Chapter 7

The Biology of Malaria Gametocytes

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Additional information is available at the end of the chapter

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Abstract

Gametocytes are sexual precursor cells of the malaria parasite that mediate the transmission of the parasite from its mammalian host to the *Anopheles* mosquito. Unlike the asexual blood stages, which are responsible for the clinical outcome of malaria, gametocytes cause no clinical manifestations. However, they are very crucial for the transmission of the disease thus represent key targets for transmission-blocking interventions. Despite their essential role in malaria transmission, only in the last decade gametocytes became a hot topic of research and their biology is not well understood. This chapter provides a detailed review on the biology of the human malaria gametocytes with emphasis on aspects such as gametocyte commitment, gametocyte maturation (gametocytogenesis), gametocyte metabolism and gametogenesis. Proper understanding of these processes will deepen our knowledge on the gametocyte biology and therefore open up more avenues for the development of malaria transmission-blocking intervention strategies.

Keywords: gametocyte, gametocytogenesis, transmission, gametogenesis, sexual reproduction, mosquito

1. Introduction

Gametocytes are specialized sexual precursor cells that mediate the transmission of the malaria parasite from the mammalian host to the mosquito. Once these cells have gained maturity, they are picked up by an *Anopheles* mosquito during a blood meal. In the midgut of the mosquito, they become activated and differentiate into male and female gametes. The male gamete then fertilizes the female gamete resulting in the formation of a zygote. The zygote further develops into a motile ookinete, which penetrates the gut epithelium and subsequently develops into an

oocyst. The oocyst then matures releasing sporozoites, which migrate to the salivary gland of the mosquito. The parasite is transmitted to another mammalian host through an infected mosquito bite [1]. Gametocytes therefore provide a link in the transmission of malaria from the mammalian host to the mosquito, thereby making them prime targets for transmission-blocking intervention strategies. These strategies rely on either vaccines or drugs, which target the gametocyte or the mosquito midgut stages to block malaria transmission from the human to the mosquito, thereby preventing the spread of the disease.

Despite their essential role in maintaining the parasite life cycle, research on gametocytes has been very much left out of the limelight. Several reasons could be accounted for this: first, gametocytes cause no clinical manifestation in their host as opposed to the asexual blood stages that are responsible for the clinical features of malaria. Second, it has been a great challenge to culture gametocytes because of the cost and time-consuming cultivation procedure, and finally, it has been a hurdle to obtain pure gametocytes for molecular and metabolic studies as well as drug screening purposes. However, in the last decade, the rapid emergence of drug-resistant parasites and mosquitoes, together with the absence of a malaria vaccine, has rekindled the interest of researchers on alternative measures aimed at interrupting the parasite life cycle by targeting gametocytes. This interest has led to the improvement of gametocyte cultivation and purification methods, which have enabled a greater understanding of the gametocyte biology.

In this chapter, we focus on key aspects of the gametocyte biology such as gametocyte commitment, maturation (gametocytogenesis), metabolism and gametogenesis. This will deepen our understanding on the biology of gametocytes and thereby open up more avenues for the development of malaria transmission-blocking intervention strategies.

2. Gametocyte commitment

The asexual blood stage parasites produce the clinical form of malaria due to destruction of the erythrocytes, while replicating in the mammalian host. However, these asexual blood stages cannot be transmitted to the mosquito to continue the life cycle of the parasite. For this reason, a subset of the asexual blood stages is able to switch to the sexual pathway, resulting in the formation of the sexual precursor cells, the gametocytes. The process of switching from the asexual blood stage to the gametocyte is referred to as gametocyte commitment.

Commitment to gametocyte development is believed to take place at some point before schizogony, in which each individual schizont produces a progeny of merozoites that develop into either sexual forms or asexual blood stage parasites, which continue the erythrocytic cycle [2, 3] (**Figure 1**). In addition, merozoites released from a single sexually committed schizont can either become male or female gametocytes and the characteristic female-biased sex ratio observed in the malaria parasite is due to the production of a higher percentage of committed female schizonts than their male counterpart [3, 4].

Until recently, gametocyte commitment was mainly assigned to stress factors such as high parasitaemia, anaemia, host immune response or drug treatment, with an overwhelming

amount of studies to justify these facts. A study by Chaubey and colleagues suggested that the malaria parasite reacts to endoplasmic reticulum stress by switching to gametocytes [5]. The treatment of mice with phenylhydrazine, which induces hyper-reticulocyte formation, stimulates gametocyte formation when infected with *P. chabaudi* [6]. Lymphocyte and serum from Gambian children have also been shown to induce *P. falciparum* gametocyte production [7]. Another study showed that *P. falciparum* cultures grown in reticulocyte-rich blood from patients with sickle cell anaemia result in enhanced gametocyte formation as compared to normal blood [8]. Haemolysis of infected erythrocytes also triggers the formation of *P. falciparum* gametocytes [9]. The treatment of the malaria parasite with drugs such as steroid hormones [10], fansidar [11] and chloroquine or sulfadoxine-pyrimethamine [12] also enhances gametocyte production.

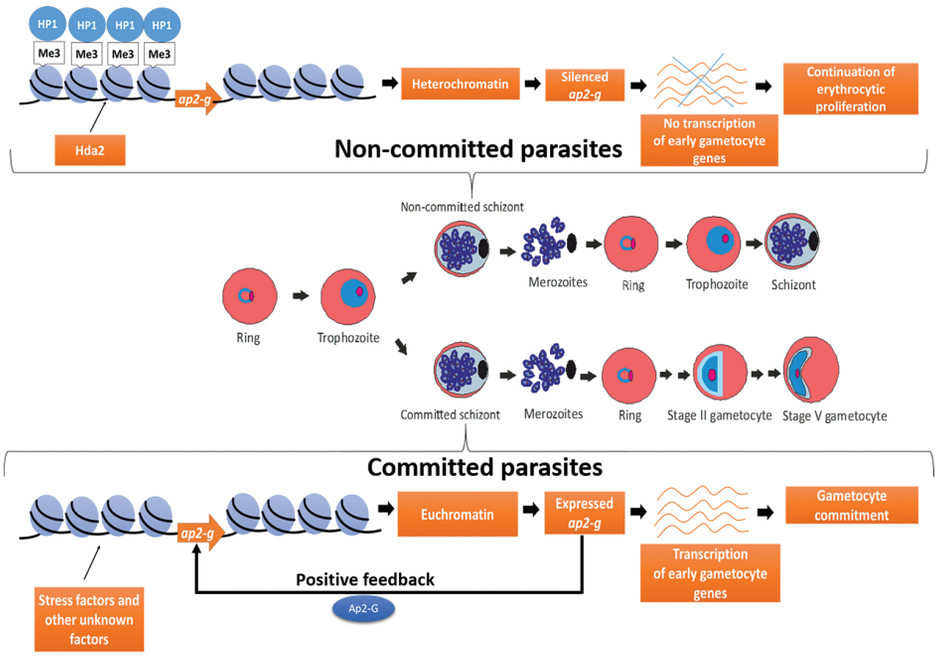

Figure 1. Proposed mechanism of gametocyte commitment. During the erythrocyte cycle, epigenetic and transcriptional regulation work hand in hand to control gametocyte commitment. Two populations of blood stage parasites exist. Non-committed parasites continue the erythrocytic replication of the parasite, and committed parasites are able to differentiate to gametocytes.

However, despite the knowledge that gametocyte commitment can be enhanced by stress factors, the mechanism by which stress induces this process is not well understood. Recent studies have also associated gametocyte commitment to the concerted action of epigenetics and transcriptional regulation.

2.1. Epigenetic mechanism of gametocyte commitment

Epigenetic mechanisms through histone modifications have been shown to regulate gene expression of important proteins in the malaria parasite [13, 14]. In *P. falciparum*, two master players in gene silencing, *P. falciparum* heterochromatin protein 1 (PfHP1) and *P. falciparum*

histone deacetylase 2 (PfHda2) were demonstrated to be implicated in the epigenetic regulation of gametocyte commitment [15, 16].

Heterochromatin protein 1 (HP1) was initially described in *Drosophila* as the main suppressor of position-effect variegation and the major component of heterochromatin gene silencing [17]. Brancucci and colleagues [16] reported that in *P. falciparum*, PfHP1 binds to histone H3-trimethylated residues on lysine 9 (H3K9me3) to maintain heterochromatin gene silencing, which results in the suppression of gametocyte commitment and variegated expression of *var* genes. *Var* genes encode for a family of 60 parasite virulent proteins in *P. falciparum* named *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is present on surface of the *P. falciparum*-infected erythrocyte of which only one is expressed at a time and this phenomenon is associated with antigenic variation. Conditional depletion of PfHP1 results in the hyper-induction of viable gametocytes, thereby demonstrating a role of PfHP1 in suppressing gametocyte commitment. Depletion of PfHP1 also results in derepression of the *ap2-g* gene, which encodes for the AP2-G transcription factor, the only member of the ApiAP2 family shown to associate with PfHP1 [18]. AP2-G was demonstrated to be important in gametocyte commitment in both *P. falciparum* and the rodent malaria parasite *P. berghei* by acting as a transcriptional switch that controls gametocyte differentiation through activating the transcription of early gametocyte genes [19, 20].

Histone deacetylases are histone-modifying enzymes that promote transcriptional silencing by removing acetyl groups from histones. The removal of the acetyl groups facilitates histone methylation and HP1 binding [18, 21, 22], thereby resulting in reduced accessibility for transcriptional factors and heterochromatin formation. A study by Coleman and colleagues [15], which complements the results of Brancucci et al. [16], showed an essential role of PfHda2 in the global silencing of *var* genes as well as controlling gametocyte commitment in *P. falciparum*. Knockdown of PfHda2 results in up-regulation of gene expression of genes associated with gametocytogenesis leading to an increase in gametocyte production. It also results in derepression of the *ap2-g* gene. The authors further reported that most of the genes dysregulated following PfHda2 knockdown were bound to PfHP1, and both proteins localize together with PfHda2-regulated genes, which were significantly enriched in H3K9me3, indicating an interaction between PfHda2 and PfHP1. The two studies therefore provide a link between PfHda2 and PfHP1 in controlling gametocyte commitment.

2.2. Transcriptional regulation of gametocyte commitment

AP2-G is a member of the apicomplexan AP2 DNA-binding protein family, characterized by the presence of an Apetala2/ethylene response factor (AP2/ERF) DNA-binding domain [23]. In the malaria parasite, other AP2 proteins have been assigned to the stage-specific transition during parasite development, for example, the development of midgut, sporozoite and liver stages [24–27].

Two studies in *P. falciparum* and *P. berghei* have shown that AP2-G triggers a transcription cascade that initiates gametocyte commitment [14, 19, 20]. To determine initially, if AP2-G is essential for gametocyte commitment and production, both studies sequenced and analysed the *ap2-g* gene from specific gametocyte-less producing strains for single nucleotide polymor-

phisms, insertions or mutations. Their results showed nonsense or missense mutations in the *ap2-g* gene providing an explanation for the inability of the strains to commit and produce gametocytes. Their initial findings were further confirmed by targeted disruption of the *ap2-g* locus in both *P. falciparum* and *P. berghei*, which resulted in the loss of sexual commitment and gametocyte production. The disruption of the *ap2-g* locus also resulted in the down-regulation of many genes highly expressed in early gametocytes such as *Pfs16* and *Pfg25/27*. The DNA motif involved in AP2-G-binding was identified as GTAC and shown to be located upstream of many genes associated with gametocytogenesis [19, 20, 28]. Because the *ap2-g* gene also possesses the GTAC domain on its promoter region, this suggests that it regulates itself through a feedback mechanism. The AP2-G DNA-binding motif was shown to interact with gametocyte-associated gene promoters in a motif-dependent manner. Mutations of the motif in the promoter region result in a blockage of the gene expression of the gametocyte-associated genes, thereby indicating that AP2-G induces the expression of genes containing the AP2-G DNA motif by binding to it at the promoter region [19].

During the malaria life cycle, therefore, two populations of the malaria parasite exist. Non-committed parasites, which continue the erythrocytic cycle, and committed parasites that form gametocytes. In the case of non-committed parasites, PHP1 binds to H3K9me3 residues thereby maintaining the *ap2-g* locus at a heterochromatic state resulting in silencing of the *ap2-g* expression. This silencing leads to the inability to initiate transcription of genes important for gametocyte commitment; therefore, the parasite continues with erythrocytic replication. PHda2 is also involved in the silencing of the *ap2-g* gene expression in non-committed parasites, probably by the removal of acetylated histone residues allowing for their methylation leading to the binding of PHP1. In committed parasites, stress factors and other unknown factors cause the removal of the histone H3 trimethylation mark, thereby preventing PHP1 from binding to the histone and therefore maintaining the *ap2-g* locus at an active state, resulting in AP2-G expression. The expression of AP2-G promotes the transcription of early gametocyte genes, which leads to gametocyte commitment and formation (**Figure 1**).

3. Gametocyte maturation

3.1. Gametocyte morphology

The morphology of gametocytes varies among the *Plasmodium* species. While mature gametocytes from *P. ovale, P. malariae, P. vivax* and *P. knowlesi* possess a round to oval shape, *P. falciparum* adopts a crescent or falciform shape when matured [29–31].

P. falciparum is named from the crescent or falciform shape of its mature stage. During gametocyte development, the parasite undergoes five distinct morphological stages (I–V) (**Figure 2**). The first observations on the morphology of *P. falciparum* gametocytes were made in the 1950s, when the five development stages were defined [30]. Later studies using light and electron microscopic approaches have defined the shape and time of appearance [32, 33]. Studies have shown that stages I–IV are sequestered into the bone marrow, while stage V circulates in the peripheral blood vessels [34–36]. Stage I gametocyte has a rounded shape,

which is indistinguishable from the young asexual trophozoite. Stage IIa, on the other hand, can be distinguished from the trophozoite by its large round format and granular distribution of pigment in several food vacuoles. In stage IIb, the parasite starts to elongate and a D-shape can be observed. Stage III gametocytes adopt an oval shape with the red blood cell slightly distorted due to further elongated of the parasite. In stage IV, it is possible to distinguish between male and female gametocytes. Both genders have an elongate thin format with pointed tips. In male gametocytes, the pigment tends to be scattered while in female it is denser. The stage V gametocyte is the most distinguishable among all stages due to the crescent or falciform shape. While the male gametocyte is thicker and the cytoplasm appears pale blue following Giemsa staining, the female is more elongate and curved with a blue-stained cytoplasm. This stage is visible in the periphery circulation after 10–12 days of maturation [29].

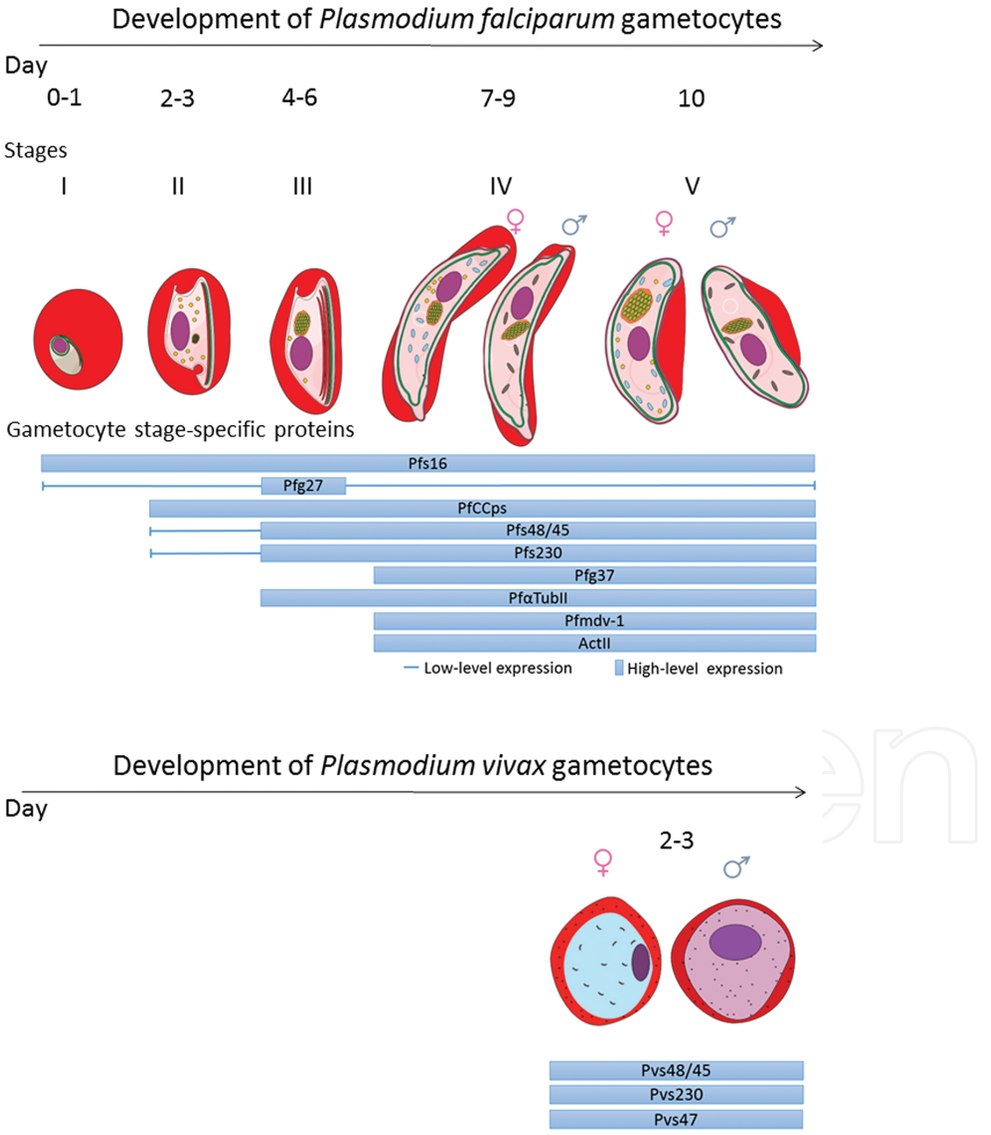

Figure 2. *P. falciparum* and *P. vivax* stage-specific protein expression during gametocyte development. The bar represents high-level expression and full line low-level expression. Only gene products were in included in this figure.

Among the other species of malaria, *P. vivax* is the most important because of its high impact on human health in South and Central America and South Asia [37]. The gametocytes of *P. vivax* are different from *P. falciparum* in their biology, dynamics and their formation, which occur within 2–3 days after asexual blood stage appearance in the circulation [38]. The male and female gametocytes of *P. vivax* possess a round shape as it is observed in *P. malariae*, *P. ovale* and *P. knowlesi*, but it is only in *P. vivax* that the parasite fills an enlarged red blood cell (**Figure 2**). In Giemsa-stained thin blood smears, female gametocytes have a blue-stained cytoplasm, and the chromatin is a purplish mass with a peripheral distribution, while male gametocytes possess a large and loose chromatin mass.

One remarkable morphological feature of gametocytes is the presence of the inner membrane complex (IMC), which underlies the plasmalemma and consists of flat membranous sacs called alveoli that are coupled to a supporting cytoskeletal network. The IMC was originally assigned to the invasive stages of *Plasmodium* as part of actin-myosin motor complex, and thus, the IMC is important for motility [39]. Recently, it was confirmed that gametocytes also possess an IMC [40–42], which is thought to be required for the stability of the crescent shape. The outer alveolar membrane contains complexes of the gliding-associated proteins GAP40, GAP45 and GAP50, which facilitate binding of myosin to this membrane. The gametocyte alveoli are subtended by a subpellicular network of interwoven alveoli filaments linked to the alveoli via intramembranous particles [39–41, 43]. Underneath the subpellicular network, an array of longitudinally oriented microtubules and F-actin fibrils are found that become reorganized in stage V gametocytes, leading to rounding-up of the gametocyte tips [44].

3.2. Gametocyte-specific proteins

Approximately 20% of all plasmodial genes are specifically expressed in the sexual stages [45, 46]. In *P. falciparum* gametocytes, the proteins are expressed at different levels in the five distinct developmental stages (**Figure 2**). Most of these proteins are associated with gametocyte development, integrity and preparation for fertilization. Furthermore, gender-related proteins are expressed in the later stages in order to support the gametes for fertilization in the mosquito midgut [1, 47–49].

In the early stage I–II gametocytes, important proteins such as Pfs16, Pfg27 and the six LCCL-domain proteins are expressed [50]. Pfs16 is present in all gametocyte stages, but it is initially expressed in stage I. It is localized in the parasitophorous vacuole (PV) where it plays a role in gametocyte development [51–53]. The main function of Pfs16 is unknown, but its disruption leads to a decrease in gametocyte production and an impairment in the ability of microgametocyte to exflagellate [54]. The protein Pfg27 is a phosphoprotein that starts to be expressed from stage II. It is known that Pfg27 forms a homodimer to act as single-stranded RNA-binding proteins *in vitro* and its gene deletion leads to the formation of abnormal gametocytes [55, 56]. The six LCCL-domain proteins (termed CCp in *P. falciparum* and LAP in *P. berghei*) possess adhesive properties and expression begins from stage II. In *P. falciparum* gametocytes, CCp proteins are localized within the PV, but after gamete emergence, the proteins are associated with the surface of gametes [47, 48, 57, 58].

Starting from stage IIb, well-known sexual-stage proteins such as the 6-cys proteins Pfs48/45 and Pfs230 and the osmiophilic body protein Pfg377 and Pfmdv-1 or sexual stage-specific cytoskeletal proteins such as α-tubII (tubulin II) and actin II are synthesized [50]. Similar to the six LCCL-domain proteins, the surface proteins Pfs48/45 and Pfs230 have adhesive properties and gene disruption of both proteins impair male gamete fertility [59, 60]. Pfg377 is known to be associated with osmiophilic bodies in female gametocyte of *P. falciparum*, and gene disruption demonstrates a fundamental role in the formation of osmiophilic bodies [61, 62]. The Pfmdv-1 protein, also known as Pfpeg3, is highly expressed in early gametocyte until stage V afterwards reaching a low level of expression. The function of *Pfmdv-1* is still unknown although disruption of the *Pfmdv-1* encoding gene leads to a 20-fold decrease in the production of male gametocytes [63]. The cytoskeletal proteins α-tubulin II and actin II are sexual stage-specific, and both are shown to be predominantly present in male gametocytes [64–66]. While α-tubulin II has been described to incorporate to axonemes of emerging male gametes, the function of actin II is still unclear, but gene disruption of both proteins leads to an impairment of male gametogenesis [65, 66]. In the mature microgametocyte, the protein PfB0400w, also known as PfMR5, can be observed. Although its function is still unknown, the expression of PfB0400w in male stage V gametocyte indicates that it may play a role in preparing gametocytes for exflagellation [50, 67].

In the gametocytes of *P. vivax*, three sexual stage-specific proteins have hitherto been identified, i.e. the 6-cys proteins Pvs230, Pvs48/45 and Pvs47, all of which are orthologues of those present in *P. falciparum* (**Figure 2**). Pvs230 has been characterized as an antigen on the surface of gametocytes and gametes while Pvs48/45 and Pvs47 have been studied recently as potential candidates for transmission-blocking vaccines. Following direct membrane feeding assays, anti-Pvs48/45 and anti-Pvs47 antibodies significantly reduced oocyst numbers in the mosquito midgut [68–70].

4. The gametocyte metabolism

During gametocytogenesis, *P. falciparum* parasites undergo intense metabolic processes including energy production as well as synthesis and degradation of various molecules such as lipids and proteins. These processes are essentials for the survival of the malaria parasite and ensure gametocyte stage development, drug resistance and transmission to the mosquito vector [30, 71, 72].

As vital molecules, lipids are responsible for many functions such as cell signalling, energy storage and the stability of membranes. Furthermore, during the various stages of the *Plasmodium* life cycle, lipids play a crucial role in the growth and proliferation of the parasite [73]. Among asexual blood stages and gametocytes, there are more than 300 lipids with known and unknown functions [74]. Although not reflected in all classes of lipids, the amount of lipids would more than double during gametocytogenesis [75]. The lipid composition of all developmental stages differs, including during gametocyte maturation. It is known that sphingolipids involved in ceramide metabolism are enriched during gametocyte maturation and the

inhibition of the ceramide biosynthetic pathway might kill gametocytes. In addition, phosphatidylserine is also highly expressed during gametocytogenesis and together with ceramides leads to gametocyte induction [74]. Neutral lipids such as cholesteryl ester and diacylglycerol (DAG) have also been shown to be significantly increased during gametocyte maturation; however, more data to better understand the metabolism and function of these lipids during gametocytogenesis are required [75].

In gametocytes, no DNA-replication occurs [76] and the nucleic acid synthesis is exclusive for RNA production. The production of RNA ceases from around day 6 of gametocytogenesis, thus the RNA and protein synthesis are more important in the early than late gametocyte stages [77, 78]. One interesting feature of malaria parasites is the expression of small subunit ribosomal RNA (SSU rRNA) genes [79], seven of them are synthesized in *P. falciparum* and one (S-type, formally known as C-type) is exclusively expressed in gametocytes. Proteomics identified 931 different proteins (72.2% of the proteins identified in *P. falciparum*) in gametocytes and within these proteins include conserved, stage-specific, secreted and membrane-associated proteins [46]. It is also known that protein expression differs between gender, female gametocytes synthetize proteins required for mitochondria and ribosomes, while male gametocytes only need proteins for DNA synthesis, axoneme formation and exflagellation [29, 80].

In eukaryotes, the mitochondria have many vital roles such as ATP synthesis, calcium homeostasis, lipid metabolism and synthesis of ion-sulphate clusters. The malaria parasites only possess a single mitochondrion and during parasite development it is observed that the mitochondria of gametocytes are longer and possess more cristae when compare to the ones of the asexual blood stages. This suggests that ATP synthesis via the electron transport chain is the main mechanism of energy production in gametocytes [29, 33]. Indeed the sexual stages appear to have seven times more cytochrome b than the asexual blood stages [81]. The cytochrome bc1 complex feeds the electron transport chain with more protons, which creates a gradient that drives ATP synthesis [82]. The cytochrome bc1 is a prime target for the antimalarial drug atovaquone that inhibits ubiquinone leading to the inhibition of *Plasmodium* cytochrome bc1 activity and loss of mitochondrial function [83–85]. Other drugs such as primaquine and the primaquine analogue bulaquine have been shown to exhibit gametocytocidal activities and primaquine is suggested to have the site of action in the mitochondria by causing its deformation. Although the mode of action is poorly understood, these drugs reduce gametocytemia in infection by *P. falciparum* and have an effect against the exo-erythrocytic stages of *P. vivax* [86].

Despite the fact that the mitochondria have been well characterized in other organisms, many mitochondrion-associated events in *P. falciparum* are still not well understood. Initially, *P. falciparum* blood stage parasites were thought to be glucose- and glycolysis-dependent for carbon metabolism, ATP production and survival [87]. However, recent reports have also shown the importance of the tricarboxylic acid (TCA) cycle for energy production, especially in gametocytes [88]. The gametocyte utilizes mitochondrial TCA to catabolize host glucose and glutamine. The carbon skeleton derived from these molecules enters in the TCA cycle via acetyl-CoA to generate proton flux and ATP via the ATP synthase. Due to the role of the TCA

cycle in parasite development, inhibition of this metabolic pathway leads to defects in gametocytogenesis. In addition, many mitochondrial enzymes involved in metabolic pathways can be targets for drug development [88].

5. Gametocyte activation and gametogenesis

5.1. The signalling cascade of gametocyte activation

The mature stage V gametocytes, which circulate in the blood of the mammalian host, are picked up by the female *Anopheles* mosquito during a bite and enter the midgut together with the blood meal. During the process, the gametocytes receive environmental signals, which indicate the switch from the warm-blooded host to the insect vector. The perception of these signals results in the immediate activation of the gametocytes followed by the onset of gametogenesis. Signals inducing gamete formation include a decrease in temperature by approximately 5°C, which is mandatory for gametocyte activation, and the presence of the mosquito-derived molecule xanthurenic acid (XA), a by-product of eye pigment synthesis [89–91].

While the plasmodial receptor or receptors important for signal perception are still unknown, the signalling pathway resulting in gametogenesis has partially been deciphered (**Figure 3**). It has previously been shown that male gametogenesis, during which the activated male microgametocyte forms eight motile flagellar microgametes (a process termed exflagellation), involves a fast increase in intracellular calcium and the second messenger cGMP [92, 93]. Two membrane-integrated guanylyl cyclases (GCα and GCβ) were identified in *Plasmodium*; however, only GCα appears to be important for cGMP synthesis in activated gametocytes [94–97]. The guanylyl cyclase can be stimulated by addition of XA. It has to be clarified, though, if XA acts directly or indirectly on GCα.

While in eukaryotes, cGMP is synthesized by guanylyl cyclases, phosphodiesterases (PDEs), which hydrolyse cGMP to GMP, are in general required for fast down-regulation of the second messenger. In *Plasmodium*, four PDEs, termed PDEα-δ, were identified, and PDEδ is highly expressed in gametocytes [98]. Zaprinast, a PDE inhibitor, is able to stimulate gametogenesis in the absence of XA due to increased cGMP levels [99]. Disruption of the gene encoding for PDEδ, however, impaired egress of *P. falciparum* gametocytes, indicating that a tight regulation of cGMP levels is important for gametogenesis [97].

The increase in cGMP activates the cGMP-dependent protein kinase PKG and chemical inhibition of PKG impairs rounding-up of the activated gametocytes. Since rounding-up is a calcium-independent step and cannot be inhibited by calcium chelators (see below), PKG acts prior to calcium increase in the activated gametocytes [97]. Gametocyte activation further leads to the generation of the second messenger molecules DAG and inositol-(1,4,5)-trisphosphate (IP_3) from phosphatidylinositol-(4,5)-bisphosphate (PIP_2) via the plasmodial phospholipase PI-PLC activity (**Figure 3**), as shown in *P. berghei*. IP_3 synthesis results in a rapid mobilization of calcium from the endoplasmic reticulum [100, 101]. PI-PLC activity itself appears to be

regulated by calcium, since it can be impaired by calcium ionophores, pointing to a calcium-regulated feedback mechanism. The increase in cytoplasmic calcium levels is further dependent on PKG activity and quantitative phosphoproteomics demonstrated that PKG is able to phosphorylate enzymes involved in the inositol phospholipid metabolism such as phosphatidylinositol-4-kinase (PI4K) and phosphoatidlyinositol-4-phosphate-5-kinase (PIP5K), which are involved in the synthesis of PIP_2 from phosphatidyl-1D-*myo*-inositol (PI) via phosphatidylinositol-4-phosphate (PI4P) [102].

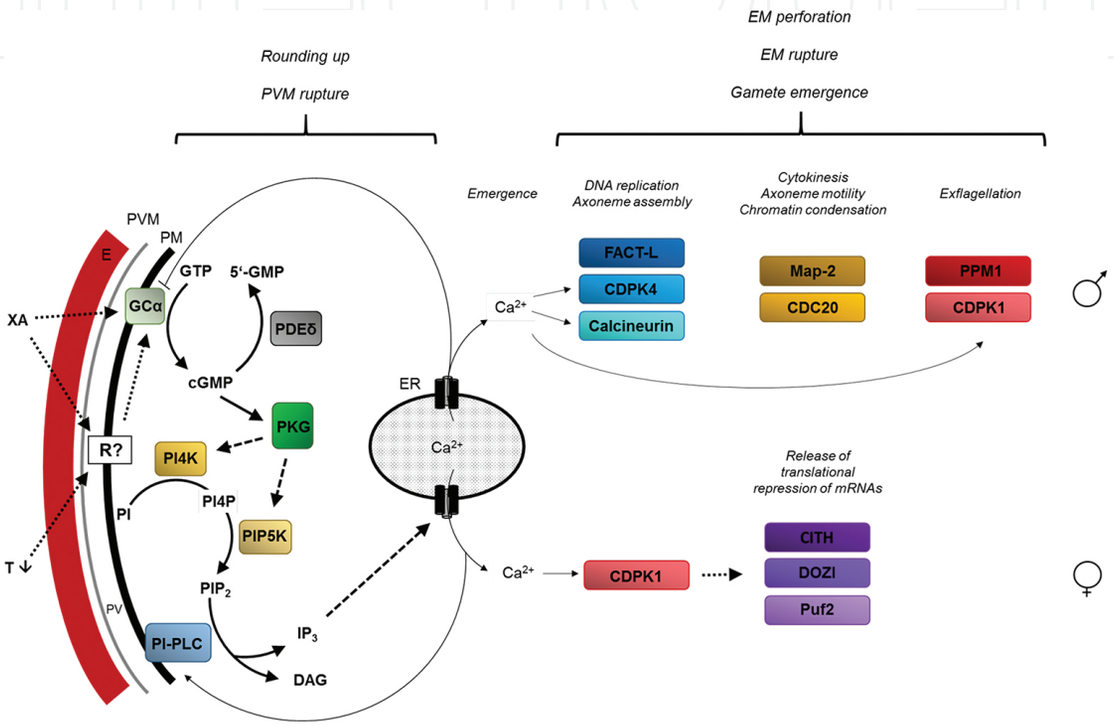

Figure 3. The signalling pathway involved in gametogenesis. Ca^{2+}, calcium ion; CDC20, cell division cycle protein 20; CDPK, calcium-dependent protein kinase; cGMP, cyclic guanosine monophosphate; CITH, homolog of worm CAR-I and fly Trailer Hitch; DAG, diacylglycerol; DNA, deoxyribonucleic acid; DOZI, development of zygote inhibited; E, erythrocyte; EM, erythrocyte membrane; ER endoplasmic reticulum; FACT-L, facilitates chromatin transcription protein L; GC, guanylyl cyclase; 5′-GMP, guanosine monophosphate; GTP, guanosine triphosphate; IP_3, inositol triphosphate; Map-2, mitogen-activated protein kinase 2; mRNA, messenger ribonucleic acid; PDE, phosphodiesterase; PI, phosphatidyl-1D-*myo*-inositol; PI4K, phosphatidylinositol-4-kinase; PI4P, phosphatidylinosito-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PIP5K, phosphatidylinositol-5-kinase; PKG, cGMP-dependent protein kinase; PPM1, metallo-dependent protein phosphatase 1; Puf2, pumilio/FBF (fem-3 binding factor) family protein 2; PV, parasitophorous vacuole; PVM, parasitophorous vacuole membrane; R, receptor; T, temperature; XA, xanthurenic acid.

In activated *P. berghei* microgametocytes, the increase in intracellular calcium was shown to activate the calcium-dependent protein kinases CDPK1 and CDPK4 and parasites lacking CDPK4 are not able to undergo DNA synthesis [103] (**Figure 3**). Further linked to this process is plasmodial FACT-L (facilitates chromatin transcription) [104]. Downstream of DNA replication two more proteins become activated, the mitogen-activated protein kinase MAP2 and the cell division cycle protein CDC20. Loss of MAP2 or CDC20 interferes with chromatin condensation, axoneme motility and cytokinesis [105–107]. Furthermore, depletion of the metallo-dependent protein phosphatase PPM1 or the calcium-dependent phosphatase

calcineurin as well as knock-down of CDPK1 result in impaired exflagellation [108–110]. While these combined studies pinpoint some key regulators of gametogenesis, the detailed pathway leading to gametocyte-to-gamete conversion is not known yet.

In female macrogametocytes, activation results in a sudden onset of protein synthesis, which is due to the CDPK1-regulated release of translational repression of messenger RNAs (mRNAs) [108]. In *P. berghei*, a total of 731 mRNAs are associated with two proteins of regulatory ribonucleoprotein complexes, i.e. the RNA helicase DOZI (development of zygote inhibited) and the Sm-like factor CITH (homolog of worm CAR-I and fly Trailer Hitch), which function in translational repression of these transcripts (**Figure 3**). These include the mRNAs encoding for P25, GAP45 and GAP50 [111–113]. For *P. falciparum*, translational repression of mRNAs was shown to involve the Pumilio/FBF (Puf) family RNA-binding protein Puf2 that is required for controlling a number of transcripts in gametocytes including those encoding for P25 and P28 [114]. The repressed mRNAs mostly encode for components of the later ookinete stage.

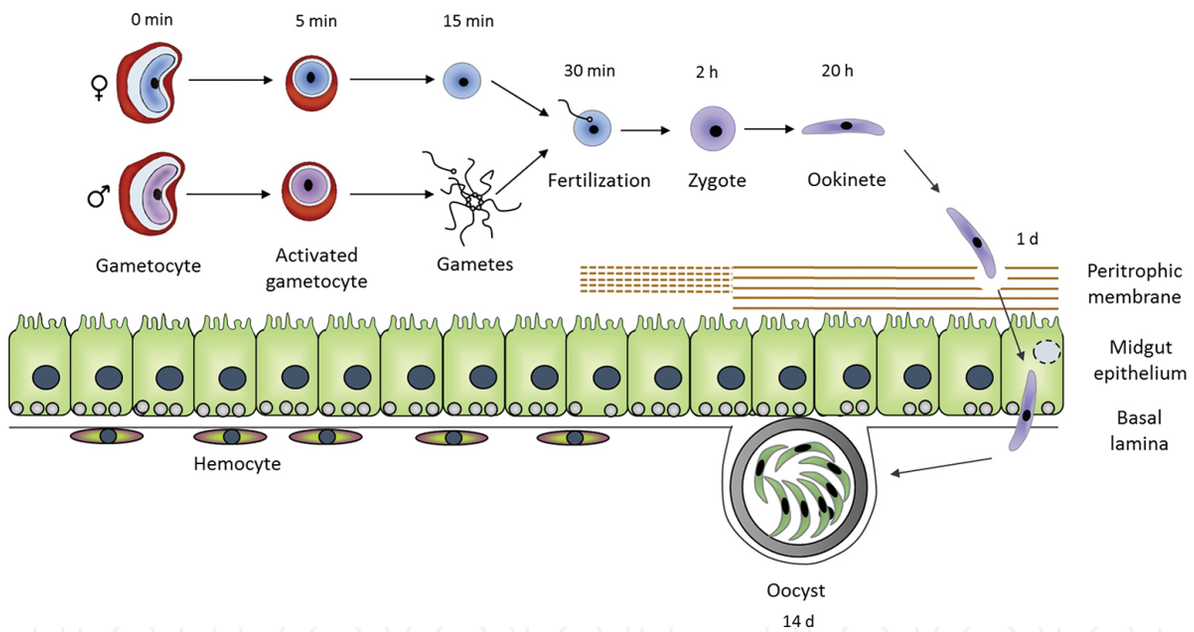

Figure 4. Development of malaria parasites in the mosquito midgut.

5.2. Gametocyte egress and gamete formation

Gametocyte activation results in three morphological changes in the parasites, i.e. rounding-up, erythrocyte egress and gamete formation, and these changes occur with approximately 15 min following perception of the environmental signals (**Figure 4**). Gametocytes exit the erythrocyte via an inside-out mode, during which the parasitophorous vacuolar membrane (PVM) ruptures prior to the opening of the erythrocyte membrane (EM). Rupture of the PVM occurs at multiple sites less than 2 min following signal perception and is accompanied by the rounding-up of the parasite. Both events are calcium-independent [91, 99]. It is suggested that

the PVM rupture is linked to the discharge of the osmiophilic body content into the PV, since an accumulation of osmiophilic bodies can be observed underneath the rupture sites [91, 115].

In a second, a calcium-dependent step, another type of vesicle discharges its content into the cytoplasm of the host erythrocyte, which includes the plasmodial pore-forming perforin PPLP2. This perforin is able to permeate the EM, resulting in the release the erythrocyte cytoplasm, eventually leaving the forming gamete enclosed by the EM [115–117]. While EM permeabilization occurs approximately 6 min post-activation, its rupture takes place 9 min later. Here, the EM opens at a single pore and releases the fertile gamete [91, 117].

The IMC disintegrates shortly after onset of gametogenesis, eventually resulting in gametes solely confined by the plasmalemma. It was shown that the transmembrane protein GAP50, which in gametocytes, is located in the outer membrane of the alveoli, relocates to the gamete plasmalemma during gametogenesis. During relocation, the N-terminal part of GAP50 becomes extracellularly exposed and once the gametes have freed from the EM binds complement factor H from the blood meal, a step crucial to protect the newly formed gametes from lysis by human complement factors [42]. Coevally with the IMC degradation long membranous tubules, termed tunneling nanotubes (TNTs), form, which protrude from the gamete surface. TNTs represent long-distance cell-to-cell connections, which were proposed to mediate intercellular contacts between gametes as a pre-requisite for mating [118]. While the question of the origin of the membranous tubules remains unanswered, one might stipulate that after IMC disintegration the alveoli membranes are used for TNT formation.

Upon activation, the male microgametocyte replicates its genome three times and mitotically produces eight flagellar microgametes [119] (**Figure 4**). Flagellum formation requires the axonemal assembly from basal bodies, which involves the centriole/basal body protein SAS-6 [120], while axoneme motility is regulated by the conserved armadillo-repeat protein PF16 [121]. Exflagellating microgametes typically adhere avidly to neighbouring erythrocytes and are hidden within such rosettes. During exflagellation, the microgamete detaches from the residual body and is freely motile in search of a female macrogamete. Once adhering to a macrogamete, fertilization begins by the fusion of the two gamete plasma membranes, and the axoneme and attached male nucleus enter the female cytoplasm. While the exact proteins involved in initial binding of male and female gametes are yet unknown, gamete fusion is mediated by the microgamete-specific protein GCS1 (generative cell specific 1), also termed HAP2 [122, 123]. Over the next 3 hours, meiosis occurs and the zygote becomes tetraploid [119]. The transformation of the zygote into an ookinete is completed at 19–36 hours post-blood meal, and the ookinete then rapidly exits the midgut lumen to settle down at the basal site of the midgut epithelium and to differentiate into an oocyst [124–127]. Parasite tetraploidy persists until sporozoite budding in the oocyst restores the haploid state [119].

6. Conclusion

Although our knowledge of the biology of gametocytes is not as advanced as that of the asexual blood stages, the great interest by researchers in recent years has considerably deepened our

understanding of the gametocyte biology. Advances in gametocyte cultivation, isolation and purification techniques as well as the impressive amount of data gained from genome-wide analyses, proteomics and microarray studies have led to the identification of some gametocyte stage-specific antigens as well as sex-specific proteins, which may be exploited as targets for malaria transmission-blocking intervention strategies. In addition, the recent discoveries on the molecular mechanism of gametocyte commitment have greatly improved our knowledge on how sexual differentiation is initiated and regulated in the malaria parasite. However, several questions are still to be answered regarding the biology of gametocytes. The mechanism by which stress factors are perceived to enhance gametocyte commitment is not well understood. Some components of the signalling cascade of gametocyte activation are yet unknown such as the receptor or receptors responsible for signal perception following gametocyte activation by XA. How gametocyte sequestration in the bone marrow takes place as well as the molecular mechanism underlying this phenomenon has to been deciphered. Also, a proper understanding of the mechanism by which the gametocyte rapidly adapts once in the mosquito vector and the basis of fertilization is needed. Furthermore, not much is known about the metabolism of gametocytes. Most of the metabolic pathways have not been described. A proper understanding of these aspects in the biology of gametocytes would greatly improve strategies aimed to target gametocytes.

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