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The developmental migration of *Plasmodium* in mosquitoes

Dina Vlachou, Timm Schlegelmilch, Ellen Runn, Antonio Mendes and Fotis C Kafatos

Migration of the protozoan parasite *Plasmodium* through the mosquito is a complex and delicate process, the outcome of which determines the success of malaria transmission. The mosquito is not simply the vector of *Plasmodium* but, in terms of the life cycle, its definitive host: there, the parasite undergoes its sexual development, which results in colonization of the mosquito salivary glands. Two of the parasite's developmental stages in the mosquito, the ookinete and the sporozoite, are invasive and depend on gliding motility to access, penetrate and traverse their host cells. Recent advances in the field have included the identification of numerous *Plasmodium* molecules that are essential for parasite migration in the mosquito vector.

Addresses
Division of Cell and Molecular Biology, Faculty of Natural Sciences, Imperial College London, London, SW7 2AZ, UK

Corresponding author: Vlachou, Dina (d.vlachou@imperial.ac.uk)

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Introduction

Malaria is a mosquito-borne parasitic disease that threatens nearly half the global population. It is caused by protozoan apicomplexan parasites of the genus *Plasmodium*; four species cause malaria in man and are transmitted by *Anopheles* mosquitoes. The parasites must complete their sexual development within the mosquito before they can infect the vertebrate hosts. An increasing number of *Plasmodium* genes that play important roles in the interaction with the mosquito have been identified. This quest has been aided by the development of methods to genetically manipulate the parasite [1,2] and by the genome sequencing of the human parasite *Plasmodium falciparum* [3,4] and other model parasites, including the rodent parasite *Plasmodium berghei* [5]. Similarly, the genome sequencing of *Anopheles gambiae* [6], the most important vector of human malaria in Africa, and the establishment of methods for stable transgenesis [7,8] and reverse genetic analysis [9] have facilitated the discovery of genes with important roles in the *Anopheles*–*Plasmodium* interplay.

In this review, we examine the complex interaction between *Plasmodium* and its mosquito host, emphasizing parasite molecules that are essential for penetration of mosquito epithelia.

Gametogenesis: the switch to the parasite sexual life

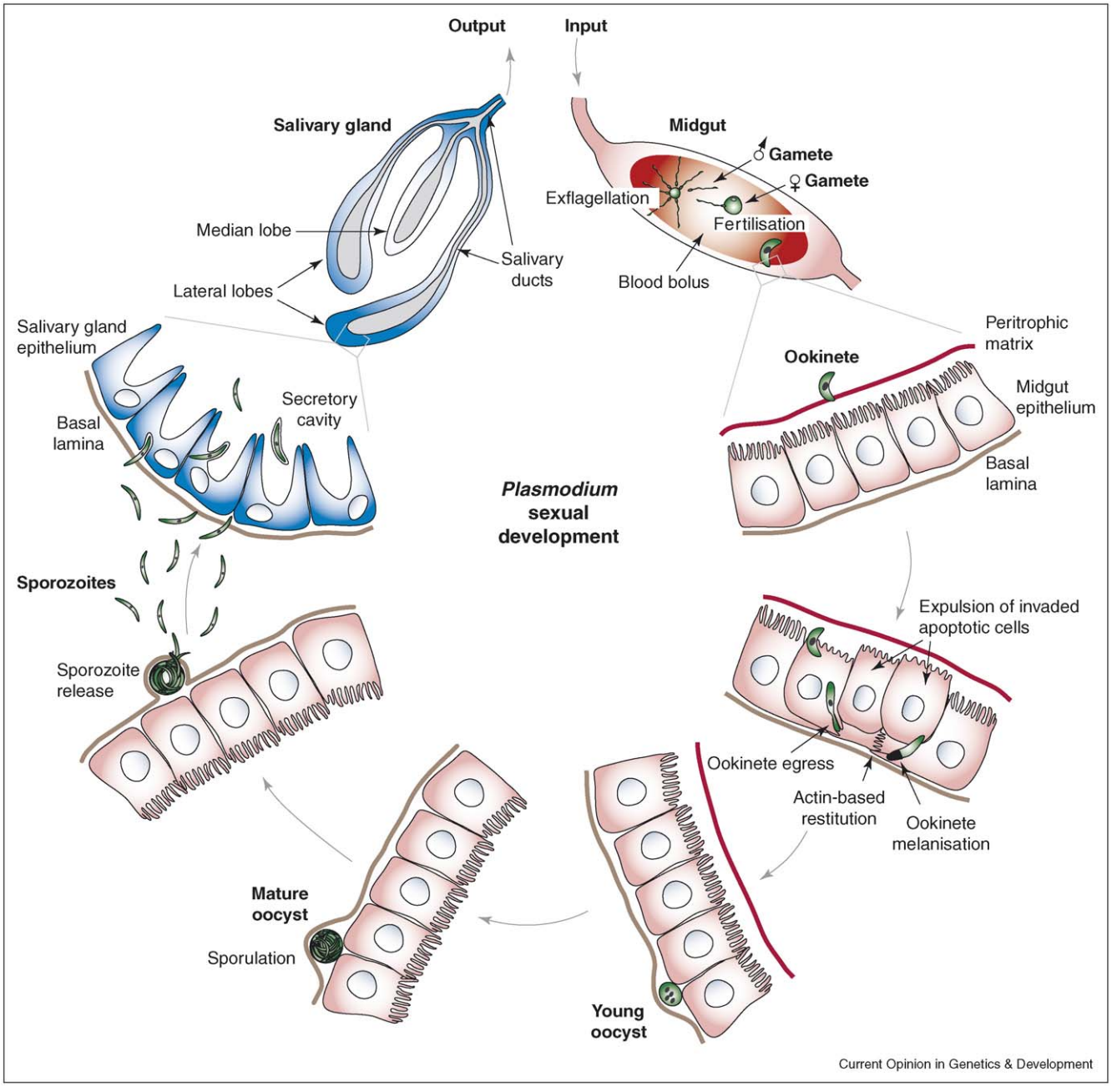
The parasite journey in the mosquito begins with the ingestion of infected blood (Figure 1). In the mosquito midgut lumen, *Plasmodium* female and male gametocytes mature into gametes after exposure to environmental and mosquito-specific factors, which can vary for different *Plasmodium* species. These include a drop in temperature by 5 °C, an increase in the pH, and exposure to xanthurenic acid [10]. A signal transduction cascade results in the release of calcium in the cytoplasm of the gametocyte (the sexual reproductive stage of the parasite), causing them to begin development and to emerge from the red blood cells. A calcium-dependent protein kinase, CDPK4, is activated in male gametocytes and regulates exflagellation and generation of eight motile gametes [11]. A mitogen-activated kinase, Pbmap2, is essential for the release of the flagellated gametes [12,13]. Peroxiredoxins, which constitute a ubiquitous family of antioxidant enzymes, are also involved in gametocyte development [14].

The ookinete-to-oocyst developmental transition and its perils

Soon after gamete fusion, the zygote, which is still in the mosquito midgut lumen, matures into a motile ookinete (Figure 1). This invasive parasite stage is able to penetrate the mosquito midgut epithelium a day after the blood meal (Figure 2a) and, upon arrival at the basal side, begins to transform into the sessile oocyst. During ookinete maturation and transition to the oocyst, the parasite undergoes meiosis, but this is not immediately followed by karyokinesis and cell division. A protein kinase, NEK4 (NIMA [never in mitosis *Aspergillus*]-related kinase 4), is essential for genome replication during meiosis [12,15].

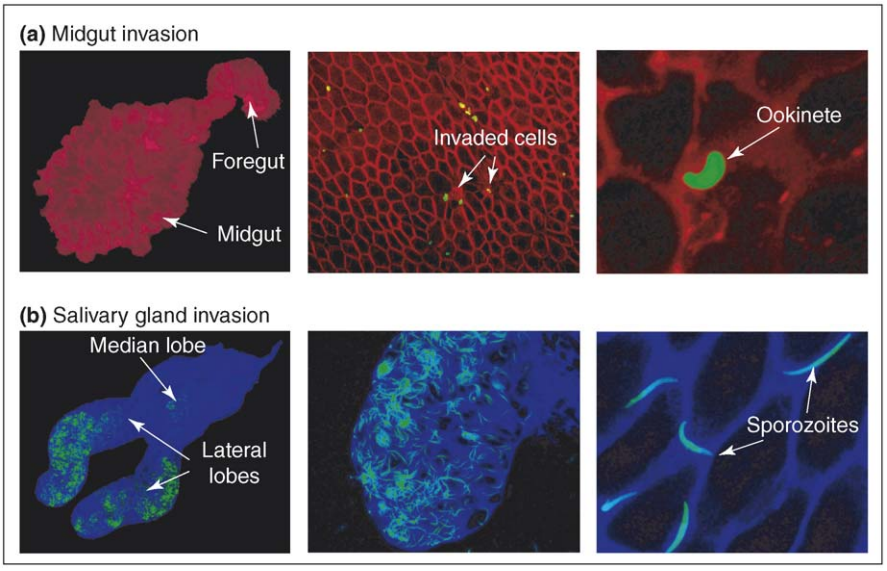
The microneme is an apical ookinete organelle implicated in host cell recognition and attachment, but also in gliding motility. It secretes proteins, many of which are associated with its surface and which, by interacting with immobile mosquito ligands and translocating to the rear end of the parasite, cause a 'grab-and-push'-style, forward parasite motion [16]. Using fluorescent *P. berghei* and real-time *in vivo* imaging, we recently described three types of ookinete movement and their relation with midgut invasion [17•]. A stationary rotation mostly occurs in the

Figure 1



Plasmodium migration in the mosquito host. The developmental lifecycle of *Plasmodium* in the mosquito starts with a female mosquito bite and blood meal on a malaria-infected vertebrate host; it ends with a new bite approximately three weeks later. Soon after blood feeding, ingested parasite gametocytes produce male and female gametes that fertilise and form the zygotes. Still in the gut lumen, the zygotes transform into motile ookinetes. Approximately one day after blood feeding, the ookinetes traverse the midgut epithelium; the invaded epithelial cells become apoptotic and are extruded from the midgut epithelium. Mosquito immune reactions result in processes, such as lysis and melanisation, mainly mediated by proteins that are present in the mosquito hemolymph. These processes drastically reduce the number of ookinetes that successfully develop to the next parasite stage, the oocyst, on the basal side of the epithelium. Approximately two weeks later — this length of time depends on the parasite–mosquito species combination — multiple nuclear divisions within each oocyst are followed by membrane partitioning and budding off of several thousand haploid sporozoites. Upon oocyst burst, this army of sporozoites is released into the haemocoel; many reach and infect the median and the distal lateral salivary gland lobes. Invasion of the salivary gland epithelial cells is thought to occur through the formation of a parasitophorous vacuole, following the interaction of sporozoites with the basal lamina and invagination of the host cell plasmalemma. A similar parasitophorous vacuole is formed around the sporozoites during their escape from the salivary glad cells into the secretory cavity of the glands. The final act of the parasites in the mosquito is the breakdown of this second vacuole and migration of sporozoites into the salivary ducts, from where they are ejected into a new vertebrate host upon the next mosquito bite.

Figure 2



Invasion of mosquito epithelia by *Plasmodium* parasite stages. Transgenic *P. berghei* parasites expressing green fluorescent protein (green) [17•,71] are used for live *in vivo* confocal imaging of infected mosquito midgut **(a)** and salivary gland **(b)** tissues. The epithelial cell membranes are stained with a lipophilic vital dye (red and blue colours) as described [17•]. The magnification is increasing in the different panels, from left to right.

midgut lumen and might not be required for invasion; a straight-segment motility requires linear translocation interrupted by turns to enable change in direction and is believed to take place in the apical crevices of the midgut epithelium; and, finally, a spiralling motility inside the midgut cells is a combination of the other two movements and enables a three-dimensional sampling of a broad territory swath. The molecular mechanisms underlying gliding motility are elucidated for sporozoites (see below) and are thought to be similar in ookinetes.

The first barrier that the ookinetes face during their migration through the midgut wall is the chitinaceous peritrophic matrix, an extracellular layer that coats the apical side of the midgut epithelium (Figure 1). Secretion of a chitinase by *P. falciparum* and *P. gallinaceum* is essential for their penetration of this barrier, but this appears to be less important in *P. berghei* [18]. A *P. berghei* calcium-dependent protein kinase, CDPK3, also plays an important role in the early events of midgut invasion; although conflicting evidence from different studies leaves its exact mode of action unclear to date [19,20], it is probably involved in ookinete motility.

Next, the ookinetes encounter the apical surface of the midgut epithelium (Figure 1). To traverse the epithelium and make contact with the basal lamina, they must penetrate the cytoplasm of the cells (Figure 2a) [17•,21–24]. A single ookinete often serially invades several cells, which become apoptotic and are extruded from the epithelium by actin-based restitution mechanisms, variable in nature between different mosquitoes [25]. This feature resembles the serial invasion of hepatocytes by sporozoites [26], although the basis of the two processes might be different. This resemblance is corroborated by the fact that the same cell-traversal protein for ookinetes and sporozoites (CelTOS) significantly promotes the ability of both these two stages to navigate across the cytoplasm of mosquito midgut cells and vertebrate hepatocytes, respectively, although it has no role in the initial invasion events [27••].

Entry of the ookinete into the epithelial cells is thought to be mediated by a specific receptor–ligand interaction, which remains to be identified. The *P. berghei* membrane attack ookinete protein (MAOP), a microneme protein with a membrane-attack complex and perforin (MACPF)-related domain, helps the parasite to enter the cells [28]. A structurally similar protein is important for sporozoite invasion of hepatocytes, further highlighting the similar mechanisms by which ookinetes and sporozoites invade their target cells [29]. Additional surface or secreted proteins have been implicated in the invasion process. CTRP, the circumsporozoite and TRAP (thrombospondin-related anonymous protein)-related protein, is a micronemal- and membrane-bound constituent that plays a role in ookinete motility and its ability to invade the midgut [30,31]. It is also found at high concentration at the point of contact between the ookinete and the basal lamina [32], and binds to laminin, the major component of the basal lamina [33], altogether suggesting a central role for CTRP during midgut invasion.

P25 is another *P. berghei* membrane-bound and surface-localised protein that binds to mosquito laminin. Its paralogue, P28, also binds laminin — with weaker affinity — and forms dimers with P25 [34,35]. Genetic evidence has highlighted an apparent functional overlap between P25 and P28: whereas single knockout parasites each display only a small reduction in the number of oocysts, the double knockouts are not only significantly impaired in their ability to develop into ookinetes but also incompetent in crossing the midgut and developing into oocysts [34,36]. Another micronemal protein, the secreted ookinete adhesive protein (SOAP), is important for ookinete invasion and oocyst development, and it too binds to laminin [37]. The interaction of ookinetes with basal lamina components is crucial for oocyst development. Ookinetes injected into the mosquito haemocoel are able to form oocysts at the basal lamina of not only the midgut but also the fat body and the malpighian tubules [38]. Similarly, *Plasmodium* is not able to develop if fed to *Drosophila melanogaster*, but if ookinetes are injected into the *D. melanogaster* haemocoel they are able to attach to the basal lamina and develop to oocysts [39]. Thus it appears that it is the actual interaction with the basal lamina that triggers oocyst development.

A large fraction of parasite losses that are encountered during the ookinete-to-oocyst transition are due to immune reactions of the host [40,41•]. An immune signalling pathway that involves the NF-κB (nuclear factor kappa B) transcription factor REL2 mediates substantial *P. berghei* killing in the midgut [42]. It controls the transcriptional activation of several genes implicated in antiparasitic immunity, such as clip-domain serine protease genes [43] and *LRIM1* (*leucine-rich immunity protein 1*) [41•]. The latter encodes a secreted leucine-rich repeat protein, and its antiparasitic function is negatively regulated by members of the C-type lectin (CTL) family. Depletion of CTL4 and CTLMA2 (C-type lectin putative mannose binding protein 2) leads to ookinete melanisation [41•], a highly regulated humoral immune response of arthropods that causes sequestration of parasites in a dense melanin coat (Figure 1). A serine protease inhibitor, SRPN2 (serine protease inhibitor 2), also negatively regulates parasite killing and melanisation, probably by inhibition of activating clip-domain serine proteases [44]. Other, enzymatically incompetent members of the clip-domain serine protease family have been shown to function either as activators or inhibitors of melanisation [45], and APL1 (*Anopheles Plasmodium*-responsive leucine-rich repeat 1), a member of the leucine-rich repeat protein superfamily, has an effect on *P. berghei* similar to that of LRIM1 [46]. One comprehensive analysis of a mosquito immune response to *Plasmodium* involved the study of the thioester-containing, complement-like protein TEP1, which binds to ookinetes as they cross the midgut and which causes their death by lysis or melanisation [40].

Cellular reactions of the invaded midgut epithelium are thought to account partly for the documented ookinete losses [47,48]. In particular, the actin polymerisation machinery of the midgut cells, which is activated during *Plasmodium* invasion, contributes to parasite killing by an as yet unknown mechanism [48]. An actin-rich structure that hoods *P. berghei* ookinetes as they traverse the basolateral plasma membrane of invaded cells (Figure 1) [17•,47] is believed to be partly responsible. Additional mosquito molecules have been implicated in this sensitive mosquito–ookinete interplay: many are transcriptionally induced during midgut invasion [48]. An intriguing example is the apolipophorins I and II, which are key components of the lipid transport machinery in the *A. gambiae* haemolymph and which promote both egg production and *Plasmodium* development [48].

Taken together, these findings indicate that in order for *Plasmodium* to successfully complete the difficult transition from the blood bolus-located ookinete to the oocyst developing at the basal lamina, it must overcome a number of challenges. Successful transition depends on *Plasmodium* motility but also on its ability to recognise, gain entry and traverse the midgut epithelial barrier — all of these while withstanding attacks from the mosquito humoral and local epithelial immune system.

Oocyst maturation and sporozoite development

The oocysts that develop in the intercellular space between the basal lamina and the midgut epithelium produce and, when mature, release some thousands of sporozoites into the mosquito haemolymph (Figure 1). Many of these sporozoites migrate into the salivary glands and are transmitted to human hosts during subsequent blood meals. Only a limited number of proteins with a role in *Plasmodium* sporogony have been identified to date.

The developing oocyst undergoes multiple nuclear divisions, which, by not being coupled to cytokinesis, result in a multinucleated parasite that gradually grows in size. In parallel to this replicative process, the oocyst plasma membrane is folded inwards and forms crevices that, extending across the oocyst cytoplasm, partition it into compartments termed sporoblasts. The developing sporozoites bud off from the sporoblasts in a process that involves mobilisation of the nucleus and other cellular organelles into each budding sporozoite. Cytokinesis of the budding sporozoites results in the formation of a mature oocyst that contains haploid sporozoites. The circumsporozoite protein (CSP) is the main surface protein of the oocyst and the salivary gland sporozoites. Its localisation at the oocyst surface through glycosylphosphatidylinositol-anchoring is essential for cytokinesis and sporozoite formation, and CSP-depleted oocysts are void of sporozoites [49,50]. The amount of CSP directly correlates with the degree of sporozoite formation [51].

Other important proteins for oocyst development are members of the limulus clotting factor C, Coch-5b2 and LglI (LCCL)–lectin adhesive-like protein (LAP) family [52,53]. *P. berghei* oocysts lacking LAP1 display significantly reduced ability to produce sporozoites [54]. Interestingly, the targeted disruption of either of two *P. falciparum* LAP family members (PfCCp2 and PfCCp3/PSLAP) result in normal levels of sporozoites that are, however, unable to enter the salivary glands [55]. A *P. falciparum* cysteine protease known as falcipain-1 is also important for oocyst development; however, it is unknown at which stage this protease acts [56].

Sporozoite release from the oocyst and migration to the mosquito salivary glands is the next important step in parasite transmission (Figure 1). Recent data showed that sporozoite egress from the oocyst is an active process, which in *P. berghei* is mediated at least partly by the egression cysteine protease, ECP [57••]. In addition, a region of CSP that contains a series of positively charged amino acids is also necessary for successful sporozoite egression [58].

Sporozoite motility and salivary gland invasion

To date, it is unclear how sporozoite migration towards the salivary glands is achieved. Recent evidence suggests that chemotaxis has a role [59]; however, other studies propose that this is a passive process, mediated solely by haemocoel circulation [60].

The founder member of the TRAP family, to which the ookinete specific protein CTRP also belongs, is essential for sporozoite gliding motility [61]. Interestingly, both CTRP and TRAP are also implicated in invasion of the midgut and salivary gland cells, respectively. Thus, related motility proteins are used by parasite stages to invade two different types of cells, in both the vertebrate and the invertebrate host. A third member of the family, the merozoite TRAP homologue (MTRAP), is involved in invasion of erythrocytes [62], again demonstrating the use of paralogues to support parasite invasion in distinctive cell types. CSP is also important for sporozoite gliding motility [61].

The force for *Plasmodium* motility is generated by an actomyosin motor that is located apically between the plasma membrane and the inner membrane complex [61]. Old observations and new molecular evidence have provided insights into the link between surface molecules and the actomyosin motor [63]. The cytoplasmic tail of TRAP binds aldolase, a molecule with known actin-binding properties. In parallel, the tail of Myo-A, the myosin component of the actomyosin motor, binds to the Myo-A tail domain-interacting protein (MTIP), which localises to the inner membrane complex of sporozoites. These studies, in conjunction with the finding that the *Plasmodium* actin filaments are very short and transient [64,65], have led to an updated model for gliding motility [63]. The short actin scaffolds are captured through aldolase at the parasite apical end by transmembrane receptors of the TRAP family. The latter interact with host ligands, providing a link between the parasite motility machinery and its substrate in the host. Intracellularly, this complex binds to Myo-A, which in turn interacts with the inner membrane complex through MTIP [66]. By passing the actin scaffolds back onto another Myo-A molecule, the parasite glides forward. The same mechanism is thought to generate the force for penetration of the host–cell membrane during invasion.

The direction of parasite migration is a major difference between midgut and salivary gland invasion: ookinetes traverse the midgut from the apical to the basal surface, whereas sporozoites traverse the salivary glands from basal to apical surface (Figures 1 and 2b). Thus, the sporozoites have to traverse the basal lamina of the salivary gland before they encounter its epithelium. Interestingly, it is proposed that the interaction with the basal plasma membrane leads to the formation of a parasitophorous vacuole, which disintegrates inside the cytoplasm, thereby freeing the parasites [67]. By contrast, no parasitophorous vacuole is formed during midgut invasion. Invasion of the salivary gland cells causes morphological changes associated with both cellular disorganization and the appearance of vesicles. Despite these differences, the molecular mechanisms of midgut and salivary gland invasion display further similarities. SM-1 (salivary gland and midgut peptide 1), which inhibits the invasive ability of the ookinete, also blocks salivary gland invasion [68]. This implies that the recognition and initial binding of the salivary gland epithelium is mediated by a common, as yet unknown, ligand (or ligand paralogues). MAEBL (merozoite apical erythrocyte-like protein) is a sporozoite protein with an important role in the recognition and binding of the salivary gland epithelium [69], but, in contrast to TRAP, its disruption does not affect motility. CSP has also been implicated in the ability of the parasite to bind to the salivary gland epithelium [61].

A second parasitophorous vacuole is thought to envelop the sporozoites during their escape into the secretory cavity of the glands. Inside the cavity, disintegration of the vacuole frees the sporozoites, which migrate by gliding motility into the fine secretory ducts that then converge into the common ducts [70•]. Only sporozoites that migrate to the ducts can be transmitted upon mosquito salivation; the vast majority of sporozoites, which are mainly found in the secretory cavities, are either stored for future bites or lost [60].

Conclusions and perspectives

Plasmodium must complete its sexual development in the mosquito before it can be transmitted to its vertebrate host and cause malaria disease. Navigation of several

mosquito tissue compartments requires the on and off switch of expression of parasite proteins that can help the parasite to traverse various barriers, interact with ligands on the mosquito host-cells, support movement of the parasite or help it evade mosquito immunity. Progression from compartment to compartment in the vertebrate and insect hosts often entails the use of different paralogues, testifying to an apparent reduplication of key genes during the evolution of the complex, multi-invasive lifestyle of the parasite. However, there is still much more to be discovered: for example, the parasite sporogonic development in the oocyst is still something of a 'black box'. Recent advances in technologies for the analysis of parasite gene function, in conjunction with the vast amount of information produced by the genome projects and associated technologies, are expected to provide insights into the molecular mechanisms that regulate the complex parasite development in the mosquito.

Malaria persists as one of the most devastating diseases affecting mankind. The successful migration of the parasite between two hosts, and across different compartments in each one, seems a most improbable journey and yet has been elaborated during the evolution of this complex parasitic life cycle. As we gain understanding of the molecular mechanisms underpinning this migration, blocking it in the mosquito host might prove to be one of the elements for successful control of malaria transmission.

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