

## Review Article

# The Coming-Out of Malaria Gametocytes

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The tropical disease malaria, which results in more than one million deaths annually, is caused by protozoan parasites of the genus *Plasmodium* and transmitted by blood-feeding Anopheline mosquitoes. Parasite transition from the human host to the mosquito vector is mediated by gametocytes, sexual stages that are formed in human erythrocytes, which therefore play a crucial part in the spread of the tropical disease. The uptake by the blood-feeding mosquito triggers important molecular and cellular changes in the gametocytes, thus mediating the rapid adjustment of the parasite from the warm-blooded host to the insect host and subsequently initiating reproduction. The contact with midgut factors triggers gametocyte activation and results in their egress from the enveloping erythrocyte, which then leads to gamete formation and fertilization. This review summarizes recent findings on the role of gametocytes during transmission to the mosquito and particularly focuses on the molecular mechanisms underlying gametocyte activation and emergence from the host erythrocyte during gametogenesis.

## 1. Introduction

With an annual death toll of more than one million people, the tropical disease malaria is considered one of the most significant infectious diseases worldwide. Malaria is caused by protozoan parasites of the genus *Plasmodium* and transmitted by blood-feeding Anopheline mosquitoes. During their life cycle, plasmodia alternate between the human host and the insect vector, and thus the transmission stages of the parasite had to develop mechanisms for rapid adaptation to the new environment in order to coexist with the respective host.

Like most apicomplexan parasites, plasmodia further switch between tissue-specific multiplication cycles and a phase of sexual reproduction, which mediates the transition from the human to the mosquito and thus plays a crucial part in the spread of the disease. The malaria sexual phase begins with the differentiation of gametocytes in human erythrocytes, followed by their uptake during the blood meal of the mosquito and the formation of gametes within the insect midgut. The transformation of the fertilized zygote into the infective ookinete subsequently marks the end of the malaria sexual phase (reviewed in [1]).

Historically, scant research has been devoted to the malaria sexual stages, namely, gametocytes, gametes, and zygotes, since they neither contribute to the clinical picture of patients nor do they play a role for vector control. However, within the last two decades the dramatic increase of drug resistance in malaria parasites has forced researchers to broaden their consideration of tactics to combat the disease, including transmission blocking strategies aimed at the sexual stages. Such transmission blocking strategies, on the level of either drugs or vaccines, are designed to disrupt parasite reproduction and further development in the mosquito midgut, thus breaking the life cycle of the parasite. Research on the malaria transmission stages, however, was formerly hampered by cost- and time-consuming cultivation, as well as by the technically challenging infections of mosquitoes with parasites. This was particularly true for work on *P. falciparum*, the causative agent of malaria tropica.

In recent years knowledge on the malaria sexual phase has benefited from a dramatic resurgence provided by proteomic, microarray, and annotation projects that arose out of the genome sequence projects for multiple malaria species (e.g., [2–7]). As a result, a number of new sexual stage antigens have been identified, and progress has been

made in the identification and functional characterization of enzymes and regulatory proteins that are involved in gametocyte differentiation and fertilization (reviewed in [1]).

Nowadays, three main questions regarding the malaria sexual phase are in the focus of interest. (1) Which are the mechanisms that cause a subset of erythrocytic parasites to enter the sexual stage pathway and to differentiate to gametocytes?. (2) How do gametocytes become activated within the mosquito midgut and how do they transform into gametes?. (3) In which way do the sexual stages interact with factors of the mosquito midgut? This review addresses the role of gametocytes during malaria transmission and particularly discusses the recent findings on gametocyte activation following entry of the mosquito midgut, as well as their egress from the host erythrocyte and transformation into gametes. Additional aspects of gametocytogenesis, sexual stage proteins, and malaria transmission can be found in other recent reviews [1, 8–11].

## 2. Gametocyte Differentiation in the Human

The gametocytes are the only stages within the life cycle of malaria parasites that are able to mediate the transition from the human host to the insect host. The development of asexual blood stage parasites to intraerythrocytic gametocytes, which is referred to as gametocytogenesis, starts approximately 7–15 days after the appearance of parasites in the human blood. It is not known to which degree gametocytes develop stochastically, with a small proportion of committed parasites leaving the asexual cycle and entering the sexual pathway, versus gametocytogenesis as a response to complex environmental signals during infection. Gametocytogenesis was previously shown to be influenced by different kinds of stress, including parasite density, anemia, host immune response or drug treatment (e.g., [12–20]; reviewed in [8, 10]). Up to date, little is known about parasite genes that regulate gametocytogenesis, but it was observed that reduced levels of gametocytes in parasite cultures are often associated with the loss of genetic information following subtelomeric deletion in the right arm of chromosome 9 [21].

While in most *Plasmodium* species the sexual stages mature within less than two days, a time period of about 10 days is required for gametocyte development in the human malaria pathogen *P. falciparum* [22]. Gametocyte maturation can be classified into five morphological stages (stages I–V) [23], and mature *P. falciparum* gametocytes show an eponymous falcipare form. In these stages, the host erythrocyte has conformed to the crescent shape of the parasite and is reduced to a small cytoplasmic hem. The intraerythrocytic gametocyte lies within the parasitophorous vacuole (PV) and is shielded from the erythrocyte cytoplasm by the PV membrane (PVM), which is located adjacent to the parasite plasma membrane (PPM) (Figure 1(a)). Underneath the PPM is a typical gametocyte feature, the pellicular complex, which consists of a subpellicular membrane (SPM) vacuole subtended by an array of longitudinally oriented microtubules [24]. These structures probably

give the gametocyte stability, and the electron-dense SPM disappears during gametogenesis (Figures 1(a), 1(b), 1(c), and 1(d)). Besides morphological changes, maturation of gametocytes also includes alterations on the molecular level in order to prepare the parasites for the rapid adaptation to the mosquito midgut. For instance, a large amount of mRNA is transcribed and stored in the cytoplasm of female gametocytes, as shown for the transcripts of the sexual stage surface proteins *Pbs25* and *Pbs28* in the rodent malaria model *P. berghei* [25], which will be transcribed only in the mosquito vector (see below).

The gender is predetermined in the developing gametocytes [26]. Gender specificity becomes established in the schizont committed to gametocytogenesis [27], and the gender ratio is typically female-biased with one male for about five female gametocytes, depending on the respective parasite clone [28]. This difference might be explained by the fact that one male gametocyte forms approximately eight microgametes, thus establishing a roughly 1 : 1 ratio of micro- and macrogametes in the mosquito midgut, thereby leading to most efficient fertilization in a monoclonal infection [8, 28]. Recently, Reece et al. demonstrated in the mouse model that parasites were able to adjust the sex ratio according to parasite density and the number of parasite clones coinfecting the mammalian host [29]. For instance, a less female-biased sex ratio would increase the probability of a successful fertilization of females of the respective clone, when competing with others [29–32]. In contrast, in vitro studies on *P. falciparum* did not show an adjustment of sex ratio to gametocyte density. However, an impact of the sex ratio on the infection rate, depending on gametocyte density, was observed [33].

The fact that single, haploid asexually replicating malaria parasites are able to develop into gametocytes of both sexes in the absence of sex chromosomes indicates that gametocyte gender determination is governed by differential gene expression [34]. Gametocyte stages I to IV were reported to sequester in the bone marrow and spleen, while terminally differentiated stage V gametocytes are then released in the peripheral blood system [35, 36] and only become infectious to mosquitoes after a further two or three days of circulation [37, 38]. Bloodstream gametocytes might not be distributed homogeneously, as evidenced by a significant aggregation pattern observed in midgut smears of *P. falciparum*-fed mosquitoes [39], and it is an intriguing hypothesis that parasites increase the likelihood of fertilization in the mosquito midgut by promoting uptake in preformed complexes of gametocytes.

## 3. Gametocyte Activation in the Mosquito Midgut

While feeding on an infected human, the female mosquito takes up malaria gametocytes together with the blood meal. By entering the midgut, the parasites receive environmental signals, which indicate the switch from warm-blooded host to insect vector and which initiate the development

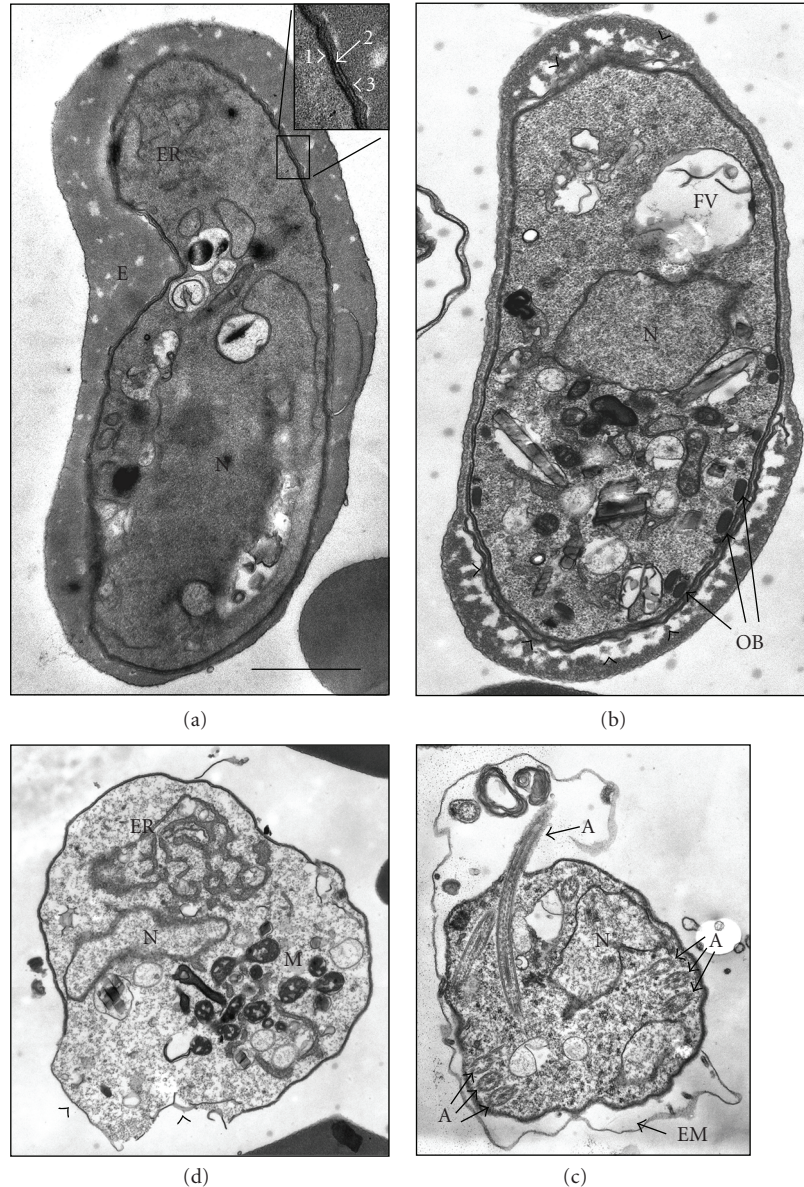


FIGURE 1: Ultrastructural changes in *P. falciparum* gametocytes during activation. (a) Transmission electron micrograph of a mature, non-activated gametocyte. The erythrocyte is reduced to an electron-light hem. The PVM is located adjacent to the PPM and the PV is therefore not discernable. No osmiophilic bodies are detectable, indicating that a male microgametocyte is pictured. Inset shows the pellicular membrane complex, depicting the SPM (1), the PPM (2) and the PVM (3). (b) A female macrogametocyte two-minute postactivation. The gametocyte is in the process of rounding up, thereby losing its crescent shape. The osmiophilic bodies become closely associated to the parasite surface. At the poles, the PVM separates from the PPM and the erythrocyte is in the process of degrading (arrowheads). (c) Ultrastructure of an exflagellating microgametocyte. The axoneme of a forming microgamete is visible as longitudinal section, and several other cross sections of axonemes are detectable inside the microgametocyte. The PVM has disappeared and the EM ruptured. (d) A female macrogamete after emergence. This stage is marked by pronounced ER and the highly branched single mitochondrion. The SPM is in the process of disintegrating (arrowheads). A, axoneme; EM, erythrocyte membrane; ER, endoplasmic reticulum; FV, food vacuole; M, mitochondrion; N, nucleus; OB, osmiophilic body; PPM, parasite plasma membrane; PV, parasitophorous vacuole; PVM, PV membrane; SPM, subpellicular membrane. Bar, 1  $\mu$ m.

of gametes. Such signals include a drop of temperature by approximately 5°C [40], and the presence of the mosquito-derived molecule xanthurenic acid (XA), a byproduct of eye pigment synthesis [41, 42]. An additional signal reported to induce gametocyte activation is an increase of pH from 7.2 to

about 8 [40, 43], but such a pH shift was later discussed to be an artificial inductor of exflagellation [9]. While XA appears to initiate a number of signaling events in the parasite (see below), the quest for a receptor that binds XA was hitherto unsuccessful.

Gametocyte activation is routinely measured by the formation of exflagellation centers, although a time period of almost 15 minutes lies between these two events [44]. This is due to the fact that exflagellation can easily be observed under the light microscope and quantified by counting of exflagellation centers. Exflagellation is the process when the activated male microgametocyte forms motile flagellar microgametes, which detach from the residual body by binding to erythrocytes (see below). Two previous studies showed that induction of exflagellation involves a fast increase in intracellular calcium and cGMP [45, 46]. An initial benchmark in elucidating sexual stage signaling was the identification of two guanylyl cyclases ( $G\alpha$  and  $G\beta$ ) as integral membrane proteins in *P. falciparum* [47], which are activated by addition of XA (Figure 2) [48]. Noteworthy, the subsequent disruption of the  $G\beta$  ortholog in *P. berghei* resulted in normal exflagellation, but motility-impaired ookinetes, indicating that the role of  $G\beta$  is not essential for gametocyte activation [49]. The increase of cGMP triggers the activation of a cGMP-dependent protein kinase, PKG. Activation of PKG leads to rounding up of the gametocyte, a process that appears to be independent from calcium increase [50]. Gametocyte exflagellation further involves the presence of the second messengers diacylglycerol and inositol triphosphate ( $IP_3$ ), hydrolysis products of phospholipase C activity [51]. The latter eventually mediates the release of intracellular calcium from the endoplasmic reticulum (ER) (Figure 2).

It is not yet known how the signaling pathway involving  $IP_3$  and calcium release and the pathway involving cGMP and PKG activation are linked together, and whether PKG has an additional effect on calcium release from the ER (Figure 2). Current data suggest that at least three effector pathways exist (discussed in [50]): (1) a PKG-dependent, calcium-independent pathway that mediates rounding up of the activated gametocytes, (2) a calcium-dependent pathway that initiates microgamete formation, and (3) a calcium-dependent pathway that regulates emergence of activated gametocytes of both genders.

#### 4. Gametocyte Egress from the Host Erythrocyte

Following uptake by the mosquito, both male and female gametocytes round up and then escape from the enveloping erythrocytes within about 10 minutes postactivation (Figures 1(b), 1(c), and 1(d)). In this period the microgametocyte replicates its genome three times in order to produce eight motile microgametes (reviewed in [1, 9]). Egress of the activated gametocyte from the host erythrocyte has been linked to the presence of osmiophilic bodies, gametocyte-specific secretory organelles that were first identified by electron microscopy due to their electron-dense features (Figure 1(b)) [24, 52]. They appear first in stage IV gametocytes and are particularly present in the female sexual stages. The osmiophilic bodies migrate to the PPM during activation and disappear within a few minutes post-activation, coevally with the rupture of the PVM (Figures 1(b), 1(c), and 1(d)) (G. Pradel, unpublished observations).

Osmiophilic bodies contain a gametocyte-specific and highly hydrophilic protein, *Pfg377*, which is considered a marker for these organelles [53, 54]. Only recently has the function of *Pfg377* been investigated by reverse genetic methods. Gene-disruption studies showed that female *P. falciparum* gametocytes lacking this protein reveal a reduced number of osmiophilic bodies and fail to egress from the host erythrocyte, pointing to a pivotal role of this protein in gametocyte emergence [55].

Another protein, which was only recently identified, MDV-1/Peg3, has also been implicated with gametocyte egress. In *P. falciparum*, expression was reported to be initiated in stage I gametocytes in association with all membranous structures of the PVM and to persist until gametocyte maturation [56–58]. First gene disruption studies on *P. falciparum* described a reduced formation of particularly male gametocytes [57]. Two subsequent studies, however, indicated a role of the protein post-activation of gametocytes. Lal et al. [59] reported the presence of MDV-1/Peg3 in *P. berghei* gametocytes of both sexes and a subsequent focal localization at the anterior pole of the developing ookinete. Studies on parasites in which the respective gene was knocked out resulted in reduced ookinete formation. A study by Ponzi et al., on the other hand, showed that *P. berghei* MDV-1/Peg3 was associated with the gametocyte osmiophilic bodies [60]. Gametocytes lacking this protein failed to egress from the host erythrocyte, thus resulting in reduced fertilization and ookinete formation. Ponzi et al. [60] therefore suggested that MDV-1/Peg3 plays a major role in disrupting the PVM and the erythrocyte membrane (EM).

Independent from the life-cycle stage, host cell egress of malaria parasites involves rupture of two membranes, PVM and EM. The time line of rupture, however, was recently object to several brisk discussions. Particularly two models are currently under investigation, the inside-out model, in which the PVM ruptures prior to the EM [61], and the outside-in model, in which the EM is degraded first [62, 63] (reviewed in [64]). The timeline of parasite egress was hitherto mainly investigated in the asexual blood and liver stages, and no data are available for the egress of gametocytes. In the above mentioned study, Ponzi et al. [60] suggested that MDV-1/Peg3 is involved in PVM destabilization and that EM rupture depends on the absence of the PVM. In accord with this hypothesis, new studies from our laboratory indicated that the PVM disappears within a few minutes after gametocyte activation, and that the rupture of the EM follows several minutes later (G. Pradel, unpublished observations), thus supporting the inside-out model of egress.

The coming-out of malaria parasites from the host cell requires protease activity. A number of new studies engaged with the identification of proteases that mediate emergence of asexual blood stage merozoites. Data point to the involvement of the cytoskeleton-degrading malaria proteases falcipain-2 and plasmepsin II [64]. Particularly SERA (serine-rich antigen) proteins, which were identified in the PV of blood stage schizonts (e.g., [65, 66]), are supposed to mediate PVM rupture. It was shown for

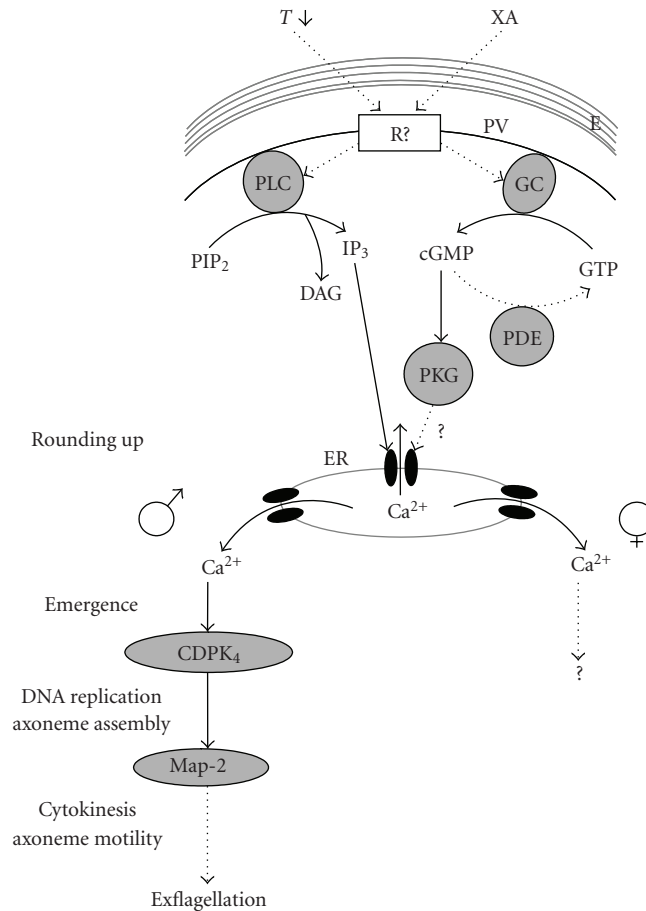


FIGURE 2: Schematic overview of signaling pathways identified to date that are involved in gametocyte activation (modified from [83]). Gametocyte activation is induced by a decrease in temperature and the presence of the mosquito-derived molecule XA. So far, a receptor involved in activation has not been identified. Activation effects PLC and GC, resulting in an increase of IP<sub>3</sub> and cGMP. The latter activates a PKG. IP<sub>3</sub> mediates release of intracellular calcium from the ER, which activates CDPK<sub>4</sub> and consequently Map-2 in the male microgametocyte, eventually leading to exflagellation. A possible link between PKG and calcium release has not yet been confirmed. Signaling pathways in the activated macrogametocyte that are downstream of calcium release were not yet identified. Black lines indicate direct interactions and dashed lines indicate indirect interactions. Ca<sup>2+</sup>, calcium ion; CDPK, calcium-dependent protein kinase; cGMP, cyclic guanosine monophosphate; DAG, diacylglycerol; DNA, deoxyribonucleic acid; E, erythrocyte; ER, endoplasmic reticulum; GC, guanylyl cyclase; GTP, guanosine triphosphate; IP<sub>3</sub>, inositol triphosphate; Map-2, Mitogen-activated protein kinase 2; PDE, phosphodiesterase; PIP<sub>2</sub>, phosphatidylinositol-4,5-bisphosphate; PKG, cGMP-dependent protein kinase; PLC, phospholipase C; PV, parasitophorous vacuole; R, receptor; T, temperature; XA, xanthurenic acid.

SERA-5 of *P. falciparum* that the protease is proteolytically activated by the serine-like subtilisin protease *PfSUB1* [67]. While no detailed studies were yet performed on malaria gametocytes, it is worth mentioning that transcripts of select representatives of the above mentioned protease families are expressed in these stages, including falcipain-1, plasmepsin VI, SERA-6, SERA-7, and *PfSUB3* [68, 69].

A popular strategy in investigating protease activity during rupture is the treatment of parasites with protease type-specific inhibitors. Again, these studies were mostly performed on blood stage parasites, particularly using cysteine protease inhibitors like E64. Treatment with this inhibitor, however, resulted to date in contradictory results, and it was reported that the inhibitor blocked

either degradation of the PVM [62, 63] or rupture of the EM [70]. A similar egress study using protease inhibitors was recently performed on activated *P. berghei* gametocytes and showed that exflagellation can be blocked by the cysteine/serine protease inhibitors TPCK and TLCK [71]. Our laboratory subsequently confirmed these results in *P. falciparum*. Treatment of activated gametocytes with TPCK, TLCK, PMSE, or two novel falcipain-targeting cysteine protease inhibitors during activation reduced the formation of microgametes [72]. Furthermore, the aspartic protease inhibitor EPNP appeared to interfere with rounding up of gametocytes. The exact modes of action for these proteases during gametocyte egress from its host cell remain to be investigated.

## 5. Exflagellation and Gamete Formation

is the process in which the newly formed microgametes adhere to neighboring erythrocytes, thus forming rosettes called exflagellation centers, and then detach from the residual body of the activated male microgametocyte. The exflagellating microgamete adheres to sialic acids and glycoporphin A of the erythrocyte surface [73] and this binding is probably mediated by *Pfs230*, an abundantly expressed adhesion protein that is associated with the gamete surface [74]. Interestingly, *Pfs230* is proteolytically processed during gametocyte activation, and this processing can be inhibited by the metalloprotease inhibitor 1,10-phenanthroline [75, 76]. The same inhibitor blocks exflagellation by leaving the microgamete amotile [72], and it is tempting to speculate that processing of *Pfs230* increases the adhesive properties of this protein, which are needed for the binding of the exflagellating microgamete to erythrocytes [74].

Astonishing advances have been made in unveiling the signaling cascades during gametogenesis of plasmodia. This might be explained by the fact that sexual stage proteins can easily be disrupted because of their nonessentiality for parasite proliferation, and thus a functional characterization can be obtained by phenotype analysis of parasites, in which the respective genes have been knocked out. Genome annotation has revealed an extensive catalog of parasite-encoded kinases, the malaria kinome, with at least 86 hypothetical kinases identified in *P. falciparum* [77, 78]. Several orthologs of these kinases were disrupted in *P. berghei*. An initial elegant study showed that the calcium-dependent protein kinase *PbCDPK4* is involved in sexual stage signaling and regulation [79] (Figure 2). The kinase becomes activated by calcium increase following XA activation, resulting in genome replication in microgametocytes [79]. In *P. falciparum*, *PfCDPK4* was reported to be gametocyte-specific and activated by phospholipase C [80]. In a subsequent step, the mitogen-activated protein kinase *Pbmap-2* controls formation of male gametes at the stage of cytokinesis [81–83]. Downstream of these events, the protein kinases *Pbnek-2* and *Pbnek-4* trigger genome replication to the tetraploid level in the zygote stage [81, 84, 85]. Furthermore, *PbCDPK3* is required for ookinete motility and engagement with the mosquito midgut epithelium [86, 87]. When highlighting these novel signaling pathways during gametogenesis, it has to be taken under consideration, however, that in some cases the results obtained for *P. berghei* and *P. falciparum* might differ. For example, a recent reverse genetics approach on the *P. falciparum* ortholog *Pfmap-2* pointed to an essential function of this kinase for the parasite asexual blood cycle [88], contradictory to the abovementioned results on *Pbmap-2*. This indicates that insights gained by studying the rodent malaria model *P. berghei* cannot be as easily applied to human malaria pathogens as has so far been assumed.

Ingestion by the blood-feeding mosquito triggers molecular changes in the sexual stages of *P. falciparum*, with approximately 20% of stage-specific genes being activated during sexual stage development and parasite transmission [3, 5, 6]. This molecular switchover adjusts the gametocytes to the invertebrate host and on one hand initiates

reproduction but on the other hand prepares the emerging gametes for the hostile environment of the mosquito midgut. Gametocyte development and gamete formation are particularly accompanied by the coordinated expression of numerous surface-associated proteins, including the EGF domain-containing proteins *Pfs25* and *Pfs28*, the cysteine motif-rich proteins *Pfs230* and *Pfs48/45*, as well as the multiadhesion domain *PfCCp* proteins. These proteins and their potential as transmission blocking targets were discussed previously [1] and will therefore not be focus of this review. Noteworthy is that the majority of these surface proteins have adhesive properties and can be divided in two classes. One class of sexual stage proteins, including *Pfs230*, *Pfs48/45*, and the six *PfCCp* proteins, is expressed within the PV of the developing gametocyte and subsequently present on the gamete surface, but expression of these proteins usually ceases during fertilization (Figure 3). The expression of the second class of surface proteins starts at the time point of fertilization, as was shown for *Pfs25* and *Pfs28*, and expression often persists until the ookinete has formed [1]. The reason for this sudden onset of protein expression during fertilization is the translational repression of messenger RNA encoding for these proteins. This was interalia shown for the repression of *Pbs25* and *Pbs28* by the *P. berghei* RNA helicase DOZI (development of zygote inhibited) as part of a ribonucleoprotein complex [25]. Furthermore, transcript of the transcription factor AP2-O is present in female gametocytes. The factor, however, is only translated in the ookinete stage, where it then activates a set of genes encoding for adhesion proteins important for midgut invasion [89].

The reason for such a high number of adhesive proteins in the malaria parasite sexual stages remains elusive, but a new study from our laboratory might provide a first step towards answering this question. We showed that the six *PfCCp* proteins, which are characterized by a high number of adhesion modules, assemble to form multiprotein complexes during their expression in the PV, and these complexes are subsequently present on the surface of the newly emerged macrogametes [90]. Preliminary data point to an additional involvement of other surface-associated adhesion proteins in these complexes, like the transmembrane protein *Pfs48/45*, which might link the complex to the gamete surface [91] (S. Scholz, A. Kuehn, N. Simon, and G. Pradel, unpublished observations). We hypothesize that these protein complexes cover the macrogamete in the form of a sticky coat and that they are involved in important adhesive processes during malaria transmission to the mosquito. The complexes might play a role in promoting contact between the emerging gametes within the blood meal or in protecting the gametes from the aggressive environment of the mosquito midgut. Noteworthy, the gametes and zygotes are the only stages within the parasite's life cycle that, for more than one day, have to persevere outside a host cell. Here they are exposed to factors of the blood meal, including midgut bacteria and digestive enzymes, as well as components of the human immune system. This exposure results in an approximate 300-fold loss of parasite abundance during transmission to the mosquito, and the malaria transmission stages are

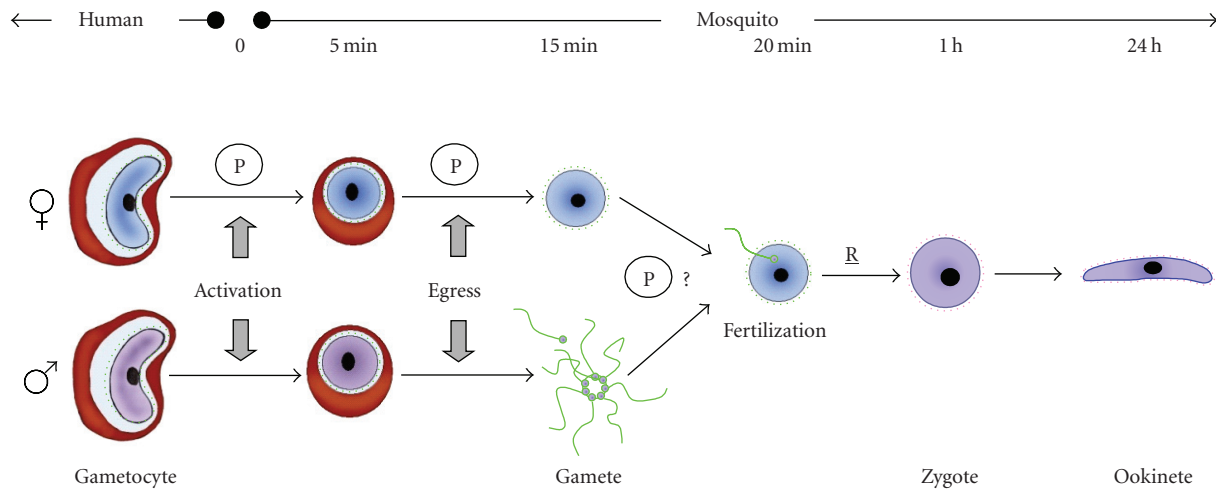


FIGURE 3: Morphological changes of malaria parasites during transmission from the human host to the mosquito vector. The intraerythrocytic gametocyte stages mature in the human host and are taken up by the blood-feeding female mosquito. By entering the mosquito midgut, the gametocytes become activated and round up, before emerging from the enveloping host erythrocyte. Proteases (P) are involved in these processes. During gametogenesis, the female macrogametocyte transforms into a macrogamete, while the activated male microgametocyte forms eight microgametes. Within approximately twenty-minute post-activation, the motile microgamete fertilizes the macrogamete and the resulting zygote transforms within a day into the infective ookinete. Two classes of sexual stage proteins are expressed in association with the parasite surface. A first class of proteins (shown in green) is expressed in the parasitophorous vacuole of the developing gametocyte, where some of them assemble to form adhesive multiprotein complexes. The proteins are later exposed on the surface of the newly emerged gametes, but expression ceases during fertilization. Expression of a second class of surface-associated proteins (shown in pink) is repressed in the gametocyte stage, but repression is released during fertilization (R) and protein expression persists to the ookinete stage.

therefore considered bottleneck stages of the parasite's life cycle [92].

## 6. Mating of Malaria Parasites

During exflagellation, the microgamete detaches from the residual body and is freely motile, moving via sinusoidal or helical waves [93]. It is not known whether the microgamete meets the macrogamete by coincidence, whether it actively scans the blood meal, or whether it migrates along a gradient of an attractant that is released by the macrogamete. Interestingly, we recently identified filamentous protrusions of the *P. falciparum* gamete surface, which form immediately upon activation and which appear to establish long-distance contacts between parasites in the mosquito midgut (G. Pradel, unpublished observations). These filaments fit the typical characteristics of so-called nanotubes, novel organelles that were recently described for a number of animal cells (reviewed in [95, 96]). It has been proposed that nanotube-like filaments can be formed by almost all cells serving as a medium for exploring the extracellular environment [94] and therefore are likely to represent ancient features of unicellular eukaryotes. Nanotubes were reported to have a function in communication between cells, including calcium signaling and organelle transfer [95, 96]. We therefore hypothesize that the "nanotubes" of malaria gametes might be tools to facilitate association within the midgut in order to increase the chance of parasite mating.

Once the microgamete adheres to a macrogamete, fertilization begins by fusion of the plasma membranes. Two recent studies on *P. berghei* described the identification of the microgamete protein GCS1 (generative cell specific 1), also termed HAP2, which enables gamete fusion, and disruption of the respective gene results in male sterility and blocked fertilization [97, 98]. GCS1/HAP2 is a conserved protein of algae and plants, where it is involved in pollen tube guidance and seed formation [99, 100], and was also identified in protozoan parasites [97, 98, 101]. Importantly, GCS1/HAP2 does not mediate the initial binding between the two mating partners, which appears to involve other adhesion proteins [98].

Cell fusion is followed by nuclear fusion, and over the next 3 hours, meiosis occurs and the zygote becomes tetraploid [102]. During the following 24 hours, the zygote transforms into the infective ookinete stage, thus marking the end of the malaria sexual phase. The ookinete is motile and possesses an apical complex which enables it to disrupt and traverse the midgut epithelium, before settling down between epithelium and basal lamina. Parasite tetraploidy persists throughout the ookinete stage until sporozoite budding in the oocyst restores the haploid state [102].

## 7. Concluding Remarks

Despite intense work on the sexual stages of malaria parasites, they represent the least understood stages of the

parasite's life cycle. Gametocyte differentiation and gametogenesis have mostly been studied in the human malaria pathogen *P. falciparum*, and for these sexual stages, a variety of proteins have been identified and characterized. On the other hand, the implication of malaria sexual stage proteins for malaria transmission was preferentially investigated in the murine *P. berghei* model, which is more easily accessible for genetic manipulations and transmission studies. Up to date it is challenging to combine information gained by both systems to receive the big picture on the malaria sexual phase. We expect that in the near future research on the sexual stages of malaria parasites will be dominated by two major tasks: (i) the big hunt for "the gene", which enables the blood stage parasite to enter the sexual pathway and (ii) the analysis of the molecular mechanisms and signaling events of sexual stage parasites during fertilization in the mosquito midgut.

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