Microreview

The development of malaria parasites in the mosquito midgut

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Summary

The mosquito midgut stages of malaria parasites are crucial for establishing an infection in the insect vector and to thus ensure further spread of the pathogen. Parasite development in the midgut starts with the activation of the intraerythrocytic gametocytes immediately after take-up and ends with traversal of the midgut epithelium by the invasive oocinetes less than 24 h later. During this time period, the plasmodia undergo two processes of stage conversion, from gametocytes to gametes and from zygotes to oocinetes, both accompanied by dramatic morphological changes. Further, gamete formation requires parasite egress from the enveloping erythrocytes, rendering them vulnerable to the aggressive factors of the insect gut, like components of the human blood meal. The mosquito midgut stages of malaria parasites are unprecedented objects to study a variety of cell biological aspects, including signal perception, cell conversion, parasite/host co-adaptation and immune evasion. This review highlights recent insights into the molecules involved in gametocyte activation and gamete formation as well as in zygote-to-oocinete conversion and oocinete midgut exit; it further discusses factors that can harm the extracellular midgut stages as well as the measures of the parasites to protect themselves from any damage.

Introduction

Malaria, a vector-borne blood disease caused by protozoan parasites of the genus Plasmodium, results in 214 million infections and claims 438 000 deaths every year. Children are particularly susceptible to malaria, and in 2015, an estimated 306 000 children under 5 years of age were killed, mostly in the African region (WHO World Malaria Report, 2015). Once transmitted to the human by a blood-feeding female Anopheles mosquito, the parasites initially multiply in the human liver, before they progress to the pathologic blood stages. These blood infections can last for months, and only once sexual precursor cells, the gametocytes, have matured, the malaria parasites are able to leave the human host and to continue the life-cycle in the insect vector.

In the mosquito midgut, the parasites are able to differentiate into their sexual forms, the female macrogametes and male microgametes, and to then undergo sexual reproduction in order to newly combine their chromosomal sets. The midgut phase lasts for approximately 20 h and includes two phases of stage conversion, the rapid conversion of gametocytes into fertile gametes upon activation and the conversion of zygotes into the motile and invasive oocinetes that once formed, immediately exit the gut lumen by traversing the midgut epithelial cell layer. Subsequently, the oocinetes settle down at the basal site of the midgut epithelium and convert to sessile oocysts, in which sporogonic replication takes place. This replication phase requires roughly 2 weeks and results in the formation of infective sporozoites that migrate to the salivary glands to be released into the human dermis with the next bite of the mosquito, wherewith the life-cycle of Plasmodium is completed (reviewed in Aly et al., 2009; Ghosh and Jacobs-Lorena, 2009; Kuehn and Pradel, 2010; Ménard et al., 2013).

The plasmodial midgut stages are attractive objects to study a variety of cell biological aspects, including cell conversion, signal perception, parasite/host co-adaptation and immune evasion. Boosted by technical advances in parasite genetics and live-cell imaging and complemented with data gained by transcriptomics and proteomics, in recent years, novel insights into the mosquito midgut phase of the malaria parasite were obtained. Two models were used in the majority of these studies, the rodent malaria parasite Plasmodium berghei and the in vitro cultivable human parasite Plasmodium falciparum.

This review summarizes the recent findings on the mosquito midgut stages of Plasmodium. In this context,
three consequent steps are considered, the signalling cascade leading to gametogenesis, the formation of gametes and the zygote-to-ookinete conversion. A fourth aspect deals with the interplay between parasites and midgut factors, including the gut microbiota and epithelium, components of the human blood meal and insect immune molecules.

The initiation of gametogenesis

Sexual precursor cells, the intraerythrocytic gametocytes, develop in the human blood in response to stress factors (reviewed in Pradel, 2007; Kuehn and Pradel, 2010). A time period of about 10 days is required for gametocyte development in *P. falciparum*, during which they pass five morphological stages, termed stages I–V. Once the mature stage V gametocytes are ingested with the blood meal of an *Anopheles* mosquito, they are activated in the mosquito midgut by environmental stimuli, and gametogenesis is initiated (Fig. 1). Signals inducing gamete formation include a drop of temperature by approximately 5 °C, which is mandatory for gametocyte activation, and the presence of the mosquito-derived molecule xanthurenic acid (XA), a metabolic intermediate of the tryptophan catabolism. An additional trigger of gametogenesis is the increase of extracellular pH from 7.2 to about 8 (Kawamoto *et al.*, 1991; Billker *et al.*, 1997, 1998; Garcia *et al.*, 1998; Sologub *et al.*, 2011).

Up to now, a plasmodial receptor that binds XA has not been identified. XA, however, was shown to trigger guanylyl cyclase activity in gametocyte membranes (Muhia *et al.*, 2001). In *P. falciparum*, two integral guanylyl cyclases have been identified, GCα and GCβ (Carucci *et al.*, 2000). While GCα was refractory to deletion in both *P. falciparum* and *P. berghei*, pointing to an essential role during erythrocytic replication, the gene coding for GCβ was successfully disrupted in both species. Parasites lacking GCβ were not impaired in gametogenesis, while in

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**Fig. 1.** The signalling pathways involved in gametogenesis. Ca²⁺, 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; IP₃, inositol triphosphate; Map-2, Mitogen-activated protein kinase 2; mRNA, messenger ribonucleic acid; PDE, phosphodiesterase; PI, phosphatidylinositol 4-phosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP₃, phosphatidylinositol 3-phosphate; PIP₅K, phosphatidylinositol 4-phosphate 5-kinase; PI-PLC, phosphoinositide-specific phospholipase C; PKG, cGMP-dependent protein kinase; PPM1, metallo-dependent protein phosphatase 1; PuF₂, Pumilio/FFB (fer-3 binding factor) family protein 2; PV, parasitophorous vacuole; PVM, parasitophorous vacuole membrane; R, receptor; T, temperature; XA, xanthurenic acid.
P. berghei revealed a severe defect in ookinete motility (Hirai et al., 2006; Taylor et al., 2008), indicating that GCₐ, not GCβ, is important for gametocyte activation.

Guanylyl cyclase activation leads to the synthesis of the second messenger cyclic GMP (cGMP), which was found to be dependent on the presence of either Mg²⁺ or Mn²⁺. Ca²⁺, on the other hand, was shown to inhibit guanylyl cyclase activity (Muhia et al., 2001). In eukaryotic cells, the intracellular cGMP concentrations are generally regulated by cGMP-synthesizing guanylyl cyclases and cGMP-hydrolyzing phosphodiesterases (PDE). The genome of P. falciparum encodes for four putative cyclic nucleotide PDEs, PDEα–β (Young et al., 2005). Chemical inhibition of PDE activity stimulates rounding up of mature gametocytes in the absence of XA, thereby verifying that increased cGMP-levels trigger gametogenesis (McRobert et al., 2008) (Fig. 1). Interestingly, deletion of the gene coding for PDEβ leads to an impaired ability of P. falciparum to undergo gametogenesis, indicating that the tight regulation of intracellular cGMP concentrations is crucial for gametocyte activation and that premature, abnormally high cGMP levels have a deleterious effect on this process (Taylor et al., 2008).

Downstream signalling of gametogenesis involves activation of the cGMP-dependent protein kinase (PKG) by cGMP. Chemical inhibition of PKG-activity arrests gametogenesis of P. falciparum in a dose-dependent manner, whereas mutant strains expressing an inhibitor-insensitive PKG are not affected by inhibitor treatment (McRobert et al., 2008). Because rounding-up of gametocytes is a Ca²⁺-independent step and cannot be inhibited by chelators, PKG acts prior to Ca²⁺ increase in the activated gametocytes (Fig. 1).

Increase in cytosolic Ca²⁺ occurs approximately 10 s after XA-mediated activation, as shown for P. berghei gametocytes (Bilker et al., 2004), resulting in male gametogenesis. Ca²⁺ mobilization is linked to the phosphoinositide-specific phospholipase C (PI-PLC). Stimulation of PI-PLC upon gametocyte activation by XA leads to the hydrolysis of phosphatidylinositol-(4,5)-bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol-(1,4,5)-trisphosphate (IP₃) (Martin et al., 1994; Raabe et al., 2011) (Fig. 1). IP₃ is suggested to be responsible for the opening of Ca²⁺-channels in the endoplasmic reticulum, although no orthologue of an IP₃ receptor channel has been identified in Plasmodium yet. PI-PLC activity itself appears to be regulated by Ca²⁺, because it can be impaired by Ca²⁺-ionophores, pointing to a Ca²⁺-regulated feedback mechanism.

Evidence suggests that the Ca²⁺ levels are further regulated by PKG. The enzyme controls the synthesis of phosphatidylinositol 4-phosphate (PI4P) from phosphatidyl-1D-myoinositol (PI) and the following conversion to PIP₂ via phosphorylation of the phosphoinositol kinases involved in these steps, phosphatidylinositol 4-kinase (PI4K) and phosphatidylinositol 4-phosphate 5-kinase (PIP5K) (Brochet et al., 2014). PIP5K activity is further controlled by a small G protein of the ADP-ribosylation factor (ARF1) family (Leber et al., 2009).

The increase of intracellular Ca²⁺ during induction of gametogenesis is sensed by specific Ca²⁺-dependent protein kinases, the CDPKs. In female macrogametocytes, CDPK1 initiates the release of the translational repression of messenger RNAs (mRNAs) (Sebastian et al., 2012). Translational repression in macrogametocytes has been linked to components of regulatory ribonucleoprotein complexes. In P. berghei, a total of 731 mRNAs associate with 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 transcript repression, including mRNAs encoding for the GPI-anchored female-specific protein P25 and the glideosome-associated proteins GAP45 and 50 (Mair et al., 2006, 2010; Guerreiro et al., 2014). For P. falciparum, translational repression of mRNAs was shown to involve the Pumilio/FBF (Puf) family RNA-binding protein Puf2. Puf2 deletion leads to increased mRNA and protein levels in gametocytes, whereas overexpression of Puf2 further reduces their mRNA and protein levels (Miao et al., 2013). The repressed mRNAs mostly encode for proteins important for zygote-to-ookinete conversion (Table 1).

Ca²⁺ is also important for microgametogenesis, because CDPK1 knock down leads to a delayed exflagellation in P. berghei (Sebastian et al., 2012). Another Ca²⁺ effector in male parasites is CDPK4, and P. berghei parasites lacking CDPK4 are not able to undergo DNA synthesis, which is a prerequisite for the following three mitotic divisions that result in the formation of eight male gametes (Bilker et al., 2004). Chemical inhibition of CDPK4 was shown to inhibit egress of P. falciparum microgametes in vitro and transmission of P. berghei to the mosquito in vivo (Ojo et al., 2012, 2014) (Table 1).

Further, a plasmodial FACT (facilitates chromatin transcription) protein (termed FACT-L) is linked to DNA replication in the males (Laurentino et al., 2011). Downstream of these events, the loss of mitogen-activated protein kinase Map-2 and cell division cycle protein CDC20 interferes with chromatin condensation, axoneme motility and cytokinesis during the formation of the male flagellar microgametes (Rangarajan et al., 2005; Tewari et al., 2005; Guttery et al., 2012a). Also, depletion of the metallo-dependent protein phosphatase PPM1 in P. berghei results in impaired exflagellation (Guttery et al., 2014), while depletion of the Ca²⁺-dependent phosphatase Calcineurin affects male gametogenesis and subsequent fertilization in the rodent malaria parasite (Philip and Waters, 2015) (Fig. 1).
### Table 1. Summary of published data on proteins involved in malaria parasite development in the mosquito midgut

| Protein | Function | Species | Gender* | Reference |
|---------|----------|---------|---------|-----------|
| Actin 2 | Male gametogenesis; ookinete formation | *P. berghei* | M | Deligianni et al., 2011; Andreadaki et al., 2014 |
| Calcinurin | Male gametogenesis; fertilization | *P. berghei* | M | Philipp and Waters, 2015 |
| CDC20 | Essential in ABS; *P. berghei* ookinete development; release of translational repression of mRNAs (F); exflagellation (M) | *P. berghei* | | Guttery et al., 2012a |
| CDPK1 | Ookinete motility; mosquito midgut invasion | *P. berghei* | | Sebastian et al., 2012 |
| CDPK3 | *P. falciparum* microgamete egress; *P. berghei* DNA synthesis; transmission to the mosquito | | | Ishino et al., 2006; Siden-Kiamos et al., 2006 |
| CDPK4 | *P. falciparum* ookinete motility; mosquito midgut invasion | *P. berghei* | M | Billker et al., 2004; Ojo et al., 2012, 2014 |
| CeiTOS | Ookinete midgut traversal | *P. berghei* | | Kariu et al., 2006 |
| Chitinase | Hydrolysis of peritrophic membrane during ookinete midgut traversal | *P. berghei* | | Vinetz et al., 1999, 2000; Langer et al., 2000; Dessens et al., 2001; Tsai et al., 2001 |
| CITH | Translational repression of mRNAs | *P. berghei* | F | Mair et al., 2006, 2009 |
| CTRP | Ookinete motility | *P. berghei* | | Trottein et al., 1995; Dessens et al., 1999; Yuda et al., 1999; Templeton et al., 2000; Li et al., 2004 |
| DHHC2 | Lipidation modification during zygote-to-ookinete conversion | *P. berghei* | | Santos et al., 2015 |
| DOZI | Translational repression of mRNAs Essential in ABS; DNA replication (M) Phosphate group transfer during ookinete formation | *P. berghei* | F | Mair et al., 2006, 2010 |
| FACT-L | | *P. berghei* | | Laurantino et al., 2011 |
| GAK | | *P. berghei* | | Tewari et al., 2010 |
| GAP45 | Ookinete development Part of the IM; relocalization to the plasmalemma during gametogenesis; receptor of factor H in gametes | *P. berghei* | | Sebastian et al., 2012 |
| GAP50 | | *P. berghei* | | Simon et al., 2013 |
| GCα | Essential in ABS; signalling during GC activation; cGMP synthesis | *P. falciparum* | | Carucci et al., 2000; Muhia et al., 2001 |
| GCβ | Ookinete motility | *P. berghei* | | Hirai et al., 2006 |
| GCS1 (HAP2) | Gamete fusion Osmiophilic body protein; GC egress; PV rupture | *P. berghei* | | Hirai et al., 2008; Liu et al., 2008 |
| GEST | Osmiophilic body protein; GC egress; PV rupture | *P. berghei* | | Talman et al., 2011 |
| IMC1a, b, h | Alveolins important for mechanical strength of ookinete | *P. berghei* | | Volkmann et al., 2012 |
| ISP1 and 3 | Association with apical complex in retorts Microgamete-macrogamete attachment | *P. falciparum* | F | Poulin et al., 2013 |
| LCCL-domain proteins | | *P. berghei* | | Pradel et al., 2004; Simon et al., 2009, 2016 |
| Map-2 | Chromatin condensation; axoneme motility; cytokinesis | *P. berghei* | M | Rangarajan et al., 2005; Tewari et al., 2005 |
| MDV-1/Peg3 | Osmiophilic body protein; GC egress; PV rupture | *P. falciparum* | | Furuya et al., 2005; Silvestrini et al., 2005; Lanfrancotti et al., 2007; Ponzì et al., 2009; Olivieri et al., 2015 |
| Nek-2 and Nek-4 | Genom replication | *P. falciparum* | Nek-4: F | Reisinger et al., 2005, 2009 |
| P25 | Ookinete surface protein; ookinete survival in the mosquito midgut, traversal of the epithelium; ookinete–oocyst transformation | *P. berghei* | F | Tomas et al., 2001 |
| P28 | Ookinete surface protein; ookinete survival in the mosquito midgut, traversal of the epithelium; ookinete–oocyst transformation | *P. berghei* | F | Tomas et al., 2001 |
| PDEδ | Hydrolysis of cGMP; rounding up of activated GC | *P. falciparum* | | McRobert et al., 2008 |
| PF16 | Axoneme motility | *P. berghei* | | Straschi et al., 2010 |
| Pg377 | Osmiophilic body protein; GC egress | *P. falciparum* | F (P. f.) | Alano et al., 1995; Severini et al., 1999; Olivieri et al., 2015 |
| PI4K | Essential in ABS; phosphorylation of PI during GC activation | *P. berghei* | | Brochet et al., 2014 |
| PIP5K | *P. berghei* essential in ABS; phosphorylation of PI4P during GC activation | *P. berghei* | | Leber et al., 2009; Brochet et al., 2014 |
| PI-PLC | PIP2 degradation into DAG and IP3 during GC activation | *P. berghei* | | Martin et al., 1994; Raabe et al., 2011 |
| PK7 | | *P. berghei* | | Tewari et al., 2010 |
Table 1. (continued)

| Protein    | Function                                                                 | Species      | Gender* | Reference                        |
|------------|--------------------------------------------------------------------------|--------------|---------|----------------------------------|
| PKG        | Gametogenesis; regulation of Ca\(^{2+}\) levels; P. berghei: ookinite gliding | P. falciparum |         | McRobert et al., 2008; Moon et al., 2009; |
| POS1-10    | Ookinite surface protein                                                  | P. berghei   |         | Brochet et al., 2014            |
| PPKL       | Ookinite development and motility                                         | P. berghei   | F       | Kaneko et al., 2015             |
| PPLP2      | GC egress; EM perforation                                                | P. falciparum |         | Guttery et al., 2012b           |
| PPLP3-5    | Ookinite midgut traversal                                                | P. falciparum | PPLP4: F| Kadota et al., 2004; Ecker et al., 2007, 2008; Kaneko et al., 2015; Wirth et al., 2015 |
| PPM1       | Exflagellation                                                           | P. berghei   |         | Guttery et al., 2014            |
| PPM2       | Ookinite development                                                     | P. berghei   |         | Guttery et al., 2014            |
| Ps230      | Rossetting; micromegakine-macrogamete attachment                         | P. falciparum |         | Eksi et al., 2006               |
| Ps47       | Microgamete-macrogamete attachment; evasion of TEP1-related killing in the mosquito midgut | P. falciparum |         | van Schaijk et al., 2006; Molina-Cruz et al., 2013 |
| Ps48/45    | Microgamete-macrogamete attachment                                        | P. falciparum |         | van Dijk et al., 2001           |
| PSOP1, 2, 6, 7, 12 | Ookinite-secreted proteins                                           | P. berghei   |         | Kaneko et al., 2015             |
| Pufr2      | Translational repression of mRNAs                                        | P. falciparum |         | Miao et al., 2013               |
| SAS-6      | Flagellum formation                                                      | P. berghei   | M       | Marques et al., 2015            |
| SHLP1      | Ookinite development                                                     | P. berghei   | F       | Patzweitz et al., 2013           |
| SOAP       | Ookinite midgut traversal                                                | P. berghei   |         | Hessens et al., 2003            |
| WARP       | Ookinite midgut traversal                                                | P. berghei   |         | Yuda et al., 2001; Li et al., 2004 |

*Proteins specifically expressed in male (M) or female (F) sexual stages. ABS, asexual blood stages; Ca\(^{2+}\), calcium ion; CDC20, cell division cycle protein 20; CDPK, calcium-dependent protein kinase; CelTOS, cell-traversal protein for ookinetes and sporozoites; cGMP, cyclic guanosine monophosphate; CITH, homolog of worm CAR-1 and fly Trailor Hitch; CTRP, circumsporozoite and thrombospondin-related adhesive protein-related protein; DAG, diacylglycerol; DHHC2, palmitoyl-S-acetyl-transferase; DJ2I, development of zygote inhibited; EM, erythrocyte membrane; FACT-L, facilitates chromatins transcription protein L; GAK, cyclin-G-associated kinase; GAP, glideosome-associated protein; GC, gametocyte; GCo/β, guanylyl cyclase; GCS1/HAP2, generative cell specific 1; GEST, gamete egress and sporozoite traversal; IMC, inner membrane complex; IP3, inositol triphosphate; ISP, IMC sub-compartment proteins; LCLL, Limulus coagulation factor C; Map-2, Mitogen-activated protein kinase 2; MDV-1/Peg3, male development-1/protein of early gametocyte 3; Nek, NIMA-related kinase; PDE, phosphodiesterase; P. f., Plasmodium falciparum; PI, phosphatidylinositol; PI4K, phosphatidylinositol 4-kinase; PI4P, phosphatidylinositol 4-phosphate; PI5P, phosphatidylinositol 4,5-bisphosphate; PIPL, phosphatidylinositol 4-phosphate 5-kinase; PIP2, phosphatidylinositol bisphosphate; PK7, protein kinase 7; PKG, cGMP-dependent protein kinase; POS, putative ookinite surface-associated protein; PPKL, kelch-like domain-containing phosphatase; PPLP, plasmodial perforin-like protein; PPM, metallo-dependent protein phosphatase; PSOP, putative secreted ookinite protein; Pufr2, Pumilio/PBF (em-3 binding factor) family protein 2; PV, parasitophorous vacuole; SAS-6; centrosome/basal body protein; SHLP1; Shewanella-like protein phosphatase 1; SOAP, secreted ookinite adhesive protein; TEP1, theoesther-containing protein 1; WARP, von Willebrand factor A domain-related protein.

The formation of gametocytes

The activation of gametocytes results in a first rapid step of stage conversion, during which the activated gametocytes round up, egress from the enveloping red blood cells and transform into fertile female macrogametes and male microgametes (Fig. 2). Gametocytes egress from the erythrocyte via an inside-out mode, during which the membrane of the PV (PVM) ruptures prior to the opening of the erythrocyte membrane (EM). PVM rupture occurs at multiple sites and is a Ca\(^{2+}\)-independent event that takes less than 2 min following parasite uptake by the mosquito (Sologub et al., 2011). Electron-dense vesicles, the osmiophilic bodies, can be observed accumulating underneath the rupture sites, which disappear simultaneously with the disintegration of the PVM, while the activated gametocytes are rounding-up (Sologub et al., 2011; reviewed in Wirth and Pradel, 2012).

Osmiophilic bodies have originally been assigned to female gametocytes (Sinden, 1982; Aikawa et al., 1984); in P. berghei, they were meanwhile also reported in the males. However, they appear to be smaller and less abundant than female osmiophilic bodies (Olivieri et al., 2015) (Fig. 2). Female osmiophilic bodies contain gametocyte-specific proteins like Pg377 (Alano et al., 1995; Severini et al., 1999; Olivieri et al., 2015), MDV-1/Peg3 (Furuya et al., 2005; Silvestrini et al., 2005; Lanfranchi et al., 2007; Ponzi et al., 2009) and GEST (gamete egress and sporozoite traversal; Talman et al., 2011), which are discharged into the PV following gametocyte activation. Gametocytes lacking MDV-1/Peg3 or GEST are impaired in egress and stuck within the PV (Ponzi et al., 2009; Talman et al., 2011). Pg377, on the other hand, appears to play a role in egress of P. berghei but not of P. falciparum gametocytes (Suaréz-Cortés et al., 2014; Olivieri et al., 2015) (Table 1).
In a second, Ca^{2+}-dependent step, a second type of vesicles is released into the cytoplasm of the host erythrocyte. These egress vesicles contain the plasmodial pore-forming perforin PPLP2, which is able to perforate the EM. This event occurs approximately 6 min post-activation of the gametocyte and results in the release of the erythrocyte cytoplasm; hence, the gamete is then contained by the EM only (Deligianni et al., 2013; Wirth et al., 2014). Approximately 15 min after uptake by the blood-feeding mosquito, the EM opens and releases the fertile gamete (Sologub et al., 2011; Wirth et al., 2014; reviewed in Wirth and Pradel, 2012). Gametocytes possess an inner membrane complex (IMC) underneath their plasmalemma, which consists of flat membranous sacs, the alveoli, and supporting structural elements and which is thought to be required for the stability of the crescent-shaped parasite stages (Dearley et al., 2012; Kono et al., 2012; Simon et al., 2013; reviewed in Harding and Meissner, 2014; Boucher and Bosch, 2015). During gametogenesis, the IMC disintegrates, resulting in gametes solely confined by the plasmalemma. Hitherto, the fate of the alveoli during breakdown is unknown; noteworthy, the isochronal formation of nanotubes in the developing gametes was

![Diagram](image-url)

**Fig. 2.** Molecules involved in malaria parasite development in the mosquito midgut. Ca^{2+}, calcium ion; CCp-MCP, CCp-based multiprotein complex; CelTOS, cell-traversal protein for ookinetes and sporozoites; CTL4, C-type lectin 4; CTRP, circumsporozoite and thrombospondin-related adhesive protein-related protein; DAG, diacylglycerol; EM, erythrocyte membrane; FBN9/39, fibrinogen immunolectin 9/39; GAP50, glideosome-associated protein 50; GCS1/HAP2, generative cell specific 1; GEST, gamete egress and sporozoite traversal; IP_{3}, inositol triphosphate; LRRDD7, leucine-rich-repeat domain-containing protein 7; MDV-1, male development-1; Peg3/4, protein of early gametocyte 3/4; PIP_{2}, phosphatidylinositol-4,5-bisphosphate; PPLP2/3/4/5, Plasmodium perforin-like protein 2/3/4/5; Plasmodium porin-like protein 2/3/4/5; POS1-10, putative ookinete surface-associated protein 1-10; PSOP1/2/6/7, putative secreted ookinete protein 1/2/6/7; PVP, parasitophorous vacuole; SOAP, secreted ookinete adhesive protein; TCC, terminal complement complex; TEP1, thioether-containing protein 1; WARP, von Willebrand factor A domain-related protein; XA, xanthurenic acid.

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previously reported. Nanotubes are long membranous tubules that originate from the gamete surface and represent long-distance cell-to-cell connections proposed to facilitate contact between gametes as a pre-requisite for mating (Rupp et al., 2011). While the question of the origin of the membranous tubules remains, one explanation might be that the alveolar membranes are reused for nanotube formation.

The activated microgametocyte replicates its genome three times, progressing from haploid to octaploid (Janse et al., 1986, 1988) and mitotically produces eight flagellar microgametes in a process termed exflagellation (Fig. 2). Flagellum formation requires axonemal assembly from basal bodies, which involves the centriole/basal body protein SAS-6 (Marques et al., 2015). Axoneme motility, on the other hand, is regulated by the conserved Armadillo-repeat protein PF16, and *P. berghei* parasites lacking this protein show abnormal flagellar movements (Straschil et al., 2010). Exflagellating microgametes typically adhere avidly to neighbouring erythrocytes and are hidden within such rosettes. In *P. falciparum*, rosetting involves among others the abundantly expressed surface-associated six-cys motif protein Ps230 (Eksi et al., 2006), as well as sialic acid and glycoporphs of the erythrocyte membrane (Templeton et al., 1998).

During exflagellation, the microgamete detaches from the residual body and is freely motile in search of a macrogamete. Several proteins have been linked to microgamete attachment to macrogametes, including the LCCL-domain proteins (termed CCp proteins in *P. falciparum*) and the six-cys motif proteins Ps47, Ps48/45 and Ps230 (van Dijk et al., 2001; Pradel et al., 2004; van Schaijk et al., 2006; Simon et al., 2009, 2016). The latter two are considered as promising candidates for transmission blocking vaccines (reviewed in Pradel, 2007). It has recently been shown that in *P. falciparum* Ps230, Ps48/45 and the six CCp proteins assemble to multi-protein complexes, which are linked via P25 to the macrogamete surface (Simon et al., 2009, 2016; reviewed in Kuehn et al., 2010).

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 protein GCS1 (generative cell specific 1; also termed HAP2), and disruption of the respective gene in *P. berghei* resulted in male sterility and blocked fertilization (Hirai et al., 2008; Liu et al., 2008). Following gamete fusion, nuclear fusion ensues, and over the next 3 h, meiosis occurs, and the zygote becomes tetraploid (Janse et al., 1986), a process involving the NIMA-related kinases Nek-2 and Nek-4 (Reininger et al., 2005, 2009). Parasite tetraploidy persists throughout the oocyst stage until sporozoite budding in the oocyst restores the haploid state (Janse et al., 1986) (Table 1).

### The zygote-to-oocinete conversion

Development of zygotes and their transformations into oocinetees is accompanied by strict regulations of transcript expression. A *de novo* synthesis study in zygotes identified 91 proteins synthesized only after fertilization, most of which are involved in motility and invasion (Sebastian et al., 2012). A recent cross-fertilization study further showed that the zygote/oocinete stage exhibits a maternal phenotype either from maternal mRNA inheritance or transcription of the maternal alleles, while the respective paternal alleles are silenced in these stages (Ukegbu et al., 2015). Gene expression is further regulated by the apicomplexan transcription factor AP-O, which associates with more than 500 genes important for oocinete development, motility, midgut penetration and protection against mosquito immunity (Yuda et al., 2009; Kaneko et al., 2015). Among the genes identified are the ones encoding for IMC components like GAP40, 45 and 50, micronemal secretory proteins like the perforins PPLP3-5, the putative oocinete-secreted proteins PSOP1, 2, 6, 7 and 12, the secreted oocinete adhesive protein SOAP, the von Willebrand factor A domain-related protein WARP and for oocinete surface-associated proteins like POS1-10 or P25 and P28 (Kaneko et al., 2015). P25 and P28 are targets of highly effective transmission-blocking antibodies, which interfere with oocyst development. The proteins exhibit multiple and partially redundant functions, for example, they play a role in oocinete survival in the mosquito midgut, traversal of the epithelium and oocinete–oocyst transformation (Tomas et al., 2001; reviewed in Pradel, 2007).

During the transformation of zygotes into the invasive oocinetees, intermediate stages, the retorts, develop, and here, the apical complex as well as the IMC is newly formed. When the ubiquitously expressed kinase CDPK1 is downregulated in the midgut stages of *P. berghei*, these arrest in the zygote stage and lack IMC components and micronemal proteins like SOAP and the thrombospondin-related adhesive protein-related protein CTRP. Similarly, downregulation of GAP45 expression in the midgut stages resulted in zygotes unable to transform into oocinetees (Sebastian et al., 2012). Two IMC sub-compartment proteins, ISP1 and 3, associate with the forming apical complex in *P. berghei* retorts, which are N-myristoylated, phosphorylated and membrane-bound (Poulin et al., 2013). Indeed, lipidation modifications appear to be important in general during zygote-to-oocinete conversion, and a palmitoyl-S-acyl-transferase DHHC2 is crucial for this process (Santos et al., 2015). Further, the sexual stage-specific actin isoform actin 2, which was originally
reported to play a role in male gametogenesis of *P. berghei*, is additionally important for ookinete formation (Deligianni et al., 2011; Andreadaki et al., 2014). Also, deletion of three ookinete-specific alveolins, IMC1a, b and h, resulted in reduced mechanical strength of this midgut stage (Volkmann et al., 2012) (Table 1).

Besides lipidations, post-transcriptional modifications via phosphate group transfer are important for ookinete formation. *P. berghei* parasites lacking the protein kinase PK7 or the cyclin G-associated kinase GAK show severely reduced ookinete numbers (Tewari et al., 2010). Further, deletion of the Shewanella-like protein phosphatase (SHLP1), PPM2 or the kelch-like domain-containing phosphatase PPKL results in impaired *P. berghei* ookinete development, and phenotypes include impaired IMC and microneme formation (Guttery et al., 2012b, 2014; Philip et al., 2012; Patzewitz et al., 2013) (Table 1).

The maturation of ookinetes is completed between 19 and 36 h post-blood meal, and the ookinetes then quickly exit the midgut lumen (Aikawa et al., 1984; Sinden et al., 1985; Vlachou et al., 2004). Ookinete motility appears to be regulated by cGMP levels, because disruption of the gene encoding GC in *P. berghei* impaired ookinete gliding (Hirai et al., 2006; Moon et al., 2009). A similar phenotype was observed, when an inhibitor of the cGMP-dependent protein kinase PKG was added to the midgut stages; hence, an essential role of this kinase in ookinete motility is suggested (Moon et al., 2009). Also, the activity of CDPK3 is required for *P. berghei* ookinete motility and engagement with the mosquito midgut epithelium (Ishino et al., 2006; Siden-Kiamos et al., 2006), suggesting that besides cGMP, Ca\(^{2+}\) is important for ookinete motility. In accord with these findings, quantitative phosphoproteomics comparing *P. berghei* ookinetes sensitive and resistant to PKG inhibitors demonstrated that the kinase is involved among others in phosphorylation of IMC components like GAP45 and IMC1b as well as of enzymes involved in the inositol phospholipid metabolism, like phosphoinositol kinases, which in consequence results in the maintenance of high cytosolic Ca\(^{2+}\) levels (Brochet et al., 2014) (Table 1).

An impressive number of micronemal proteins important for midgut traversal were identified in the past (Fig. 2). CTRP, once secreted, inserts into the ookinete surface to form a molecular link between the epithelium and the actin/myosin-motor and hence to mediate motility (Trottein et al., 1995; Dessens et al., 1999; Yuda et al., 1999; Templeton et al., 2000; Li et al., 2004). Furthermore, three of the five plasmodial perforins were assigned to mediating midgut traversal via breaching of the epithelial membranes, that is, PPLP3-5. Other microneme proteins assigned to midgut traversal of *P. berghei* ookinetes include SOAP, WARP and CelTOS (cell-traversal protein for ookinetes and sporozoites) (Yuda et al., 2001; Dessens et al., 2003; Kadota et al., 2004; Li et al., 2004; Kariu et al., 2006; Ecker et al., 2007, 2008; Kaneko et al., 2015; Wirth et al., 2015) (Table 1).

One last challenge that the ookinete has to master before exiting the midgut lumen is the peritrophic membrane. This structure, secreted by the midgut epithelium, forms within 1–2 days post-blood meal. It consists of chitin and cross-linked proteins and functions in protecting the midgut from food particles as well as microbial infections (reviewed in Lehane, 1997). In order to breach through the peritrophic membrane before it has fully matured, the ookinetes secrete a chitinase able to hydrolyse the polysaccharide (Vinetz et al., 1999, 2000; Langer et al., 2000) (Table 1). Lack of this enzyme leads to reduced ookinete midgut infection (Dessens et al., 2001; Tsai et al., 2001).

### The effect of midgut factors on parasite development

The mosquito midgut represents a major bottleneck during the life-cycle of *Plasmodium*. Here, the midgut stages have to persevere for more than 20 h outside a protective host cell and have to defend themselves against factors of the blood meal like components of the human immune system, the natural midgut microbial flora and the innate immune system of the mosquito (Fig. 2). This exposure leads to an approximate 300-fold loss of parasite abundance during the transmission to the mosquito (Vaughan et al., 1994).

After the blood meal, human complement is active in the mosquito midgut for 1 h and during this period represents a severe threat for the emerging gametes (Simon et al., 2013). The gametes, though, are able to evade human complement by binding the complement regulatory protein factor H from the blood meal, in consequence inactivating complement factor C3b and thus preventing complement-induced lysis. Interestingly, a new study showed that factor H is also captured by the *Anopheles* midgut epithelium to inactivate the human complement cascade that would be able to harm the mosquito (Khattab et al., 2015).

Interestingly, the glideosome-associated protein GAP50 was identified as a factor H-binding receptor of gametes. GAP50, a transmembrane protein, is originally present in the outer alveolar membrane of gametocytes but relocates to the plasmalemma during gametogenesis. During relocation, the N-terminal part of the protein, which originally extends into the alveolar lumen, becomes extracellularly exposed and is then able to highjack factor H from the blood meal (Simon et al., 2013). It is noticeable that after EM perforation by PPLP2, the forming gametes remain sheltered by the EM for about 10 min before they eventually egress from the host cell, and this prolonged stay might represent a strategy of the parasite to transform into fertile gametes unscathed.
Other midgut factors leading to parasite killing are mosquito-derived immune response molecules targeting the ingested *Plasmodium* parasites. Their activation is triggered by the recognition of pathogen-associated molecular patterns (PAMPs) present on the pathogen surfaces like the ones of midgut bacteria or malaria midgut stages via pattern recognition receptors (PRRs). This process leads to the upregulation of different effector molecules from diverse gene families, among others encoding for leucine rich-repeat (LRR) domain-containing proteins, fibrinogen-related proteins (FREPs) and C-type lectins (CTLs) as identified by microarray analyses and RNAi-based studies (Christophides et al., 2002; Dimopoulos et al., 2002; Osta et al., 2004; reviewed in Cirimotich et al., 2010).

Microarray analyses identified several mosquito-derived molecules like the thioester-containing protein 1 (TEP1), the fibrinogen immunolectin 9 (FBN9), the fibrinogen immunolecctin 39 (FBN39), the Leucine rich-repeat domain-containing protein 7 (LRRDD7) and the C-type lectin 4 (CTL4) that exhibit anti-plasmodial and antibacterial activity (Dong et al., 2006). This suggests that the mosquito uses besides the direct anti-plasmodial immune response also the anti-bacterial immune response, initiated through increased multiplication of bacteria after the blood meal, to fight against *Plasmodium* infection, which makes the bacterial midgut flora an important regulator of the permissiveness of *Anopheles* to *Plasmodium* parasites (Dong et al., 2006, 2009).

Several studies showed that the malaria vector *Anopheles gambiae* defends itself against invading *Plasmodium* parasites by activating a complement-like pathway with the complement C3-like protein TEP1, which is responsible for the killing and clearance through lysis and melanization of *Plasmodium* ookinetes that traverse the midgut epithelium (Blandin et al., 2004; Fraiture et al., 2009; Povelones et al., 2011). TEP1 is secreted into the hemolymph by the phagocytic hemocytes and knockdown of TEP1 leads to a fivefold increase of developing oocysts. For the binding of TEP1 to the parasite surface, two members of the LRR protein family, LRIM1 and APL1C, are required, which form a disulfide-bonded complex (Fraiture et al., 2009; Povelones et al., 2011). This complex helps to stabilize and promote the binding of a proteolytically cleaved mature TEP1 (Povelones et al., 2011). Noteworthy, *P. falciparum* is able to evade this TEP1-related killing with the help of the surface-associated Ps47 via suppression of nitration processes involved in the activation of the complement-like pathway in the mosquito (Molina-Cruz et al., 2013).

Because the parasite midgut stages are directly exposed to midgut bacteria, the microbiome composition is of large interest. 16S rRNA analyses using field-caught mosquitoes identified mainly members of the class *Proteobacteria* with species of the genus *Enterobacter*, *Serratia* and *Pantoea* (Cirimotich et al., 2011a; Boissière et al., 2012). Furthermore, in lab-reared *A. gambiae* and *Anopheles stephensi* mosquitoes Gram-negative bacteria belonging to the genus *Elizabethkingia* were identified as the most prominent species, and the prevalence of *Elizabethkingia* is accompanied with the decrease of the microbial diversity during larvae-to-adult conversion (Boissière et al., 2012; Ngwa et al., 2013). A negative correlation between the amount of gut bacteria and *P. falciparum* infection rates was previously shown and removal of the natural midgut flora via antibiotic treatment leads to a higher susceptibility of *A. gambiae* to *P. falciparum* (Pumpuni et al., 1993; Dong et al., 2009; Meister et al., 2009; Cirimotich et al., 2011b). For example, the bacterium *Serratia marcescens* reduces infection by rodent *Plasmodium* oocinetes in field-caught *A. gambiae* (Bando et al., 2013). While no differences in oocinete numbers between mosquitoes harbouring *S. marcescens* and aseptic mosquitoes were observed in the midgut lumen, there were approximately eightfold more oocinete counts within the midgut epithelium of aseptic mosquitoes, indicating that the bacterium influences oocinete invasion of the epithelium.

In some cases, the molecular mechanisms of bacterial defeat of plasmodial intruders were investigated in more detail. The *Enterobacter* isolate *Esp Z* inhibits plasmodial development in the mosquito midgut by producing reactive oxygen species both *in vivo* and *in vitro* (Cirimotich et al., 2011b). Further, the midgut bacterium *Elizabethkingia meningoseptica*, isolated from lab-reared *A. stephensi* mosquitoes, was demonstrated to produce an anti-microbial compound active against *Plasmodium* parasites. Ethyl acetate extracts of this bacterium exhibit anti-plasmodial effects against the asexual blood stages as well as against gametocyte and mosquito midgut stages of *P. falciparum* (Ngwa et al., 2013).

**Conclusions**

The mosquito midgut stages of malaria parasites mark the interface between human host and insect vector. Due to this unique role, they represent optimal objects to study a broad range of biological processes, including stage conversion and parasite/host co-adaptation. As bottleneck stages of the life-cycle, they are also prime targets for transmission-blocking intervention strategies aimed to inhibit spread of the disease by the mosquito. In the past, several players of gametogenesis and zygote-to-okinete conversion have been identified, some of which represent targets for transmission-blocking vaccines or drugs. However, so far, the identified players represent isolated jigsaw pieces and more work is needed to piece together the puzzle in order to get the big picture on malaria parasite development in the mosquito midgut.
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