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Invited Review

Malaria parasite colonisation of the mosquito midgut – Placing the *Plasmodium* ookinete centre stage

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ABSTRACT

Vector-borne diseases constitute an enormous burden on public health across the world. However, despite the importance of interactions between infectious pathogens and their respective vector for disease transmission, the biology of the pathogen in the insect is often less well understood than the forms that cause human infections. Even with the global impact of *Plasmodium* parasites, the causative agents of malarial disease, no vaccine exists to prevent infection and resistance to all frontline drugs is emerging. Malaria parasite migration through the mosquito host constitutes a major population bottleneck of the lifecycle and therefore represents a powerful, although as yet relatively untapped, target for therapeutic intervention. The understanding of parasite–mosquito interactions has increased in recent years with developments in genome-wide approaches, genomics and proteomics. Each development has shed significant light on the biology of the malaria parasite during the mosquito phase of the lifecycle. Less well understood, however, is the process of midgut colonisation and oocyst formation, the precursor to parasite re-infection from the next mosquito bite. Here, we review the current understanding of cellular and molecular events underlying midgut colonisation centred on the role of the motile ookinete. Further insight into the major interactions between the parasite and the mosquito will help support the broader goal to identify targets for transmission-blocking therapies against malarial disease.

1. Introduction

The parasites that cause malarial disease, from the genus *Plasmodium*, have a fixed requirement for the active infection of both vertebrate and mosquito tissues throughout their developmental lifecycle. Many of the core molecular and cellular processes required for navigating and establishing infection in these different microenvironments are conserved. This includes the molecular basis of motility in each stage (Baum et al., 2008) and steps required for cell penetration and vacuole formation during erythrocyte and hepatocyte invasion (Besteiro et al., 2011). At the same time tropism to the different human and mosquito tissues requires features that are specialised for each parasite stage. Some are universal, such as the linkage of stage-specific surface adhesins belonging to the thrombospondin-related anonymous protein (TRAP) family with the conserved internal motor (Baum et al., 2008; Morahan et al., 2009). Others are found only in individual lifecycle stages, such as those that tell a parasite where it is and how to behave (Singh et al., 2010; Armistead et al., 2011; Coppi et al., 2011). For example both sporozoites and merozoites form a tight junction during invasion but the ookinete, which lacks rhoptries, traverses the midgut without forming a vacuole. In combination, this complex kaleidoscope of conserved and variable biology through the parasite lifecycle represents a formidable and changing landscape for therapeutic intervention strategies. It is therefore not surprising that every stage of host-parasite biology will need to be targeted if we are to achieve global control or even eradication of malarial disease (Alonso et al., 2011).

Several powerful arguments can be made that the best opportunities for success in malaria control (or even eradication) lie in targeting the parasite as it passes through the mosquito and not the human host (Sinden, 2004). One core argument behind this reasoning is a major parasite population bottleneck that occurs during transmission through the mosquito (Sinden and Billingsley, 2001; Dawes et al., 2009). Approaches that are pursuing transmission targets include utilisation of mosquito immunity, other mechanisms of vector resistance (Whitten et al., 2006; Dinglasan and Jacobs-Lorena, 2008; Cirimotich et al., 2010) or the release of genetically modified or non-infectious mosquitoes (e.g. Moreira et al., 2009). On the parasite side, amongst the most promising transmission-blocking strategies are vaccines designed to elicit

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human antibody responses, which are taken up in the mosquito blood meal and block gametocyte fusion or colonisation of the midgut (reviewed in Saul, 2007). Recently this strategy has been extended to drugs that may act according to the same basic principle (e.g. Adjalley et al., 2011). Further progress in this area will certainly require improved fundamental understanding of the biology of the parasite as it passes through the mosquito, including the fine dissection of the molecular basis of sexual development, sporozoite maturation and the mosquito response to infection, all of which have been areas of increasingly active research during the last decade (e.g. Blandin and Levashina, 2004; Lasonder et al., 2008; Tewari et al., 2010). However, understanding at the molecular level for the process of oocyst formation, which is the smallest population bottleneck of the lifecycle, has lagged significantly behind. Here we review the current understanding of the steps that lead to successful midgut colonisation with a focus on the role of the ookinete, the largest motile malaria parasite lifecycle stage, and the cell responsible for establishing midgut infection and oocyst formation.

2. An overview of the mosquito infection cycle

The complete developmental process that leads to generation of new infectious sporozoites (sporogony) begins with the blood meal of a female mosquito (summarised in Fig. 1). On ingestion of infected blood, male and female gametocytes are taken up, emerge from within their infected erythrocytes and undergo fertilisation, zygote formation and development into the motile ookinete (10–25 h post-gamete fusion depending on the *Plasmodium* sp.). Once formed, the ookinete migrates out of the central blood bolus and faces one or two key physical barriers blocking its path to colonisation of the midgut. The first is a de novo formed chitin and protein-rich peritrophic matrix that variably assembles in response to feeding that surrounds the blood bolus. The second is the midgut epithelium itself. Once over these significant barriers, the ookinete then differentiates and begins development into a vegetative oocyst, resting between the luminal side of the basement membrane and the epithelium. Following rounds of nuclear division (over 10–14 days), hundreds of sporozoites are then released into the body cavity or haemocoel, eventually migrating to the salivary glands where they reside ready for release at the next blood feed. For further details on different aspects of the sporogonic cycle from sexual development through to salivary gland infection (summarised in Fig. 1) see recent reviews (Baton and Ranford-Cartwright, 2005; Vinetz, 2005; Vlachou et al., 2006; Aly et al., 2009; Ghosh and Jacobs-Lorena, 2009).

3. Signalling events during midgut colonisation

The transformations that occur at each step of the sporogonic cycle are poorly understood, but involve a complex mix of cellular and extracellular signals. Within seconds of the blood meal gametogenesis begins, involving a switch that is known to include simultaneous exposure to various stimuli (Billker et al., 1998; Arai et al., 2001). These include a drop in blood meal temperature to 5 °C below that of the warm blooded vertebrate host and a rise in pH from 7.4 to approximately 7.5–7.6 (under in vitro conditions as much as pH 7.8–8.0 can be tolerated) (Fig. 2) (Billker et al., 2000). Concomitant exposure to a midgut metabolite, xanthurenic acid, also appears to play a key role in the activation process (Billker et al.,

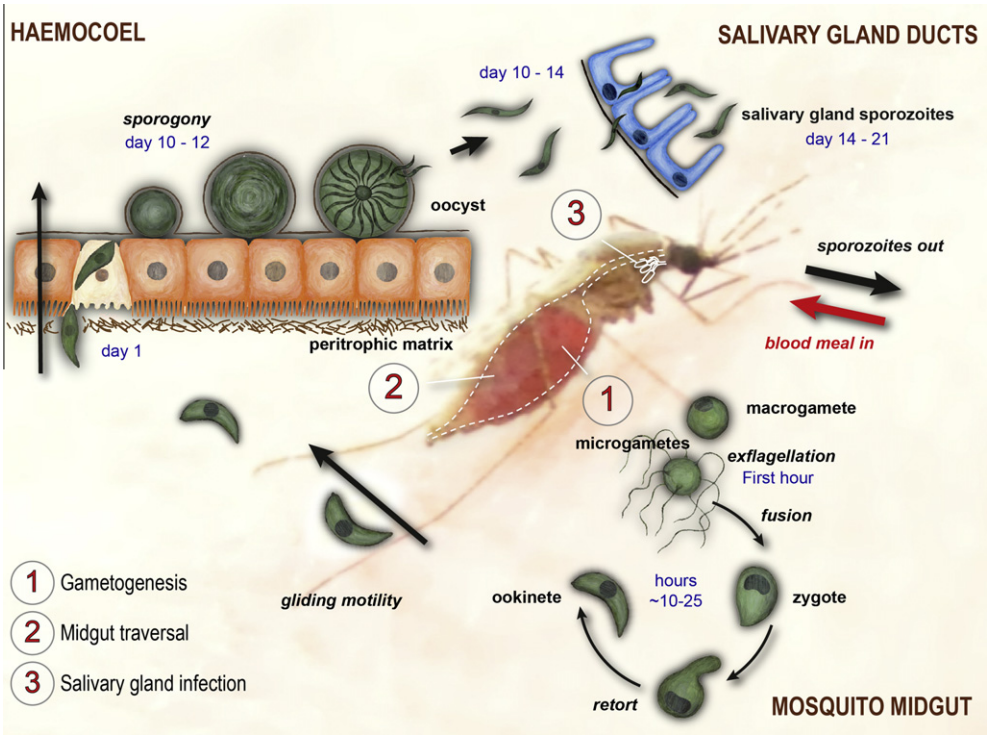

Fig. 1. The *Plasmodium* lifecycle stages within the mosquito. The transmission stages of the lifecycle begins with a female mosquito bite and the ingestion of infected blood from a vertebrate host. Three key transformation processes define the phase: gametogenesis (1), midgut traversal (2) and salivary gland infection (3). Ingested gametocytes are taken up with the blood meal and within 1 h undergo differentiation into both male and female gametes that fertilise to form zygotes in the gut lumen. The zygotes transform through a retort form, finally forming mature motile ookinetes over 24 h. Within 48 h ookinetes then traverse the midgut epithelium. Invaded epithelium cells undergo apoptosis and are extruded from the midgut wall (not shown). When an ookinete has reached the basal lamina, it then differentiates, losing its elongated shape, to form a young oocyst. Approximately 2 weeks later (depending on parasite–vector combination) multiple rounds of nuclear division occur in each oocyst. Membrane rupture releases sporozoites into the haemocoel. Sporozoites that reach and invade mosquito salivary gland epithelial cells migrate into the salivary gland ducts where they wait to be released into a new vertebrate host during the next blood meal.

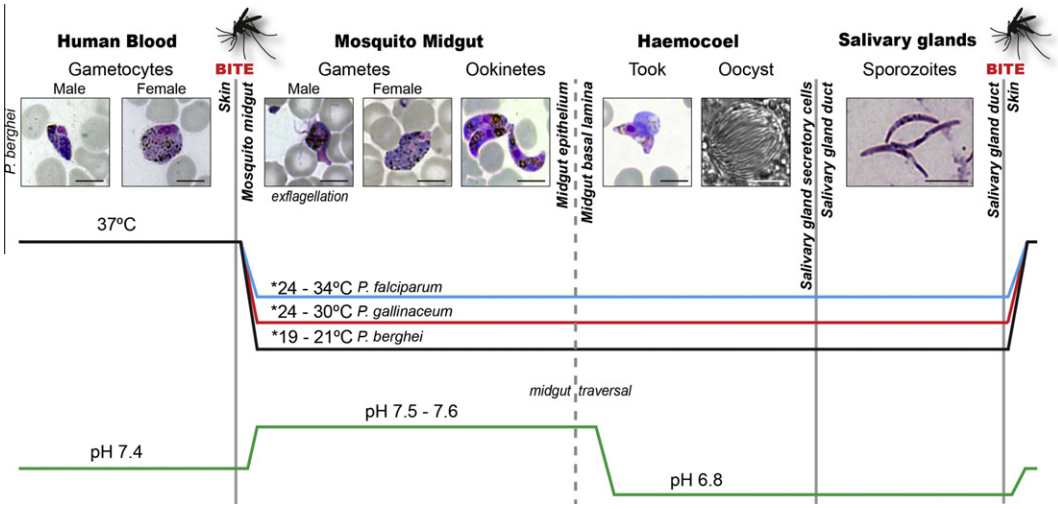

Fig. 2. A time series of *Plasmodium* development in the mosquito host. In the vertebrate host *Plasmodium* gametocytes are formed at 37 °C and pH 7.4. The female mosquito midgut temperature and pH are very different from the vertebrate host. Following ingestion, gametocytes are induced to produce gametes under the condition of increasing pH (Billker et al., 2000) and decreasing temperature depending on the *Plasmodium* sp. (coloured lines). *Note some caution is required with exact estimates of temperature since sporozoite-infected mosquitoes can be left for long periods below as little as 10 °C. These conditions remain during the transformation of zygote into ookinete within the midgut. Following traversal, oocyst development commences (through the transforming ookinete or "took" phase; Carter et al., 2007). The temperature relative to the *Plasmodium* sp. remains constant but the pH drops to approximately pH 6.8, with maturation completed in this environment. Mature sporozoites are released into the haemocoel and then targeted to the salivary glands, where on the next bite they are ejected into the skin of a human host. Representative Giemsa-stained images (excluding oocyst, phase contrast) of *Plasmodium berghei* mosquito stages are shown (scale bar = 5 μm).

1998), which stimulates both gamete forms to egress from the infected host cell. The male gamete undergoes a dramatic process of exflagellation whereby flagellated microgametes break free of the male gametocyte before finding their female macrogamete counterpart (Sinden et al., 1976). Kinase-dependent signalling pathways are increasingly being implicated in this and other midgut developmental processes. For example, full maturation of flagellated microgametes has been shown to be dependent on a Serine/Arginine-rich (SR) protein kinase (SRPK) (Tewari et al., 2010), a calcium-dependent protein kinase, CDPK4 (Billker et al., 2004) and a mitogen-activated kinase, MAP2 (Tewari et al., 2005). Post-zygote formation appears to require its own set of cellular regulators including two members of a Serine/Threonine NIMA-related (never in mitosis/Aspergillus1) protein kinase family, Nek-2 and Nek-4, which are both essential at or before the genome replication step that precedes meiosis (Reininger et al., 2005, 2009). During complete formation of the motile ookinete, calcium/calmodulin-dependent protein kinases have been implicated in the morphological development of the zygote into the elongated ookinete (Silva-Neto et al., 2002), whilst knockouts for kinases pk7 and gak both failed to progress to the ookinete stages in vitro (Tewari et al., 2010). Although complete regulation of cell movement in the ookinete (or indeed any motile stage) is still not known, active ookinete gliding has been shown to be dependent on dynamic actin and myosin (Siden-Kiamos et al., 2006, 2011), and involve signalling pathways dependent on calcium (Ishino et al., 2006; Siden-Kiamos et al., 2006a, 2008), guanosine 3′,5′-cyclic monophosphate (cGMP) (Moon et al., 2009), and guanylate cyclase (Hirai et al., 2006). Recently, with description of the proteome of the ookinete microneme (Lal et al., 2009) and other developments in genomics and proteomics (Hall et al., 2005; Zhou et al., 2008), other signalling molecules and associated pathways are likely to be revealed that regulate a range of events from ookinete formation to oocyst maturation and ultimately sporogony. We note here that most of our understanding of mosquito-stage developmental biology comes from the mouse malaria parasite, *Plasmodium berghei* (with other data from *Plasmodium galineceum* and limited data from *Plasmodium falciparum* and *Plasmodium vivax*), due to the ease of in vitro ookinete generation and broad genetic tractability in this species (Hall et al., 2005). Recent improvements in working with the transmission stages of human species, namely *P. falciparum* and *P. vivax* (Bounkeua et al., 2010; Ghosh et al., 2010; McClean et al., 2010) will likely change this imbalance in future years.

4. Ookinete cell biology

Compared with their motile counterparts involved in salivary gland, hepatocyte and erythrocyte infection (sporozoites and merozoites, respectively), ookinetes are the largest motile cell of the malaria parasite lifecycle. With some degree of species variability, they measure between 10 and 12 μm in length by 2–3 μm wide and, like the other polarised and motile lifecycle stages, retain the core cellular architecture of all Apicomplexa (Canning and Sinden, 1973; Vinetz, 2005). This includes the presence of the specialised apical complex and pellicular architecture associated with apicomplexan motility and host–cell invasion (Morrissette and Sibley, 2002). The pellicle is composed of three membranes: the plasma membrane and the two associated inner membranes, which form one flattened vacuole that is located beneath the plasma membrane and referred to as the inner membrane complex (IMC). The intervening space between the plasma membrane and IMC is referred to as the supra-alveolar space (Raibaud et al., 2001). Underlying the IMC is an array of microtubules, the subpellicular network (Morrissette and Sibley, 2002), and a lattice network of intermediate filaments, comprised of a family of related IMC proteins recently reclassified as alveolins (Gould et al., 2008). Whilst the precise molecular linkages between microtubules and intermediate filaments with the IMC membranes are not known, they appear to be connected to the innermost membrane of the IMC by 9 nm intra-membraneous particles that stud the underlying membrane surface (Raibaud et al., 2001). These particles have been postulated to be involved in both the transport of motor proteins and non-micronemal surface proteins (Raibaud et al., 2001) and as potential anchors for the IMC-bound motor complex involved with gliding motility. Recent identification of a family of IMC-bound six-pass transmembrane proteins, termed the glideosome associated protein with multiple-membrane spans (GAPM)

family, may constitute the molecular basis for these structures (Bullen et al., 2009). Ookinetes are highly motile and whilst they do not attain the high speeds observed in the sporozoite (Vanderberg, 1974; Munter et al., 2009) they move on average at approximately 6–8 μm per min under in vitro motility conditions (Tan et al., unpublished data) (Vlachou et al., 2004). The basis of this motility is very likely conserved with that found underlying cell motility in all apicomplexan parasites, called gliding motility (Baum et al., 2008), with recent evidence confirming the role of myosin A as the motor protein driving cell movement (Siden-Kiamos et al., 2011). Actin dynamics are also clearly important, as demonstrated by drug sensitivity of ookinete gliding (Siden-Kiamos et al., 2006b; Angrisano et al., 2012a). Efforts to visualise filaments of actin in the supra-alveolar space (their predicted location according to current understanding of gliding motility (Baum et al., 2008)) have not been forthcoming in any lifecycle stage. Recent high resolution imaging approaches towards visualising actin certainly confirms the idea that actin filaments occur in regions associated with motility, supporting the notion that ookinetes use the generic gliding motor for midgut penetration (Angrisano et al., 2012a,b; Siden-Kiamos et al., 2012).

The ookinete apex, similar to that of its apicomplexan counterparts, is intimately involved with secretion of adhesins and invasins associated with motility, host-cell recognition and midgut traversal. However, whilst the canonical apical architecture is found, the ookinete has several unique features. Foremost, and in contrast to merozoites and sporozoites, the ookinete lacks the full complement of secretory organelles (micronemes, rhoptries and dense granules), retaining only the numerous miniature club-shaped micronemes (Sinden, 2004; Lal et al., 2009). This observation correlates well with the observed absence of parasitophorous vacuole formation during midgut traversal and the implicated role of the rhoptries and dense granules in establishment of the parasitophorous vacuole within an infected cell (Blackman and Bannister, 2001). The micronemes house the bulk of adhesins associated with host cell recognition, penetration and linkage with the internal actomyosin motor, in line with the ookinete's primary role to reach and traverse the midgut wall (Lal et al., 2009). Another organelle, found only in ookinetes and young oocysts, is the crystalloid. These are short-lived cytoplasmic aggregations of small irregular spherical particles, 25–40 nm in diameter, that form in the developing ookinete and disappear after ookinete-to-oocyst transition (Canning and Sinden, 1973; Sinden et al., 1985). Although poorly understood, these geometrically arranged pigment-surrounded particles have been postulated to form a reservoir of protein, synthesised by the macrogametocyte, which provides vital resources for the development, infectivity and transmission of sporozoites (Dessens et al., 2011). Recent identification of a family of proteins grouped by the presence of an LCCL domain (named after the best characterised family members, Limulus factor C, Coch-5b2, Lgl1 domain) that localise to the crystalloid are providing much needed molecular insight into the potential function of these little understood organelles (Carter et al., 2008; Saeed et al., 2010).

A final unique feature of the ookinete is its distinct cytoskeletal organisation at the anterior end of the cell, which consists of a complex, multilayered structure known as the collar or canopy (Canning and Sinden, 1973; Aikawa et al., 1984; Sinden et al., 1985). This electron dense region covers the anterior apex of the cell, extending a short distance over the shoulder, but does not reach the main body itself. Ultrastructurally, the collar may connect with the apical polar rings, which act as a microtubule organising centre for the radiating sub-pellicular microtubules, but its precise function (and the reason for its specificity to the ookinete) is not known. Given its likely rigid nature via connections with the cytoskeleton, the collar may act as a reinforced spearhead as the ookinete drives through the midgut epithelial layer (Canning and Sinden, 1973). Association of chitinase (see Section 5) with this structure (Langer et al., 2000) would support such a penetrating role.

5. The basis for ookinete traversal

The precise steps of midgut traversal are still incompletely understood, with the parasite having to navigate across several different barriers (Zieler et al., 1998; Vinetz, 2005) (Fig. 1). The first definitive barrier is the chitin-containing peritrophic matrix which forms, 16–30 h post-feeding, around the ingested blood meal bolus. This completely encapsulates the blood meal and disintegrates following digestion (Shahabuddin and Kaslow, 1994). To penetrate this first barrier towards midgut colonisation, the ookinete secretes micronemal chitinases (and potentially other proteases (Vinetz, 2005)) into the extracellular milieu, permitting passage of the parasite through the peritrophic matrix to reach the mosquito midgut epithelium (Shahabuddin et al., 1993; Vinetz et al., 1999). The differential reliance on chitinase for different parasite species (as determined by knockout (Dessens et al., 2001; Tsai et al., 2001)) is likely explained by their different timing of maturation, with early maturing ookinete species (*P. berghei*, and *Plasmodium yoelii*) reaching the midgut before an intact peritrophic matrix forms, whilst other late developers (*P. falciparum* and *P. galinaceum*) meet the fully formed matrix (Vinetz, 2005). Chitinase is initially formed in a pro-domain containing form (pro-chitinase), which is matured by the specific action of a trypsin protease in the midgut that permits peritrophic matrix degradation (Huber et al., 1991; Shahabuddin et al., 1993). Although the origin of tryptic digestion is not clear (either midgut or parasite-derived (Vinetz, 2005)), exposure to the harsh environment of the midgut milieu is clearly a significant challenge to the motile cell. Surface proteins, in particular the glycosyl-phosphatidylinositol (GPI) anchored epidermal growth factor domain containing proteins P25 and P28 (see Section 6.1), may confer on the ookinete resistance to proteolytic activity within the mosquito stomach (Tomas et al., 2001).

Once the ookinete has conquered the peritrophic matrix, it must then traverse the substantial barrier of the midgut epithelium. When examined in vitro, ookinetes specifically adhere to the lumenal surface of dissected midgut tissue but not to the reverse side, suggesting that there may be (as yet unknown) mechanisms of ookinete recognition for the epithelial brush-border (Zieler et al., 1998). The process of traversal, unlike invasion of erythrocytes and hepatocytes, does not involve eventual formation of a parasitophorous vacuole. As such the ookinete effectively remains extracellular but must still overcome the substantial cellular barrier before it can differentiate and begin oocyst formation under the basement membrane. This is matched by the absence of rhoptries and dense granules – organelles intimately associated with vacuole formation (Baum et al., 2008). The ookinete appears to begin cellular traversal by recognising a non-sialic acid carbohydrate on the epithelial cell surface (Zieler et al., 1999). However, whilst controversial, it appears that no specific variety of epithelial cell is required, with the ookinete instead gliding across the tissue surface and then abruptly driving through or between any single columnar midgut epithelial cell (Zieler and Dvorak, 2000; Vlachou et al., 2004). Invaded host epithelial cells are severely damaged and become apoptotic after encountering an ookinete, although whether this is merely a response to infection or parasite-initiated is not clear (Han et al., 2000; Vlachou et al., 2004). In analyses with *Anopheles stephensi* midguts, invaded cells expel the apoptotic midgut cell via a dynamic actin purse string wherein healthy cells either side of the damaged cells squeeze together to push the apoptic cell out (Baton and Ranford-Cartwright, 2005). Likewise, in

Aedes aegypti infections (with *P. galinaceum*), the midgut is repaired by a unique actin zipper mechanism that involves the formation of a cone-shaped actin aggregate at the base of the cell that closes sequentially, expelling the damaged cell into the midgut lumen, again bringing together neighbouring healthy cells (Gupta et al., 2005). The use of heterologous parasites with non-native mosquito species likely clouds some of our understanding of this process.

Ookinete traversal is accompanied by the launch of a substantial mosquito immune response, involving proteins in the mosquito haemolymph that cause parasite lysis and melanisation (Whitten et al., 2006). When combined with the challenges of navigating the digestive environment of the mosquito and traversal of the peritrophic matrix and epithelium, it is perhaps not surprising that so few ookinetes successfully reach the basal sub-epithelial space to form oocysts. Indeed, the drastic reduction in ookinete numbers between those that start out and those that successfully transform into oocysts creates an enormous bottleneck in the *Plasmodium* lifecycle. Whilst the exact numbers are hard to estimate and depend on the parasite–vector combinations, it has been calculated that of the 1×10^5 gametocytes ingested in a blood meal, an average of perhaps less than 1,000 ookinetes develop and of those fewer than five may survive to develop into oocysts (Sinden and Billingsley, 2001; Dawes et al., 2009).

6. Ookinete proteins involved in midgut colonisation

The recent efforts in genomics and proteomics, together with other genome-wide approaches to catalogue all parasite proteins and their functions, have revealed a large number of as yet unknown ookinete molecules that may play key roles in the mechanics of traversal and inevitable receptor–ligand interactions that facilitate this process (Hall et al., 2005; Ecker et al., 2008; Zhou et al., 2008; Lal et al., 2009). A few of the better-understood ookinete-specific proteins, with particular focus on those involved in midgut survival, recognition and traversal, are presented in Table 1.

6.1. The ookinete surface

The GPI-anchored P25 and P28 proteins are structurally related membrane-bound proteins that are secreted onto the ookinete surface and have putative binding functions, such as to laminin (Vlachou et al., 2001). These key surface proteins may play essential roles in protecting the ookinete from midgut degradation. The structure of P25 from *P. vivax* would support this notion (Saxena et al., 2006), forming a triangular prism that tiles the surface of the ookinete, perhaps constituting a protective sheet. However, P25 and 28 also show evidence for a role in the process of midgut traversal and, similar to their GPI-anchored counterparts in merozoite and sporozoite stages (Cowman and Crabb, 2006; Aly et al., 2009), may be important for initial interactions with the epithelial cell surface. Deposition of P28 at the site of ookinete entry certainly suggests a possible role in cell recognition (Tomas et al., 2001). Each is somewhat functionally overlapping but knockout of both gives rise to a marked reduction in the capacity of ookinetes to traverse the midgut and transform into oocysts, although it does not completely abolish ookinete entry into midgut epithelial cells (Siden-Kiamos et al., 2000; Tomas et al., 2001).

6.2. Ookinete cell motility

One of the best-characterised proteins of the ookinete is the circumsporozoite TRAP-related protein (CTRP) which was first described in *P. falciparum* (Trottein et al., 1995) and later found in all other *Plasmodium* spp. (Dessens et al., 1999; Yuda et al., 1999; Templeton et al., 2000; Kaneko et al., 2006). This ookinete-specific member of the TRAP-related family is secreted from micronemes and is essential for ookinete motility (Dessens et al., 1999; Yuda et al., 1999; Templeton et al., 2000). The CTRP short cytoplasmic tail retains the classical features of TRAP-like proteins, pulling down aldolase in in vitro assays and therefore likely linked to the internal gliding motor (Heiss et al., 2008). The extracellular portion of the protein is composed of a series of repeated adhesive domains (von Willebrand or factor type-A related and thrombospondin type-1 related), suggestive of a role in recognising surface epitopes on target midgut epithelial cells. In support of this, once secreted, CTRP is found most abundantly at the site of contact between the apical end of an ookinete and the basal lamina of an epithelial cell (Limviroj et al., 2002). Furthermore, it has been shown to be a potential binding ligand for midgut epithelial lamnin and collagen (Arrighi and Hurd, 2002; Mahairaki et al., 2005). Recent genetic dissection of CTRP demonstrates that whilst the A domains of the protein are critical for ookinete gliding motility (the thrombospondin domains are surprisingly redundant), CTRP is not required for oocyst formation, demonstrating a clear focussed role in motility and mosquito cell recognition (Nacer et al., 2008; Ramakrishnan et al., 2011).

6.3. Midgut epithelium recognition

Several other micronemal proteins play varied roles in midgut colonisation. This includes the aforementioned chitinase (see Section 5), *Plasmodium* von Willebrand factor A domain-related protein, WARP (Li et al., 2004), which, like CTRP, shares some features with the TRAP family of proteins whilst being soluble, secreted ookinete apical protein (SOAP) (Dessens et al., 2003) and more recently described putative secreted ookinete proteins (PSOPs) (Ecker et al., 2008). WARP has been hypothesised to act as an adhesive substrate connecting the ookinete surface and midgut molecules during ookinete midgut traversal (Yuda et al., 2001). SOAP is highly conserved amongst *Plasmodium* spp. and encodes a cysteine-rich soluble protein, again found associated with the parasite surface (Nacer et al., 2008). SOAP has been shown to form high molecular mass complexes via disulphide bonds and interacts strongly with mosquito laminin. Targeted disruption of the SOAP gene gives rise to ookinetes that are impaired in their ability to invade the mosquito midgut (Dessens et al., 2003). Amongst unrelated PSOP proteins (Ecker et al., 2008), two show key features suggestive of a role in midgut recognition and traversal. Knockout of PSOP2 reduces the number of ookinetes produced and a disruption of PSOP7 gives rise to ookinetes unable to invade the mosquito midgut (Ecker et al., 2008). Of note, PSOP7 has a C-terminal carbohydrate binding domain, suggesting it may be acting as a parasite cell adhesin or recognition protein (Ecker et al., 2008). For many of the micronemal proteins described, even though it is possible for knockouts to form oocysts (via direct haemocoel injection e.g. Nacer et al., 2008), their precise roles in reaching the midgut epithelium and traversing this key tissue barrier are not known. Their high numbers however, clearly highlight the importance of adhesive proteins to midgut recognition and traversal.

A final protein that has been recently implicated in direct interactions with the midgut epithelium is enolase, which is secreted onto the surface of ookinetes (Ghosh et al., 2011). Once secreted, it plays a dual role in midgut invasion by acting as a ligand that interacts with the epithelium whilst at the same time capturing plasminogen (the most abundant serine protease in mammalian blood) whose conversion to plasmin promotes the invasion process. Thus it may be that *Plasmodium* ookinetes co-opt a host protease to facilitate degradation of the midgut for traversal (Ghosh et al., 2011). A recent report that actin, another normally

Table 1
Plasmodium ookinete proteins involved in interactions with the mosquito during midgut colonisation.

Name	GeneID [a,b]	Localisation	Putative function	KO species	KO phenotype[c,d]	Reference[e]
Chitinase	PBANKA_080050 PFL2510w	Microneme	Penetration of the peritophic matrix	*P. berghei* *P. falciparum*	Impairment of the oocysts formation with varying effects in different parasite species	Dessens et al. (2001), Tsai et al. (2001)
P25	PBANKA_051500 PF10_033	Ookinete surface	Possible role in ookinetes entry into the mosquito midgut and in protection of ookinete surface from stomach enzymes	*P. berghei*	Single knockout shows slight reduction of ookinete and oocyst formation	Siden-Kiamos et al. (2000), Tomas et al. (2001)
P28	PBANKA_051490 PF10_0302	Ookinete surface	Possible role in ookinetes entry into the mosquito midgut and in protection of ookinete surface from stomach enzymes	*P. berghei*	Same as single knockout of P25. But double knockout of P25 and P28 strongly inhibits ookinete motility and entry into the mosquito midgut.	Siden-Kiamos et al. (2000), Tomas et al. (2001)
Enolase	PBANKA_121430 PF10_0155	Ookinete surface	Interaction with the midgut epithelium and plasminogen		ND[c]	Ghosh et al. (2011)
Membrane attack ookinete protein (also called *Plasmodium* perforin like protein 3)	PBANKA_082420 PFI1145w	Microneme	Invasive motility	*P. berghei*	Migration to the midgut epithelium and attachment to the cell surface at the apical tip, cannot enter cytoplasm	Kadota et al. (2004)
Plasmodium perforin like protein 5	PBANKA_071160 PF08_0052	Uncharacterised (secreted or surface)	Invasive motility and traversal	*P. berghei*	Normal numbers of ookinetes are produced,however, most do not develop into oocysts.	Ecker et al. (2007)
Subtilisin-like protease 2	PBANKA_091170 PF11_0381	Microneme	Disrupt actin cytoskeleton of the midgut epithelial cell		ND	Han et al. (2000)
Circumsporozoite and thrombospondin-related adhesive-related protein	PBANKA_041290 PFC0640w	Microneme	Adhesion and invasion of the mosquito midgut epithelium	*P. berghei* *P. falciparum*	Reduced motility of ookinetes, failure to invade the midgut epithelium or trigger the mosquito immune system. No development to oocysts. Direct haemocoel injection yields viable infective sporozoites [d]	Dessens et al. (1999), Yuda et al. (1999), Templeton et al. (2000), Nacer et al. (2008)
Secreted ookinete adhesive protein	PBANKA_103780 PF14_0040	Microneme	Invasive motility and interaction with laminin	*P. berghei*	Reduced capacity to invade mosquito midgut, reduced oocysts formation. Direct haemocoel injection yields viable infective sporozoites	Dessens et al. (2003), Nacer et al. (2008)
von Willebrand factor A-domain-related protein	PBANKA_122890 PF08_0136b	Microneme	Attachment and motility of ookinetes	*P. berghei*	No clear phenotype	Yuda et al. (2001)
Putative secreted ookinete protein 2	PBANKA_114370 PF13_0355	Microneme	Invasive motility	*P. berghei*	Ookinete numbers reduced compared to wild type with strongly reduced numbers of oocysts	Ecker et al. (2008)
Putative secreted ookinete protein 7	PBANKA_135340 MAL13P1.203	Microneme	Invasive motility	*P. berghei*	Normal production of ookinetes, but, reduced ookinete invasion and traversal of the midgut wall. Strongly reduced numbers of oocysts	Ecker et al. (2008)
Cell-traversal protein for ookinetes and sporozoites	PBANKA_143230 PFL0800c	Microneme	Cell traversal (mosquito and hepatocyte stages)	*P. berghei*	Mutant ookinetes penetrate epithelial cells but fail to migrate through cytoplasm. No development to oocysts. Direct haemocoel injection yields viable infective sporozoites (these also arrest at hepatocyte traversal)	Kariu et al. (2006)

[a] PBANKA, *Plasmodium berghei* strain ANKA.
[b] PF. *Plasmodium falciparum* 3D7.
[c] No data.
[d] Direct injection into haemocoel, where ookinetes are placed into the haemocoel to induce oocyst formation independent of midgut traversal.
[e] References relating to knockout (KO).

cytosolic protein, also lines the surface of the ookinete and interacts with enolase (Hernandez-Romano et al., 2011) is intriguing. However, this requires validation given conflicting data about the spatial distribution of actin during ookinete motility and the absence of evidence for its surface localisation in these studies (Angrisano et al., 2012a,b; Siden-Kiamos et al., 2012).

6.4. Midgut traversal

There has been much debate in the field on the molecular basis of midgut traversal, focussing on whether the ookinete crosses the epithelial barrier via an inter-cellular (i.e. passage between two cells) or intra-cellular (i.e. passage through a cell) route. In all

likelihood the process involves both events although debate continues as to whether lateral movement between cells is an adaptive process (the 'Time Bomb Model'; Han et al., 2000) or simply the result of midgut epithelial cell extrusion arising from host cell apoptosis (the 'Cellular Treadmill Model'; Baton and Ranford-Cartwright, 2005). The limited number of studies that have captured real time invasion events (Zieler and Dvorak, 2000; Vlachou et al., 2004), and differences between species and the parasite/mosquito combination (most studies are not carried out in a natural vector for each parasite species) probably account for much of the variability in ookinete routes of traversal seen. Irrespective of the ongoing debate, one final class of proteins that plays an essential role in midgut colonisation is those involved in breaking down cellular barriers during mosquito cell traversal. One group that plays a key role is the *Plasmodium* perforin-like proteins (PPLP). These proteins are conserved across *Plasmodium* spp. and characterised by the presence of a membrane attack perforin (MACPF) – like domain, which in other proteins has been shown to play a role in the formation of transmembrane pores in lipid bilayers (Kaiser et al., 2004). Ookinetes express three perforin-like proteins, two of which have been shown to be essential for mosquito midgut invasion (Kadota et al., 2004; Ecker et al., 2007), with the third as yet not fully characterised (Ecker et al., 2008). The first, the *Plasmodium* membrane-attack complex and perforin related domain protein (MAOP, also called PPLP3) appears to be related to pore-forming proteins involved in cell traversal of liver stages of the lifecycle; a pre-requisite stage before hepatocyte infection (Kaiser et al., 2004; Ishino et al., 2005). Knockout parasites for MAOP are unable to traverse the midgut and instead build up at sites of entry leaving the midgut epithelium largely intact (in contrast to the damaged epithelium that is successfully invaded by wild-type ookinetes) (Kadota et al., 2004). Such a phenotype would fit with MAOP functioning as a MACPF family protein that creates pores in the membranes of target cells for traversal. PPLP5 appears to be equally essential for mosquito infection (Ecker et al., 2007). Crossing an epithelial cell will also involve necessary disruption of the actin cytoskeleton. It has been shown that the ookinetes secrete a subtilisin-like protease (Sub2) into the cytoplasm of the invaded epithelial cells. This protease has been observed in association with actin aggregates and postulated to act by proteolytically cleaving the actin cytoskeleton (Han et al., 2000). There are clear similarities between the processes of cell traversal in the midgut and traversal required for liver-stage infections (Prudencio et al., 2006), highlighted by the shared expression of a protein termed CelTOS, the cell-traversal protein for ookinetes and sporozoites (Kariu et al., 2006). This micronemal protein has a critical role in both traversal processes, with CelTOS disrupted parasites arrested during passage to the basal lamina in the cytoplasm of the damaged midgut epithelial cells and markedly reduced infectivity of the liver (Kariu et al., 2006). Since motility and hepatocyte invasion are not affected, CelTOS clearly plays a more nuanced role in facilitating passage through the cytoplasm of the infected cell.

7. Future directions

Many of the outstanding questions in our understanding of midgut colonisation arise due to the difficulties in working on the insect stages of the parasite lifecycle. Even with access to an insectary, key biological steps as the parasite passes through the mosquito are hard to isolate in a live infection and, as such, understanding of host–parasite interactions during transmission is still poorly documented with a limited number of replicate studies for key events such as live imaging of traversal or oocyst formation (Zieler and Dvorak, 2000; Al-Olayan et al., 2002; Vlachou et al., 2004; Carter et al., 2007; Nacer et al., 2008). In addition to the challenges of dissecting parasite biology in a mosquito, working with those that carry human infections requires high level containment facilities and has a very real risk of infection for researchers working with pathogen-infected vectors. Looking towards the future, recent improvements in the ability to generate ookinetes from the *Plasmodium* spp. that infect humans (Bounkeua et al., 2010; Ghosh et al., 2010; McClean et al., 2010) and improvements to the published methods for in vitro generation of oocysts from any species (Warburg and Miller, 1992; Al-Olayan et al., 2002; Carter et al., 2007) will significantly boost our ability to dissect the molecular basis of midgut colonisation. Amongst the central unresolved questions is whether there are specific molecular signals required for ookinete recognition of, and activation for, the process of peritrophic matrix and midgut traversal or whether, as one eminent malariologist has anecdotally termed it, the ookinete is simply a "tank" that barrages through the gut on its way to the basal lamina. The ability to generate oocysts in the absence of mosquito cells or tissue certainly suggests that there are no essential signals to facilitate the process (Carter et al., 2007). A key prerequisite for this question will be improved understanding of the molecular signals that govern release of micronemes, given the role of these proteins in every stage of midgut colonisation (Table 1) (Ecker et al., 2008; Lal et al., 2009). Further refinement of our understanding of the process of traversal, the role of pore forming proteins and the elusive function of CelTOS in particular, are clearly key not only because of their potential role in colonisation but due to increased interest in their potential (and indeed most ookinete secreted or surface proteins) as targets for development of transmission blocking vaccines (Saul, 2007). Finally, improved methods of intravital imaging of ookinete motility and colonisation following the lead of pioneers in this area (Zieler and Dvorak, 2000; Vlachou et al., 2004) are clearly required to boost the number of infectious events studied and towards resolving one of the most mysterious lifecycle processes of this deadly global scourge.

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