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Microreview

The cell biology of malaria infection of mosquito: advances and opportunities

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Summary

Recent reviews (Feachem *et al.*; Alonso *et al.*) have concluded that in order to have a sustainable impact on the global burden of malaria, it is essential that we knowingly reduce the global incidence of infected persons. To achieve this we must reduce the basic reproductive rate of the parasites to < 1 in diverse epidemiological settings. This can be achieved by impacting combinations of the following parameters: the number of mosquitoes relative to the number of persons, the mosquito/human biting rate, the proportion of mosquitoes carrying infectious sporozoites, the daily survival rate of the infectious mosquito and the ability of malaria-infected persons to infect mosquito vectors.

This paper focuses on our understanding of parasite biology underpinning the last of these terms: infection of the mosquito. The article attempts to highlight central issues that require further study to assist in the discovery of useful transmission-blocking measures.

Introduction

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This paper focuses on our understanding of parasite biology underpinning the last of these terms: infection of the mosquito (Fig. 1). The article attempts to highlight central issues that require further study to assist in the discovery of useful transmission-blocking measures.

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From vertebrate host to insect vector

Noting the apparent differences in the sexual biology of malaria parasites in the subgenera *Laverania* and *Plasmodium*, where comment or data specifically refers to just one subgenus it will be stated explicitly, and where it is felt by the author to apply to both subgenera it will not be stated.

Transmission from host to vector is the exclusive role of the intra-erythrocytic gametocytes. The development of which can be divided into induction, maturation and gamete formation. The often extended periods of gametocyte induction raises the question whether any generalization can be made as to a physical site where gametocytogenesis is triggered. Rarely it is appreciated that there is evidence that merozoites released directly from the liver schizont can produce gametocytes (Suhrbier *et al.*, 1987; Sinden *et al.*, 1990), and that in the 'ancestral' haemoproteids *all* the merozoites released from the tissue schizonts make gametocytes, i.e. induction is not necessarily a consequence of prior blood infection. Past research (Smalley *et al.*, 1981) showed the young falciparum gametocytes were found in the bone marrow. Trager and Gill (1992) recognized the eminent logic of the slow-growing gametocytes of this species (subgenus *Laverania*), initiating their protracted development in hematopoietic tissues. These observations have been elegantly extended using both molecular techniques (Aguilar *et al.*, 2014; Joice *et al.*, 2014) and *in vitro* systems (Farfour *et al.*, 2012; Tiburcio *et al.*, 2012; Peatey *et al.*, 2013). The latter illustrate how both the rigidity of

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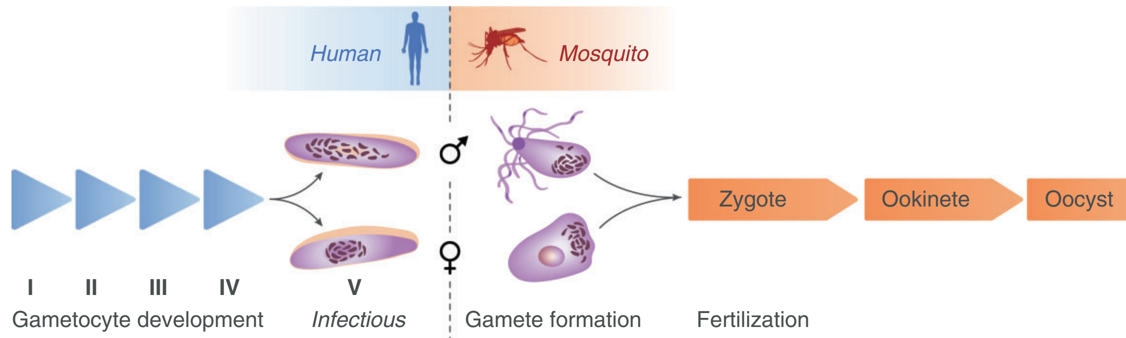

Fig. 1. Some of the earlier and significant steps in the analysis of the cell biology of sexual development of *Plasmodium* spp.

	I	II	III	IV	V	Gamete formation	Fertilization	Ookinete	Oocyst
Light microscopy 1881-	(Hawking et al., 1971; Carter and Miller, 1979)					(Laveran, 1881)	(McCallum, 1897)		(Ross, 1897)
Induction mechanisms 1926-	(Sinton et al., 1926; Kafsack et al., 2014; Sinha et al., 2014)					(Billker et al., 1998)			
Electron microscopy 1965-	(Sinden et al., 1978)					(Garnham et al., 1967)	(Sinden et al., 1976)	(Mehlhorn et al., 1980)	(Garnham, 1965)
Nuclear organization 1973-					(Janse et al., 1986b)	(Sinden et al., 1976)		(Sinden and Hartley, 1985)	(Canning and Sinden, 1973)
Transcriptome 2000-	(Lanfrancotti et al., 2007)				(Hayward et al., 2000)			(Hall et al., 2005)	
Translation repression 1990-					(Paton et al., 1993; Guerreiro et al., 2014)		(Mair et al., 2010)		
Proteins first identified 1974-	(Silvestrini et al., 2005)				(Vermeulen et al., 1985)	(Kumar and Carter, 1984)	(Liu et al., 2008)	Vermeulen et al., 1985)	(Krotoski et al., 1974)
Proteome 2005-					(Khan et al., 2005)	(Talman et al., 2014)		(Hall et al., 2005)	
Metabolome 2014-	(Lamour et al., 2014)								

the red blood cell (RBC) infected by the elongated immature *P. falciparum* (*Pf*) gametocyte, and surfin expression on the infected RBC (iRBC), might correlate with retention of the immature gametocyte in the complex extravascular environment of the bone marrow. The former attribute is unlikely to apply to the spherical parasites of the subgenus *Plasmodium* that lack the elongate sub-pellicular cytoskeleton of *Laverania*. When considering induction of gametocytogenesis in the peripheral blood, current *in vitro* evidence suggests that in *P. falciparum* induction occurs in the trophozoite of the asexual generation preceding gametocyte formation, and that the merozoites from any one committed schizont are predetermined to be either male or female (i.e. multiple fate 'decisions' have already been made prior to merozoite formation) (Bruce *et al.*, 1994). A marker of this commitment has recently been described as Pf10_0164 (ETRAMP 10.3) (Brancucci, unpubl.). In the rodent malaria parasite *P. berghei* gametocyte determination possibly occurs following invasion by the merozoite (Mons *et al.*, 1985). Myriad potential external inducers of gametocytogenesis have been described, all of which may be broadly classified as 'stress related', but molecular characterization of any consensus induction pathway still eludes us (Sinton, 1926; Bhasin and Trager, 1984; Dyer and Day, 2000; Carter *et al.*, 2013). Future experiments to understand sexual/asexual differentiation should at least entertain the hypothesis that sexual development is the ancestral – and therefore default – pathway.

Not knowing the molecular properties of the inducers, it is unsurprising that we do not know the receptors initiating the signalling pathways for gametocytogenesis. Things we do know however are that the down-regulation of the histone deacetylase gene *pfHda2*, the heterochromatin

protein PfHP1 and the ABC transporter-encoding gene *gabcg2* increase the proportion of gametocytes in bloodstage infections (Alano, 2014; Brancucci *et al*., 2014; Coleman *et al*., 2014; Tran *et al*., 2014). Perhaps the closest we have come to understanding these pathways is through the production and characterization of mutations resulting in the loss of gametocyte formation. The genetic regulation of gametocytogenesis was first appreciated in studies correlating the loss of chromosomal integrity (notably *P. falciparum* chromosome 9) with the loss of sexual potential (Birago *et al*., 1982; Janse *et al*., 1989; Day *et al*., 1993; Ikadai *et al*., 2013), but it was transposon-mediated mutagenesis (Ikadai *et al*., 2013) that identified 16 loci, nine of whose disruption led to loss of any identifiable (stage I) gametocytes [this group includes a member of the *ap2* family (PF13_0097)] and seven loci essential to formation of stage II gametocytes. Of these 16 'mutagenized' loci only five were successfully complemented. Perhaps, unsurprisingly, the former nine loci were classifiable as genes encoding regulators/signalling moieties, and the latter seven included genes with potential roles in the formation of the extensive cytoskeletal structures defining *Pf* gametocyte morphology. These conclusions are consistent with parallel observations (Young *et al*., 2005) that noted up-regulation of 246 gametocyte-specific gene classes in the mature gametocytes – shown to include kinase/phosphatase enzymes (Guttery *et al*., 2012b; Brochet *et al*., 2014). Genes that have been considered of particular note include *pfgdv1* (Eksi *et al*., 2012), *pfgig*, *pfgly* and the *plasma membrane transporter NPT1* (Baker, 2010; Boisson *et al*., 2011). Elegant recent studies have confirmed a pivotal role for *ap2-g* in gametocytogenesis (Kafsack *et al*., 2014; Sinha *et al*., 2014); transcription of *ap2-g* is regulated by PfHP1-H3K9me3 histone modification (Kafsack *et al*., 2014). Pathways regulating gametocytogenesis have been reviewed (Morahan and Garcia-Bustos, 2014); these authors suggest *ap2g* may regulate *pfgdv1*, thence *pfnek4* and *nek2* – genes that are additionally regulated by extracellular factors via cyclic adenosine monophosphate-mediated pathways. Interestingly, the protein phosphatase PPM2 (see Table 1) has been shown to have a positive role in the determination of sex ratio (female bias) (Guttery *et al*., 2014). Have we identified all the key regulator(s) of sexual differentiation, certainly not, but we are now developing the tools for doing so.

Despite long-established protocols for the culture of *P. falciparum* gametocytes *en masse* (Ponnudurai *et al*., 1983; Sinden *et al*., 1984) and the detailed descriptions of the complex morphological changes occurring in the skeletal, cytoplasmic and nuclear organization of the parasites (Sinden *et al*., 1978; Dixon *et al*., 2012; Hliscs *et al*., 2015) and in the organization of the mitochondrion and apicoplast (Okamoto *et al*., 2008), we remain disturbingly ignorant of the molecular profile of the maturing gametocyte. Transcriptomic and proteomic studies (Hall *et al*., 2005; Khan *et al*., 2005; Tao *et al*., 2014) have revealed, for example, 174 male- and 258 female-enriched proteins in mature (dimorphic/separable) *P. falciparum* gametocyte preparations. The gametocyte metabolomes are incompletely described (Lamour *et al*., 2014); indirect observations, e.g. drug sensitivity, currently suggest the immature gametocytes (stages I–III) are more readily killed by inhibitors of RNA and protein synthesis (Sinden and Smalley, 1979) and schizonticides (e.g. chloroquine) than the mature (stage IV–V) gametocytes (Smalley, 1977; Sinden, 1982; Targett *et al*., 2001), an observation previously linked to the substantial reduction in protein synthesis/ribosome population seen in the more mature forms (Sinden *et al*., 1976; 1978). To date one of the most interesting of the unexplored aspects of gametocyte biology lies in protein translation for two reasons: first, the mature female gametocyte (which retains a 'normal' ribosome population) accumulates a reported 169–731 species of translationally repressed gene transcripts (Mair *et al*., 2006; Guerreiro *et al*., 2014), which may be localized in cytoplasmic 'granules' (Thompson and Sinden,

Table 1. Suggested stages of *Plasmodium* development in the mosquito vector at which identified protein phosphatases and protein kinases are active**.**

	Female sex allocation	Male gamete exflagellation	Zygote/ookinete differentiation	Ookinete movement	Ookinete invasion	Oocyst development	Sporogony
Protein phosphatases	PPM2	PPM1	PPKL PPM2	PPKL	SHLP1	PPM5	PTPLA
Protein kinases	–	SRPK CDPK4 MAP2	NEK2 NEK4 GAK PK7	CDPK3	CDPK3	GAK PK7	CDLK

Data from Tewari *et al*. (2010) and Guttery *et al*. (2014).
CDLK, cyclin-dependent-like kinase; *CDPK3,4*, calcium-dependent protein kinase; *GAK*, cyclin g-associated kinase; *MAP2*, mitogen-activated protein kinase; *NEK2, NEK4*, NimA-related kinase; *PK7*, protein kinase; *PPKL*, protein phosphatase containing an N-terminal β-propeller formed by kelch-like motifs; *PPM1, PPM2, PPM5*, metallo-dependent protein phosphatases; *PTPLA*, protein tyrosine phosphatase-like A; *SHLP1*, Shewanella-like protein phosphatase; *SRPK*, SR protein kinase.

1994; Vervenne *et al*., 1994) where they are presumably complexed with the translational regulators DOZI and CITH (Mair *et al*., 2006; 2010; Guerreiro *et al*., 2014); but what is often overlooked is the evidence that the structure of the ribosome population itself changes as the parasite moves from vertebrate to insect host – the population transitions from A to S-form ribosomes in which different alleles encoding the SsuRNAs 30 and 31 are expressed (Waters *et al*., 1989). Why these changes occur and whether they have any relevance to the significant role of translational regulation in gametogenesis has yet to be tested.

Structural studies have shown that the mitochondrion enlarges significantly throughout gametocyte maturation (Aikawa *et al*., 1969; Sinden *et al*., 1976; Jensen, 1979; Okamoto *et al*., 2008). The seminal early electron microscopy studies of Aikawa *et al*. (1969) demonstrated beyond question that the mitochondrion (at the time considered to be numerous organelles) of the gametocytes was morphologically distinguished from those of the asexual bloodstages by the presence of prominent tubular cristae (Aikawa *et al*., 1969); subsequent studies revealed that in the ookinete and oocyst stages the cristae were even more prominently developed. Jim Jensen (1979) was the first to suggest that the multiple profiles of mitochondria seen in the gametocytes were in fact derived from a single network-like organelle. By contrast the apicoplast remains comparatively small and closely associated with the nucleus. Recent application of elegant whole-cell confocal studies combined with the use of organelle-specific fluorochrome-tagged markers has emphasized how the mitochondrion and apicoplast subsequently proliferate in the oocyst (Stanway *et al*., 2009). Recent metabolomic studies (Lamour *et al*., 2014) suggest that oxidative – and lipid – metabolism may differ significantly between the asexual and sexual bloodstage parasites. Recognizing the female gametocyte provides the majority of the biomass of the zygote/ookinete, this expansion of energy provision is understandable, but the male gametocyte forms eight microgametes devoid of both plastids, suggesting in the male cell that it performs a vital function during gametocytogenesis or during the dramatic events of gamete *formation* in the mosquito? It has been shown that the apicoplast provides isoprenoids essential for gametocytogenesis (Wiley *et al*., 2014), and up-regulation of glyoxalase provides defence against oxidative stress (Okamoto *et al*., 2008) that has recently been shown to be a component of the mosquitoes' responses to malarial infection (Shrinet *et al*., 2014). Up-regulation of genes encoding type II fatty acid, and 15 of 16 TCA enzymes in the mature gametocyte are also consistent with the cytological observations. In marked contrast it is the enzymes of the glycolytic pathway that are among the most abundant proteins in the free swimming microgamete (Wass *et al*., 2012; Talman *et al*., 2014). The male gamete lacks both plastids (Sinden *et al*., 1976; Okamoto *et al*., 2008); as a consequence their inheritance is maternal (Creasey *et al*., 1993), and the male gametes rely entirely on hexose import and glycolysis for energy to drive their vigorous swimming (Slavic *et al*., 2011; Talman *et al*., 2014). Reflecting on this body of data, it is interesting to note that some of the antimalarial drugs, e.g. atovaquone with known mitochondrial targets such as the cytochrome B1 complex and adenosine triphosphate (ATP) binding cassette transporter (Fry and Pudney, 1992; Rijpma *et al*., 2014), are exquisitely active against transmission of parasites from vertebrate to mosquito hosts, and the ookinete in particular (Fowler *et al*., 1995; Delves *et al*., 2012).

An outstanding dilemma in the understanding of gametocyte biology is whether the pattern of metabolism revealed by the studies on slow maturing/long-lived gametocytes of the subgenus *Laverania* (Smalley and Sinden, 1977; Eichner *et al*., 2001; Bousema and Drakeley, 2011) is representative of the subgenus *Plasmodium*. Evidence suggesting this may be the case stems from the observations that purified populations of viable mature infectious gametocytes of the rodent malaria parasites, like those of *P. falciparum*, can be prepared by treating mixed bloodstage infections with schizonticides (Beetsma *et al*., 1998; Rodrıguez *et al*., 2002).

The impact of gametocytes on the biology of host/parasite–vector interactions has for decades provoked intriguing discussion and experimentation. 'Is the infectious host less responsive to mosquito bites?' (Rossignol *et al*., 1985); 'Is it more attractive to mosquitoes?' (Batista *et al*., 2014); 'Is the sporozoite-infected mosquito more likely to probe?' (Anderson *et al*., 1999). At the cellular level the oft-raised question whether mature (infectious) gametocytes are preferentially retained in the capillaries of the skin where they would be accessible to the mosquito remains an interesting but still unresolved question. The potential now to use late gametocyte promoters to regulate luciferase expression in the mature gametocyte, combined with IVIS technology, might provide the quickest method of resolving this question. Should gametocytes exhibit this tropism, it would seriously impact the methodologies required to describe the infectious reservoir. However, recognizing the now widely acknowledged fact that ~ 80% of persons infected with *P. falciparum* will also be gametocyte positive (Muirhead-Thomson and Mercier, 1952a,b; Muirhead-Thomson, 1957; Bousema and Drakeley, 2011) does raise the question as to how any further knowledge, beyond correlating peripheral gametocytaemia with the probability of infecting mosquitoes (Churcher *et al*., 2013), is going to change any control strategy (Bousema *et al*.,

2012) – which must surely treat every infected individual as if they were infectious to the vector.

Sexual development in mosquito

For this author, the events of malaria microgametogenesis (exflagellation) in the mosquito vector remain one of the most beautifully orchestrated and dramatic developmental transformations in eukaryote biology.

Induction of exflagellation

Our understanding of the induction of gametogenesis at the molecular level has advanced little since its discovery in 1998 (Billker *et al.*, 1998). We know two conditions must prevail: a fall in temperature of $> 5°C$ (Roller and Desser, 1973; Sinden and Croll, 1975), suggesting an as yet undescribed role for heat shock proteins in protein remodelling, and the presence of elevated levels of the mosquito waste product xanthurenic acid (XA) (Billker *et al.*, 1998). The fall in temperature alone may trigger the secretion of the osmiophilic bodies leading to RBC breakdown (see below).

In marked contrast the downstream pathways regulating the component events of gamete formation, namely – escape from the RBC and molecular remodelling of the gamete surfaces and exclusively in the male cell – genome replication, genome separation and axoneme assembly, are now extensively although still incompletely characterized. Current models of the signalling cascade regulating gametogenesis have been outlined (Baker, 2010; Morahan and Garcia-Bustos, 2014; Sinha *et al.*, 2014). Although prior data suggested raised extracellular pH (~pH 8.0) is a useful laboratory ‘inducer’ (Nijhout and Carter, 1978), the overall pH of the bloodmeal only rises by some 0.2 pH units in the 24 h following bloodmeal ingestion (Billker *et al.*, 1997), suggesting that a transient rise in intracellular pH may form part of the signalling cascade. Studies on the role of gametocyte ion pumps (Kawamoto *et al.*, 1992; 1993; Kawamoto, 1993) nonetheless suggested that the cytoplasmic pH of the gametes might change both rapidly and significantly.

Activation (directly or indirectly) of guanylyl cyclase by XA produces cGMP that is in turn converted to guanidine 5 monophosphate by phosphodiesterase (knockout of *pde*δ ablates exflagellation). cGMP activates PKG (McRobert *et al.*, 2008) that in turn maintains elevated levels of cytosolic Ca^{2+} (see below) (Brochet *et al.*, 2014), which regulates rounding up (i.e. increase in volume = water uptake?) of the cells and the expression of at least some translationally repressed surface proteins. Deletion of *pbcax*, however, prevents the onward development of the rounded female cell into an ookinete (Guttery *et al.*, 2013).

XA additionally stimulates PIPLC to produce PIP2 and IP3; these in turn give rise to elevated cytoplasmic calcium just 10 s after activation (Billker *et al.*, 2004; Sebastian *et al.*, 2012) which, through CDPK4, results in DNA replication and axoneme assembly in the male cell, and translation release in the female. Downstream of CDPK4, and mediated by NEK1 and NEK3, MAP2 and SPRK regulate the motility of the axonemes and cytokinesis of the microgametocyte (Tewari *et al.*, 2010). The writer is amused by the observation that a viagra-like molecule (Zaprinast) can obviate the need for XA in male gamete release (McRobert *et al.*, 2008).

Escape from the RBC

Both male and female cells increase in volume (Sinden and Croll, 1975); thus, stressing the host cell, the males additionally release very motile gametes that undoubtedly assist the disruption of a weakened host cell (Sologub *et al.*, 2011; Deligianni *et al.*, 2013; Wirth *et al.*, 2014). Escape is mediated by the secretion of the osmiophilic bodies (more abundant in the sessile female; Sinden *et al.*, 1976). The biogenesis and lytic function of the osmiophilic bodies is dependent on the following moieties Pfg377: exclusively in females (Alano *et al.*, 1995; Olivieri *et al.*, 2014), MDV-1/PEG3 (Lanfrancotti *et al.*, 2007; Lal *et al.*, 2009; Ponzi *et al.*, 2009), pbGEST (Talman *et al.*, 2011) cysteine and aspartic protease, and a perforin-like protein 2 (Wirth *et al.*, 2014).

Genome replication in male gametocyte

The ploidy and replication of the gametocyte genome has been a controversial topic, resolved in large part by the studies of Cornelissen and Janse in the 1980s, who, using cytochemical techniques, showed that mature gametocytes like the merozoite are haploid, and that the male cell replicated its DNA three times in brief period (~10 min) between induction and microgamete release – exflagellation (Janse *et al.*, 1986a,b; Cornelissen, 1988). This replication is *cdpk4* dependent (Guttery *et al.*, 2012a). In *cdc20* and *map2* knockout lines subsequent mitotic separation of the replicated genomes in the male cells is blocked after metaphase (Guttery *et al.*, 2012a). The incredible speed of replication suggested that thousands of replication forks are distributed across the 14 chromosomes. DNA polymerase-α mediates replication, a process sensitive to aphidicolin but not mitomycin C (Janse *et al.*, 1986a). Electron microscopy revealed three conventional mitotic divisions within the single large male nucleus at ~ 3, 8 and 15 min following activation (conventional with the no exception that at no point do the chromosomes condense! – a fact that might correlate with the very rapid replication of the genomes). Although this

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observation is entirely consistent with the absence of histone 1 from the genome, it also raises fascinating questions as to how the initial 14, and final 112, filamentous chromosomes (whose telomeres are likely embedded in the nuclear envelope) can be moved by the kinetochores on the spindle microtubules with such speed and precision without breaking. The female gamete like the originating immature female gametocyte is haploid, but the intervening nuclear events are unclear; data suggested that the DNA content may rise above 1°C (Cornelissen, 1988) and descriptions of spindle-like structures in the developing (putative) macrogametocyte are either erroneous or suggest the female genome is somehow modified (Sinden *et al*., 1978).

Axoneme assembly in male gametocyte

The cytoplasm of the mature microgametocyte contains large quantities of tubulin, which upon activation rapidly polymerize into microtubules. Polymerization begins with the *de novo* assembly of eight basal bodies (apparently as two conventionally orientated orthogonal tetrads). Basal body structure/patterning and function requires SAS6 expression (Marques *et al*., 2015). Axoneme polymerization, being intracytoplasmic, is independent of intra-flagellar transport and consequentially very rapid; all eight axonemes reaching 14 µm in length in just 10 min, when formed, lay coiled around the persistent envelope of the single nucleus. Axonemes are largely of conventional 9 + 2 design (Sinden *et al*., 2010). The formation of the central pair is severely, although incompletely, disrupted when the armadillo repeat protein PF16 is deleted (Straschil *et al*., 2010). At the onset of exflagellation, the axonemes become motile and swim basal body first out of the parental cell. The prior attachment of each basal body to a mitotic spindle pole 'ensures' each basal body drags a haploid genome into the gamete; the gamete then swims through the dense mosquito bloodmeal using alternating periods of fast and slow rotary 'ambidextrous' sinusoidal waves (Sinden and Croll, 1975; Wilson *et al*., 2013). Microgamete motility is entirely dependent on the import of hexose from the bloodmeal (Slavic *et al*., 2011).

Protein remodelling of the gamete surfaces

The surface proteins of the male and female gametes differ both from each other and from that of the parental gametocytes. Sexual differences between gametes, e.g. expression of P48/45, P230 and HAP2 in male and P47 and the LAP/CCCP proteins in female (van Schaijk *et al*., 2006; Raine *et al*., 2007; Liu *et al*., 2008), may reflect functions in fertilization, and differences between gametocyte and gametes may indicate functions related to gamete survival in the challenging (both immunologically and enzymatically) environment of the bloodmeal. Expression of some 169–731 proteins in the female gamete, including the surface proteins P25 and P28 on the fertilized zygote, is dependent on the release of their mRNA from translation repression (Mair *et al*., 2006; 2010; Guerreiro *et al*., 2014), a process in part regulated by CDPK1 (reviewed by Morahan and Garcia-Bustos, 2014). Early defence of the gametes/zygote by expression of a complement regulator factor H-like protein (Simon *et al*., 2013) inactivates complement ingested in the bloodmeal (Grotendorst and Carter, 1987; Margos *et al*., 2001; Simon *et al*., 2013); however, the gametes have no defence against antibody attack targeted against proteins secreted onto the gamete surface (e.g. P45/48; P230), making these molecules excellent targets for 'contraceptive' vaccines (Carter *et al*., 2000; Sauerwein, 2007). P230 is reportedly responsible for the attachment of the male gametes to RBCs in the bloodmeal (Templeton *et al*., 1998), although what possible advantage this is to the parasite escapes this writer.

Fertilization

Fertilization of malaria gametes was first recognized in 1897 (McCallum, 1897) and described at the ultrastructural level some 40 years ago (Sinden *et al*., 1976; Aikawa *et al*., 1984). Gamete recognition and binding is mediated through GPI-anchored P47 on female interacting with P230, complexed to GPI-anchored P48/45 on the male cell. The 'ambidextrous' ability of P230 to bind both male- and female-specific moieties may explain the formation of intimately bound same-sex and mixed-sex clusters of gametes/gametocytes *in vitro* (Janse *et al*., 1985; Sinden and Hartley, 1985; Sinden *et al*., 1985). The fusion of the gamete membranes is now known to be mediated by male-specific HAP2/CSC1 (Liu *et al*., 2008; Mori *et al*., 2010) and can be severely disrupted by anti-HAP2 antibodies (Blagborough and Sinden, 2009; Miura *et al*., 2013). Fusion of the gamete nuclei occurs within 1 h of plasma membrane fusion (Aikawa *et al*., 1984), and is preceded by the decondensation of the chromosomes in the male nucleus. There is substantial transcriptional up-regulation of the zygote genome via AP2O (Yuda *et al*., 2009; Akinosoglou *et al*., 2015). We do not yet have definitive evidence as to when expression of male-inherited genes begins, but zygote development reportedly can progress in the absence of RNA polymerase II mediated transcription (Guerreiro *et al*., 2014).

Within the mosquito the parasite is from the time of zygote formation until budding of daughter sporozoites from the oocyst a polyploid cell, but cytological study shows that the genetic organization of the parasite, fol-

lowing fertilization, is immediately returned to a haploid state, within a single nucleus, by a conventional two-step meiotic division producing four haploid genomes in the ookinete (Janse *et al*., 1986b; Sinden, 1991). Thus, any one oocyst has the potential to contain four discrete and potentially recombinant genotypes; this has been confirmed experimentally (Ranfordcartwright *et al*., 1991).

It must be remembered that the process of gamete production and fertilization occurs in an environment composed essentially of unaltered host blood to which the standing components of the mosquito gut have been added, among which the trypsin-like proteases are significant factor (Muller *et al*., 1993). The introduction of proteolytic enzymes to the bloodmeal degrades vertebrate complement in the first 3 h (Margos *et al*., 2001), a change that is intriguingly mirrored by the short-lived initial defence of the parasite against complement (see above) – a truly fascinating interplay between host, vector and parasite.

Host antibodies persist, at potentially lethal concentrations, in the bloodmeal for at least 24 h, and can escape through the midgut epithelium into the haemocoele (possibly enhanced by the rupture of the epithelium by the invading ookinetes; Han and Barillas-Mury, 2002). Convincing evidence has been provided that cytokines, which can be present at significant levels in the hosts' blood especially following synchronous schizogony (Motard *et al*., 1990), can, when present in the bloodmeal, significantly suppress both gametogenesis and oocyst infection (Dearsly *et al*., 1990; Naotunne *et al*., 1993). The duration for which active host cytokines persist in the bloodmeal is however not known. Interesting data suggesting a dialogue between vertebrate insulin and mosquito receptors (Pakpour *et al*., 2012) raise the fascinating possibility that the antiparasitic activity of mosquito may be both directly and indirectly mediated.

The mosquito places both physical and immune barriers in the path of the parasite. The physical barrier to infection/invasion offered by the peritrophic matrix (PTM) was revealed when inhibitors of parasite chitinase (e.g. allosamidin) were shown to decrease oocyst numbers (Shahabuddin *et al*., 1993; 1995; Shahabuddin and Kaslow, 1994a,b; Zieler *et al*., 1999; 2000). Humoral insect defence mechanisms however remain potent inhibitors when the ookinete crosses the epithelial layer (James, 1928; Dimopoulos, 2003; Osta *et al*., 2004; Christophides, 2005; Povelones *et al*., 2011; Shrinet *et al*., 2014).

The ookinete

Notwithstanding the critical genetic events of meiosis (Sinden, 1985; Sinden and Hartley, 1985) – recently shown to be regulated by NIMA kinases, NEK2 and 4 (Reininger *et al*., 2005; 2009; 2012) and reviewed in Morahan and Garcia-Bustos (2014) – the key role of differentiation of the zygote into the ookinete is to produce a polarized motile cell that can escape the increasingly potent digestive enzymes of the bloodmeal to which the ookinete is susceptible (Gass, 1977; Gass and Yeates, 1979). This differentiation is both fertilization dependent and reliant upon PK7, PPKL (protein phosphatase with Kelch-like domains) and GA kinase activities (Guttery *et al*., 2012b; Philip *et al*., 2012; Morahan and Garcia-Bustos, 2014). Although formation of the single ookinete from each zygote in very large part mirrors the assembly of both the merozoite and the sporozoite from their respective 'schizonts', we know less of the cellular events. Although the assembly and cytoskeletal polarity of apicomplexan 'zoites' is clearly determined by the position of a single nuclear spindle plaque/microtubule organizing centre (Dubremetz, 1975), it is challenging to understand how/whether the ookinete nucleus, which contains *four* recombinant haploid genomes/spindle plaques, similarly dictates the polar assembly of the *single* ookinete cytoskeleton (unless polarity was determined by the early polarization of the zygote nucleus and formation of the perinuclear array of cytoplasmic microtubules; Aikawa *et al*., 1984). The functions of the elaborate, and ookinete-specific, anterior apical collar, beyond providing apparent rigidity to the ookinete penetrative apparatus, have yet to be determined. The functions of those components driving motility are better but still incompletely understood. Conventional thinking suggests motility is dependent on CDPK3, Ca^{++} and cGMP signalling pathways (Siden-Kiamos and Louis, 2008; Moon *et al*., 2009; Guttery *et al*., 2012b), controlling the activity of the extensively described actin-myosin glideosome motor (Raibaud *et al*., 2001; Opitz and Soldati, 2002; Kan *et al*., 2014) that is linked to transmembrane surface adhesins such as CTRP, a large protein composed of seven thrombospondin and six von Willebrand factor A domains, of which only the proximal A domains appear to be essential to locomotion (Ramakrishnan *et al*., 2011). The validity of the conventional glideosome model has recently been challenged by controversial data from *Toxoplasma* suggesting neither actin nor myosin is essential for locomotion (Andenmatten *et al*., 2012). The spiral architecture of the microtubular ookinete skeleton dictates both the overall shape of the ookinete and the direction of movement (essentially a left-handed corkscrew; Vlachou *et al*., 2004; Kan *et al*., 2014), which may be optimal to drive the parasite through the particulate and increasingly 'viscous' bloodmeal.

Whether the movement of the ookinete is in any way directed to the midgut epithelium is not known, but interaction with the complex layers of the midgut epithelium is certainly not understood. The epithelium is composed of

three layers: the chitinous PTM, which is secreted at the time of bloodmeal ingestion (Shao *et al.*, 2001); the tubular network (Zieler *et al.*, 2000); and the cell epithelium monolayer. Traversal of the PTM (if formed) is assisted by the secretion of one or more chitinase enzymes, one secreted as a pro-enzyme that is reportedly activated by the mosquito trypsins (Shahabuddin *et al.*, 1993). Addition of the chitinase inhibitor allosamidin to bloodmeals containing *P. gallinaceum* or *P. falciparum* reduced oocyst numbers (Shahabuddin *et al.*, 1993), and in *P. berghei* knockout of chitinase 1 reduces infectivity significantly (Dessens *et al.*, 2001).

Microscopic evidence has been interpreted as suggesting that ookinetes preferentially invade/lyse epithelial cells at the posterior of the midgut, and close to intercellular junctions. Early work suggested that the 'preferential' oocyst distribution at the posterior midgut was simply due to the sedimentation of the iRBC in the bloodmeal during the prolonged period when the bloodfed female mosquitoes rest 'head-up' after the feed. The validity of this early observation has, however, been questioned (Kan *et al.*, 2014). Studies in the 1990s showing that ookinetes in the midgut wall frequently co-located with v-ATPase activity led workers to suggest that ookinetes invaded a specific subclass of epithelial cell, which they termed the 'Ross Cell' (Shahabuddin, 2002). The study, however, failed to recognize that v-ATPase might be up-regulated following, and not before, invasion. To some, the most compelling data suggesting there is a specific epithelial ligand bound by the ookinete comes from antibody blockade studies where anti-P25, -P28 (Gozar *et al.*, 1998; Miura *et al.*, 2013), enolase (through interaction with host plasminogen; Ghosh *et al.*, 2011) and AgAPN1 antibodies (Armistead *et al.*, 2013) can inhibit invasion. However, it must be recognized that coating abundant surface moieties with antibodies does not necessarily identify specific ligands. Other molecules reducing ookinete invasion by unknown mechanisms when added to the bloodmeal include PLA2 (Rodrigues *et al.*, 2008), bee venom (Moreira *et al.*, 2002) and the peptides SHIVA (Boisbouvier *et al.*, 1998), and SM1 (Fang *et al.*, 2011). Early data (Rosales-Ronquillo and Silverman, 1974) strongly suggested that ookinetes can recognize and lyse RBC – exactly the same mechanisms now believed to mediate midgut invasion, it is thus tempting to speculate that the host molecules inducing secretion of the lytic contents of the abundant micronemes are shared between the RBC and mosquito epithelial cells (and are perhaps somewhat less specific than commonly hypothesized). In our efforts to develop ever improved transmission-blocking vaccines, we must recognize we know too little of the molecular architecture and immunogenic potential of the ookinete surface.

Twelve to 36 h following ookinete–epithelium interaction, the secretions from micronemes lyse the host cell and the ookinete migrates into the resulting 'cadaver'. Should many ookinetes invade in the same locality epithelial destruction is considerable (Ecker *et al.*, 2007), with the consequent opportunity of transfer of bloodmeal contents to the haemocoele, significant parasite density-dependent mosquito death may occur at this time. The writer is fascinated by the question of how the ookinete escapes, in a directed manner, the sac of the lysed midgut cell, particularly noting the very different molecular profiles presented by the cytoplasmic membrane surfaces potentially encountered. The 'time bomb' theory of midgut invasion (Han and Barillas-Mury, 2002) suggests the lysed cells will be ejected from the epithelium by a draw-string repair mechanism, involving substantial reorganization of the epithelial–cell actin network (Shiao *et al.*, 2006); thus, escape from the lysed cell is essential for 'infection' to be a success. We do not yet understand what, on reaching the luminal/midgut face of the basal lamina, induces the emergent ookinete to become immotile and begin differentiation into an oocyst, but it is tempting to suggest that the molecular recognition of the collagen-rich basal lamina is a regulatory factor. Note, however, that the ookinete is very motile in Matrigel® (Corning Life Sciences), an artificial intercellular matrix rich in (mouse) laminin, enactin and collagen (Moon *et al.*, 2009).

Irrespective of the extent of the damage caused by the ookinete to the epithelial cells, the mosquito mounts a significant response to the ookinete and developing oocyst within the epithelial layer. Current evidence in a rapidly changing area of study suggests that, in a nitration-dependent event (Molina-Cruz *et al.*, 2008), the mosquito thioester (complement-like) molecule TEP-1 forms a complex with LRIM1 and APL1 and the ookinete surface, causing the lethal deposition of melanin on the parasites' membrane systems (Sinden and Garnham, 1973; Fraiture *et al.*, 2009; Povelones *et al.*, 2011). The presence of the protein Pfs47 on the parasite surface reportedly reduces this nitration-dependent attack mechanism (Molina-Cruz *et al.*, 2013). Other PKC-mediated antimalarial immune response pathways have been identified (Pakpour *et al.*, 2013).

Although infection of the mosquito is theoretically 'completed' following the induction of sporogony in the ookinete under the basal lamina, the parasite nonetheless undergoes further unique and poorly understood developmental steps that intrigue the writer, topmost being the restructuring of its ribosome population. In the early 1990s pioneering work in the McCutchan laboratory recognized that the sSUrRNA from sporozoites (S) and asexual blood stages (A) differed (Zhu *et al.*, 1990; Waters, 1994). Subsequently, it was demonstrated that shortly after fertilization a third form (O) of the 18S RNA is expressed to be

replaced in the late oocyst by the S form (Li *et al*., 1997). It is interesting to note that the small ribosomal subunit protein S1 has the highest transcript abundance in the gametocyte and S2 in the ookinete; whether this is related to the skeletal 18S rRNA changes is unclear. What biological critical advantage is gained from these fundamental and fascinating changes in ribosome design are still unresolved.

The future?

Over the past 50 years the advances in our understanding of malaria cell biology have been truly amazing, what of the next 50 years? Ongoing metabolomic studies are opening a broader understanding of parasite function and might be expected to play a central role in future drug development programmes. Image resolution does not need to improve on that of the electron microscope (at the cellular level) and X-ray crystallography (at the molecular level), but without doubt the new abilities to determine in live or un-fixed specimens, the exact position of identified molecules in complex molecular/cellular machines will be utterly fascinating (Wong *et al*., 2014). This combined with *in vivo* single-cell imaging has the potential to transform our understanding of these amazing cells.

Recognizing the paucity of technologies available to cell biologists in the 1970s, it is somewhat bizarre to believe that it is almost to be taken for granted that we will have at our disposal fluorescent-tagged constructs, and knockouts of every parasite gene of interest, but to be provocative, if we are to make significant steps in our understanding of the parasites molecular machines perhaps we should already consider the construction of appropriately tagged protein libraries for the *ultrastructural* localization of molecules *in situ* by cryo-tomography. As ever we will be dependent on emerging technologies, such as single-cell 'omics', but perhaps we should think more of the difficult areas of lipid and carbohydrate biology and the roles of small nucleic acids in parasite biology.

It is now clear that in our efforts to reduce the impact of these potent pathogens, the sexual and early sporogonic development of malarial parasites offer unique opportunities to attack vulnerable bottleneck populations with both drugs (Ruecker *et al*., 2014) and 'simple' antibody-based vaccines (Miura *et al*., 2013). It should therefore be a collective priority to understand: first, the contrasting metabolic organizations of the quiescent (and comparatively schizonticide insensitive) mature gametocyte and the diverse pathways mediating gamete, zygote and ookinete development; and second, how the molecular composition and architecture of the exposed surfaces of the gametes, zygotes and ookinetes define the immunological vulnerability of both fertilization and zygote development. We live in exciting times with ever new and powerful technologies at our disposal; we must however accept our responsibility to those infected by these formidable pathogens that perhaps the most important task in front of us is to ensure the considerable knowledge we have already gathered and will continue to gather is translated into useful intervention measures, a process which of itself provides academic challenges and rewards every bit as demanding as those of basic research.

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