liver stages in cultures of pure primary hepatocytes or hepatoma cells i.e. in the absence of Kupffer cells (141, 155). However, even in the absence of the sinusoidal barrier it remains difficult to obtain high levels of sporozoite-invaded hepatocytes *in vitro*. This non-invasiveness may be attributed to several factors. *In vivo* the switch from 'migration mode' to 'invasion mode' is hypothesised to be triggered by parasite circumsporozoite protein recognition of the presence of cells covered by HSPGs (140). Using a rodent model system Coppi *et al*. (192), demonstrated that *P. berghei* sporozoites use the sulphation level of host HSPGs to navigate in the mammalian host. Sporozoites were shown to migrate through cells expressing low-sulphated HSPGs, such as those in skin and endothelium, while highly sulphated HSPGs of hepatocytes activate sporozoites for invasion. *In vitro* this may not be a feature that is easily reproducible as the cell lines used for invasion may not display optimal levels of HSPG's on their surfaces for the

parasites to switch from migration mode to invasion mode, thus, the absence of the sinusoidal layer *in vitro* may ultimately be a disadvantage as the sporozoites are not sufficiently triggered to undergo invasion mode. This may lead researchers to re-consider the design of *in vitro* systems and co-culturing with macrophages or stromal cells may be a possible improvement. Another method to overcome this is the addition of calcium ionophores (145) or uracil derivatives or forskolin (193) to stimulate sporozoite apical regulated exocytosis which decreases migration and enhances infectivity (155). However, Prudêncio *et al*. draw attention to the fact that such measures should be carefully considered as these additives may confound ones data (155).

Additional reasons why sporozoites do not invade hepatocytes *in vitro* may be as a result of cell density or cell confluence. It has been demonstrated *in vitro* that EE forms of the parasite grow to a lesser extent when the cell density is high, preferring to invade hepatocytes at approximately 60-70% confluence (155). The hepatocytes growing in our 3D system may, therefore, simply be too dense for invasion. Until a more plentiful source of sporozoites is available to us one possible change we could make to test our hypothesis that 3D cells will permit more sporozoites to invade is to change the type of 3D cell support used. By making use of a “basement membrane” in place of the non-woven fibres, such as Matrigel (BD Biosciences) or Geltrex™ Matrix (Invitrogen) we may have greater success in achieving a higher level of invasion. A basement membrane is thin extracellular matrices underlying cells *in vivo*. BD Matrigel Basement Membrane Matrix is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumour rich in extracellular matrix proteins. Its major components are laminin, collagen IV, heparan sulfate proteoglycans and entactin/nidogen. BD Matrigel Basement Membrane Matrix is effective for the attachment and differentiation of both normal and transformed anchorage dependent cell types including hepatocytes (194). The Matrigel basement membrane was used by Al-Olayan *et al.* (156) to culture *P. berghei* sporozoites *in vitro*. They hypothesised that the Matrigel mimics the basal lamina of the mosquito midgut and when co-cultured with *Drosophila melanogaster* S2 cells they were successful in generating this stage of the parasites life cycle.

In his 2004 paper entitled “Beyond the Petri dish” Zang (2) proposes that in a true 3D system the pores need to be smaller than an individual cell and that 3D matrices produced from animals (such as Matrigel) offer a good alternative to the synthetically produced scaffolds. Whilst this is not problematic for normal hepatocyte 3D cultures, in the context of sporozoite invasion of hepatocytes, the reticulated surface of the Matrigel would permit denser more uniform hepatocyte coverage of the hydrogel, thus affording the sporozoites less of an opportunity to

"escape" the matrix. It may also reduce the time such an assay takes to perform. Typically it takes 14-21 days to populate the PP or nylon scaffolds with hepatocytes to the point where almost all the fibres are covered with cells, the architecture of the Matrigel is such that the cells would form a 3D layer over the top of the matrix in a relatively short period of time. The "flatter" surface would also allow us to monitor the level of confluence more closely, but, due the 3D nature of the hepatocytes growing on this matrix the sporozoites would be still be able to migrate into the 3D cell network thus able to traverse multiple hepatocytes prior to settling in their final host cells.

In addition to the modification strategy for growing the hepatocytes in 3D, readdressing the cell-line used for these experiments may yield a solution. Whereas HC04 hepatocytes are known to support the *in vitro* growth of *P. falciparum* (113), they typically have lost some of the features of the cells they were derived from through immortalisation and several cell culture passages. By using primary cells, though more expensive and less convenient for routine work we may be able to get better "proof of principle" of our hypothesis.

Robust protocols for the generation of high yielding *in vitro* gametocyte cultures were successfully developed in this study. Further modifications to the mosquito membrane feeding protocols will be explored until sporozoite infected mosquitoes are observed. This will then allow us to fully explore the possibility of enhanced sporozoite invasion in 3D hepatocyte systems.