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Gametocytogenesis : the puberty of *Plasmodium falciparum*

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Abstract

The protozoan *Plasmodium falciparum* has a complex life cycle in which asexual multiplication in the vertebrate host alternates with an obligate sexual reproduction in the anopheline mosquito. Apart from the apparent recombination advantages conferred by sex, *P. falciparum* has evolved a remarkable biology and adaptive phenotypes to insure its transmission despite the dangers of sex. This review mainly focuses on the current knowledge on commitment to sexual development, gametocytogenesis and the evolutionary significance of various aspects of gametocyte biology. It goes further than pure biology to look at the strategies used to improve successful transmission. Although gametocytes are inevitable stages for transmission and provide a potential target to fight malaria, they have received less attention than the pathogenic asexual stages. There is a need for research on gametocytes, which are a fascinating stage, responsible to a large extent for the success of *P. falciparum*.

Introduction

Plasmodium falciparum has the morbid characteristic of being the deadliest protozoan parasite of humans. Like all malaria parasites, it is an organism with an obligatory sexual reproduction which takes place in the mosquito midgut. After several development stages, the parasite migrates to the salivary glands to be injected into the next human host. However, before it can succeed sexually in the mosquito host, *P. falciparum* undergoes a puberty-like process in the human blood: an asexual parasite goes through a series of changes, which will lead to the generation of a sexually competent parasite. This maturation has been termed gametocytogenesis, whereby male and female gametocytes (i.e. pre-gametes) are produced to later fertilize in the invertebrate host.

One of the intriguing facts about sex is that it has been established and maintained regardless of how expensive it might be to the organism bearing it [1]. Several costs are associated with sexual reproduction. There is a two-fold disadvantage as compared to asexual proliferation: the investment in securing a mate and the risk of mixing genes with another, possibly burdened, individual [2]. The fusion of genomes (syngamy), followed by meiosis, whereby chromosomal segregation and recombination occur, seems, however, to confer sufficiently powerful advantages to have driven and maintained sexual reproduction in a vast majority of eukaryotes.

Gametocytogenesis delivers a gametocyte, the only transmission stage from the human to the mosquito. Moreover, it allows sexual achievement and therefore

recombination with other genotypes. The present review focuses on specific points of commitment to sexual development – gametocytogenesis and gametocyte biology– especially those relevant to transmission and evolution of *P. falciparum* transmission strategies.

The biology of gametocytogenesis

A gametocyte is a cell specializing in the transition between the human and the mosquito host. In order to adjust to life in such drastically different environments, many changes occur in its cell biology, metabolism, gene expression and protein synthesis.

Facts and figures on gametocytogenesis

Gametocytaemia, i.e. the presence of gametocytes in the peripheral blood, arises 7–15 days after the initial asexual wave [3,4]; this maturation period has long been compared to that of the other human malaria species (1–3 days) [5]. It is well established that the ratio of gametocytes to asexual stages in *P. falciparum* is less than 1:10 [6-9]; a recent study calculates a much lower ratio (1:156) [3]. The half-life of the mature gametocyte in the blood is generally reported to be 2.4 days, based on the observations of Smalley and Sinden [10]. Eichner and colleagues [3] have, however, reported a mean circulation time of 6.4 days which is about twice the expected 3.4 days deduced from a 2.4 half-life. Some gametocytes have been found to have a longevity of up to four weeks in the bloodstream [10].

From merozoite to gametocyte

Gametocytes arise from erythrocytic asexual stages. The production of gametocytes directly from hepatic merozoites, which has been described in other species, does not occur in *P. falciparum* [11]. There has been much debate on the actual point of sexual differentiation and Bruce and colleagues [12] have shown that merozoites emerging from a single schizont developed either into further asexual stages or into gametocytes. It has been further shown that the gametocytes from one schizont are all male or all female [13,14]. This suggests that the trophozoites of the preceding asexual generation were already committed to either sexual development or continuing asexual cycling.

Morphology of gametocytes

Field and Shute [15] first described five different maturation stages of *P. faciparum* gametocytes. These different steps were further characterized by light microscopy using blood from *P. falciparum*-infected *Aotus* monkeys [16] and later by electron microscopy [17,18]. Stages I to V are described in Table 1. One of the most striking feature of gametocytes is the presence of a pellicular complex, which originates from a small membranous vesicle observed beneath the gametocyte plasmalemma in late stage I. This structure is absent from asexual stages. It consists of a subpellicular membrane vacuole subtended by an array of longitudinally-oriented microtubules [8], which strengthens the parasite, explaining the lack of amoeboid forms observed in asexual parasites [17]. The function of this structure is still unknown.

Gene expression in gametocytogenesis

Gametocytes of *P. falciparum* have been shown to exhibit a different pattern of gene expression than asexual stages, which is unsurprising if one considers the difference of fate between these two stages. Transcription and translation levels are not constant during gametocytogenesis: this was shown in drug sensitivity studies where RNA and protein synthesis levels were much more important in the early than the late gametocyte stages [8]. Furthermore, a sex-specific expression has also been discovered, with differences in RNA, mitochondria and ribosome content. The female is preparing for a continued development, whilst the male is terminally differentiated and only needs what is necessary for exflagellation (e.g. cell division cycle, dynein and α-tubulin II).

Many stage-specific RNAs and proteins have been described and have been reviewed elsewhere [19]. Most studies have concentrated on surface antigens, the majority of these characterized antigens are gamete antigens that are synthesized during gametocytogenesis. Some are released into the human host circulation in large numbers from dead gametocytes that were not ingested by mosquitoes, hence the immune response observed against some of these gamete surface antigens [20]. With the full genome sequence available, the functional characterization of individual open reading frames (ORFs) offers new insights into the biology of gametocytogenesis. Differential transcription and proteomic studies using different techniques have already been performed. The developmentally differential expression of distinct ribosomal RNA isoforms was highlighted in *P. falciparum* with at least two rDNA transcription units: one expressed predominantly in sporozoites (S-genes) and one in the asexual cycle (A-genes). Interestingly, the transition between the A and S expression starts during gametocytogenesis [21,22].

The large scale proteomic study carried out by Lasonder *et al.* [23] has identified 1,289 malaria proteins of which 315 were solely expressed in the gametocyte, 103 were shared with trophozoite and schizont stages, 163 were shared with gametes and 350 were common to all stages (trophozoites, schizonts, gametes and gametocytes). Of these specific expressed proteins, many were found to have functions as diverse as mRNA processing, ribosomal proteins, cell cycle-DNA processing, energy metabolism [24] and cytoskeletal structure [25]. The number and vari-

Table 1: Morphology of gametocytogenesis

	Stage I	Stage II	Stage III	Stage IV	Stage V
Shape (Light microscopy) [4,16, 18, 159]	IA: Indistinguishable from the small round trophozoite IB: Larger round shape, distinguished by granular distribution of pigment in food	IIA: elongates within the erythrocyte IIB: D shaped	D shaped, slightly distorted erythrocyte Pink/blue distinction of the male/female.	Elongated and thin parasite, distorted red cell Male: pigment tends to be scattered Female: pigment more dense	Sausage shaped parasite with rounded extremities Male: pigment scattered, pink Female: dense pigment, light violet (see Figure [1])
Ultrastructure [18]	-No visible alteration of the erythrocyte plasmalemma ("knobless"), opposite the knobbed asexual infected erythrocyte -Formation of a subpellicular membrane flattened vesicle and microtubule array -Sexual dimorphism in nuclear size	-Subpellicular membrane and microtubule complex in expansion (giving the D form) giving an asymmetrical cell -Nucleus in terminal site or elongated across the long axis of the cell; some spindle observed within it	-Further development of the subpellicular membrane complex distorts cell -Male nucleus is notably larger(also becoming lobbed) then that of the female, which contains slightly more ribosomes, ER and mitochondria then the male.	-Membrane and microtubule complex now surrounds the gametocyte completely (restoring symmetry) -Appearance of membrane bound osmophillic bodies -Obvious sexual dimorphism: mitochondria, ribosomes and osmophillic bodies more numerous in female, a transcription factory is observed; male nucleus is larger In male kinetochores of each chromosome are attached to an electron dense body, located over a nuclear pore, opposite of which there is a MTOC (cytoplasmic face of the pore)	-Loss of subpellicular microtubules by depolymerisation, the inner membrane remains -Microgametocyte (male) exhibit a dramatic reduction in ribosomal density, very few mitochondria, with a large nucleus with a kinetochore complex attached to the nuclear envelop -Numerous mitochondria, ribosomes and osmophillic bodies in the macrogametocyte (female); the nucleus is small with a transcription factory
Time of appearance (days) [159]	0–2	1–4	2–8	6–10	9–23
Point in the cell cycle [160]	G1	G1	G0	G0	G0

ety of the gametocyte specific protein reflects the drastic change for which gametocytes are preparing. The way in which the transcription and translation patterns shift from asexual cycling to gametocytogenesis must be exceptionally well adapted to the shift from one host to another.

On the side of the crucial identification of transcripts and protein, it is also important to unravel the regulation processes in gene expression. Given the rich A+T content of the genome [26], it has been tricky to identify promoter sequences. Transcription regulation is thought to be monocistronic. Proteomic analyses have failed to detect a stage-specific chromosomal clustering of gene expression [24]. Accordingly, the differential transcriptome analysis performed for asexual blood stages has corroborated the hypothesis of polycistronic regulation in *P. falciparum* [27]. Although a common regulation for stage-specific groups of genes is attractive, a full transcriptome analysis of both asexual and sexual blood stages is needed to gain a clearer picture of the differential expression patterns.

Are gametocytes diploid?

DNA synthesis was shown to take place at the very beginning of gametocytogenesis, between the sexually committed ring form and the stage I gametocyte [28,29]; from then on the DNA content of gametocytes is about twice the haploid amount. The gametocytes of *Plasmodium berghei* also have more DNA than the haploid content, but significantly less than the diploid amount [30]. This observation, together with the fact that genome segregation failed to be detected with Feulgen staining, indicates that the excess of DNA in *P. falciparum* may not be due to a duplication of the genome. The macrogametocyte has

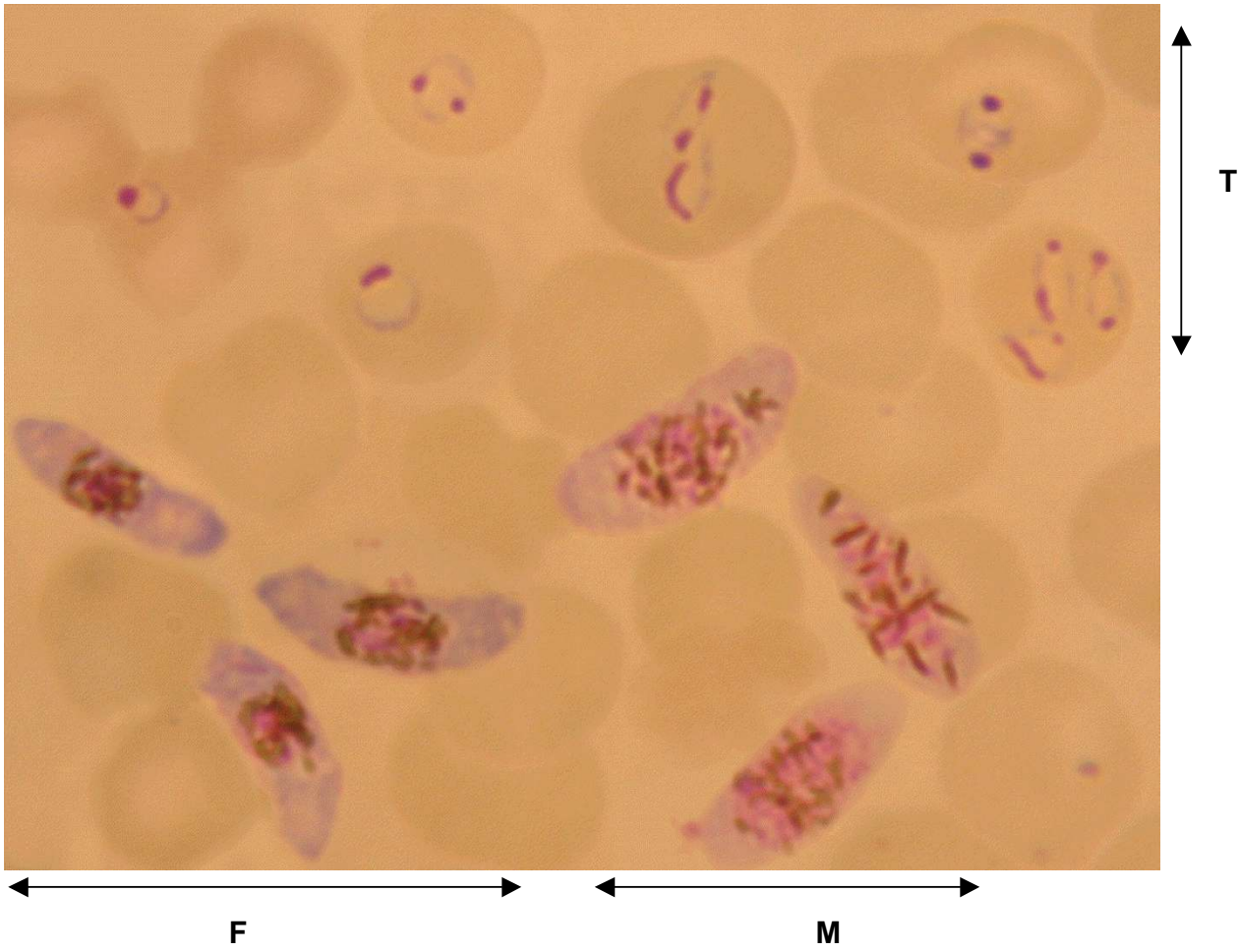

Figure 1
Mature female (F) and male (M) gametocytes and trophozoites (T) of *Plasmodium falciparum* in the blood of malaria-infected patient. This picture is a composite of several pictures originating from the same Giemsa-stained thin smear.

not been shown to be diploid, and the microgametocyte is certainly not octoploid, in contrast to that of *P. berghei* [30]. The triple duplication of the genome occurs only after activation of the microgametocyte [28]. Finally, the supplementary DNA may be accounted for by selective gene amplification. Sinden suggested that this DNA may represent amplified ribosomal RNA genes located in the nucleolus [8], as the gametocyte prepares for the expression of the S form in the mosquito stage [31]. Although rRNA could not be detected in the nucleolus of *P. berghei* by *in situ* hybridization [32], the concept of selective gene amplification pre-synthesis is an attractive hypothesis in view of the transient role of gametocytes and needs to be further explored.

Metabolic changes in sexual development

P. falciparum gametocytes switch from one environment to another and this must involve the parasite in considerable metabolic changes considering the major differences between the two microenvironments. The rich milieu of the blood is modified by mosquito factors (such as serine proteases and chymotrypsins) [33], there is a drop in temperature, a modified pH and, importantly, the parasite becomes extracellular from then on.

In the intra-erythrocytic asexual stage, energy is mainly produced by anaerobic glycolytic ATP production in the Embden-Meyerhoff-Parnas pathway [34]. It is presumed that glycolytic enzymes are also present and active in gametocyte and mosquito stages, but no evidence has supported this assumption so far, except that the mosquito stages of *Plasmodium yoelii* express lactate dehydrogenase in *Anopheles Stephensi* and *Anopheles gambiae* [35].

The mitochondria of asexual parasites and early-gametocytes have been shown to have few, if any, cristae. Stage III-IV-V macrogametocyte exhibit an increase in cristate mitochondria, whereas microgametes have very few mitochondria [17,36]. Furthermore, susceptibility to drugs and metabolic inhibitors is also reduced during the end of gametocytogenesis, except for the drugs derived from artemisinin and the 8-aminoquinoline primaquine. Although the mode of action of these drugs is still unclear, it is probably related to mitochondria and oxygen consumption in the case of primaquine [37,38]. Artemisinin is thought to inhibit a sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)-type *P. falciparum* protein, called PfATP6 [39]. It has also been shown that the *P. falciparum* mitochondrial gene encoding cytochrome b had an expression several folds higher in sexual stages than in asexual stages [40,41]. These differences in structure and chemical susceptibility have been hypothesized to be due to the development of an active tricarboxylic acid (TCA) cycle and respiration during gametocytogenesis. Although a differential oxygen consumption has not been clearly established, the possibility of a metabolically active, yet underdeveloped, TCA cycle has been suggested but remains to be proved [36,42].

Another function of the electron transport system (ETS) is the pyrimidine biosynthesis. The latter evidence of mitochondrial activity could be accounted for by this mechanism. The ETS has been shown to be active in both the asexual and sexual parasite by the identification of the enzyme and by drug sensitivity assays [43]. Functional mitochondria of both stages contribute to the *de novo* synthesis of pyrimidine [36]; the enzyme by which this process is achieved is dihydroorotate dehydrogenase (DHODase). Drugs that inhibit specifically DHODase were found to affect *P. falciparum :* atovaquone and salicylhydrox-amic acid have been reported to inhibit the survival of asexual stages and early-stage gametocytes but not that of late stages. This tends to indicate that *de novo* pyrimidine synthesis in early stages of gametocytes is sufficient for the whole maturation process [43,44].

Phosphoenolpyruvate carboxykinase (PfPEPCK) is another differentially-expressed enzyme known to catalyse CO_2 fixation. The transcription and activity of this enzyme was found to be up-regulated in gametocytes and zygotes as compared to asexual stages. It was hypothesised that this enzyme was abundantly transcribed in gametocytes in order for the post-fertilization stages to adapt to the scarcer access to glucose in the mosquito haemolymph by means of an alternative ATP source through a gluconeogenesis pathway [45,46].

Asexual blood parasites and young gametocytes rely extensively on haemoglobin digestion as a source of amino acid and iron [47]. As one might predict, this mechanism is abandoned during maturation of gametocytes, possibly between stage III and IV [46].

Whereas asexual metabolism has been quite well characterized, there is a general lack of research on the metabolism of gametocytes. Gametocytocidal drug development deserves more attention in view of their potential role in reducing transmission.

Sequestration of gametocytes

The mature asexual stages of *P. falciparum* are absent from the peripheral circulation, due to the adherence of infected erythrocytes to microvascular endothelia of many organs and tissues such as heart, lung, liver, skin and brain [48]. Sequestration allows avoidance of phagocytic clearance in the spleen during maturation [49]. A similar phenomenon is also observed in maturing gametocytes: stages I to IV are sequestered preferentially in the bone marrow and spleen [50,51], whilst stage V are released in the peripheral circulation and only become infectious to mosquitoes after a further two or three days of circulation [10,52].

The mechanism of sequestration of asexual stages is that of cytoadherence of infected red blood cells (IRBCs), mediated by a series of host receptors: intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), P-selectin, thrombospondin (TSP) and most strongly with the glycoprotein CD36 [[53-57], reviewed in [58]]. The parasite ligand for CD36, ICAM-1 and TSP has been identified as the *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1) [59]. This protein is encoded by the highly variant *var* gene family [60,61].

Day and colleagues [62] have shown that the cytoadherence of stage I and IIA to C32 melanoma cells is indistinguishable from that of the asexual stages. CD36 binding was observed, as well as knobs on the surface of erythrocyte and HRP-1 expression. Furthermore, the PfEMP-1 pattern of expression is similar in these very young gametocytes compared to the one observed in asexual stages [63]. This is further corroborated by the work of Smith and colleagues [64] who observed CD-36 PfEMP-1 mediated non-opsonic phagocytosis of stage I and II A infected erythrocytes, confirming the importance of the CD36-

PfEMP-1 interaction in the sequestration of these stages. It is interesting to note that a deletion on chromosome 9 results in loss of both adhesion of asexual stages and gametocytogenesis [65].

Stage III and IV are also sequestered in the bone marrow, yet it has been found that the mechanism mediating this process is different from that of other stages: PfEMP-1 is not expressed on the surface after stage IIB [66], sequestration in bone marrow does not involve a PfEMP-1-CD36 interaction [67] and CD36 mediated phagocytosis is greatly reduced in late stages [64]. Candidate low avidity receptors for sequestration of stage IIB to IV have been identified, they include ICAM-1, CD49c, CD166 and CD164 [67]. The gametocyte ligand for this interaction remains unidentified.

Nacher [68] suggested that the specific banana shape of *P. falciparum* gametocytes could have been selected naturally to facilitate the detachment of mature transmission stages from the bone marrow, the sausage shape parasite being more susceptible to rheological forces.

On the available evidence to-date, one can hypothesize that the asexual blood stages and stage I and IIA gametocytes use the same mechanisms for sequestration. One might ask how do the asexual and early sexual stages sequester in different locations, with the same mechanisms, and what mechanism mediates this differential sequestration? And what is the advantage of changing of sequestration mechanism in the course of gametocytogenesis?

Commitment to gametocytogenesis

Genetic variation in commitment to sexual development

Differences in the rate of sexual conversion have been reported between isolates of *P. falciparum* from different patients [69,70], and between cloned lines derived from the same isolate [71]. Individual clones *in vitro* also seem to lose spontaneously the ability to produce gametocytes after *in vitro* culture [72]; the period necessary to lose this ability is variable amongst clones, from a few weeks to more than a year [65,70,71,73]. This suggests that a genetic factor influences the rate of conversion to sexual development in *P. falciparum.* There is a selection shift when the parasite is grown *in vitro.* Transmission stages are no longer selected for, which could explain the variation in isolates but not for individual clones. The probable mechanism accounting for this variation is chromosome deletion, especially chromosome 9 subtelomeric deletion, which is commonly observed in cultured parasites [65,74]. One could also imagine single gene defects, for instance, the knock-out of the Pfg27 leads to loss of sexual phenotype [75]. The observation that *in vitro* lines that had not produced gametocytes for many years could still be environmentally stimulated [76,77] could indicate that a mechanism for loss of sexual phenotype is the loss of capacity to respond to the environment, which would be bypassed by artificial induction.

Environmental factors influencing commitment to sexual development

The first demonstration that the rate of conversion was influenced by environmental factors was that of Carter and Miller [78]. The study demonstrated in three different strains that the addition of fresh blood cells to the culture and a lower parasitaemia significantly reduced commitment to gametocytogenesis. Since then many factors have been found to influence gametocytogenesis, even if there have been problems in reproducing some of these results [79].

Host factors

The rate of commitment to gametocytes production was shown to be influenced by host factors, of which the most important is host immunity. An induction of gametocytogenesis due to increased immune pressure was demonstrated in *P. yoelii* using immunization of a mouse by a protein expressed throughout the life cycle [80] and for *Plasmodium chabaudi* in partially immunized mouse [81]. This stimulation was also found *in vitro* for *P. falciparum* using lymphocytes and serum from *P. falciparum*-infected children [82] and the culture supernatant of hybridoma cells producing anti-*P. falciparum* antibody [83]. Although these results must be taken with caution as for most of them a decrease in gametocytaemia was associated with the increased proportion of gametocyte or rate of commitment to gametocytogenesis. However, as the immune system acts upon both the supply of gametocytes (asexual parasites), it is plausible that a higher commitment to gametocytogenesis is stimulated by immune stress. Further investigations are needed.

Host steroids and corticosteroids have also been associated with a higher commitment to sexual development [84]. Furthermore, there are reports that, *in vitro,* a high proportion of reticulocytes in the blood stimulates gametocytogenesis [85,86]. Anaemia of the host is also a risk factor associated with gametocyte carriage; as anaemia is usually due to long term poorly treated or untreated infections, increased gametocyte density might be the result of the length of the infection more than that of an adaptive mechanism for optimization of sexual stage development [87,88].

Drug-treament

The induction of gametocytogenesis by treatment has been shown for both chloroquine [89-91] and for sulfadoxine+pyrimethamine [92,93]. This effect has also been shown to be enhanced in resistant strains [92,94,95].

These effects are to be taken with caution as data for untreated patients are impossible to collect, for obvious ethical reasons. The mechanisms by which this commitment would be enhanced are quite obscure; however, the "stress" of treatment on the asexual stages may act as a positive feedback on gametocytogenesis (see below). It is worth noting that artemisinin derivatives combination therapy has been shown to be gametocytocidal [96] and to reduce but not suppress transmission [95,97].

Signal transduction pathway

The fact that a population of parasites is capable of having a marked response to the environment indicates that there are means of receiving and responding to environmental signals. There is consistent evidence of the involvement of cAMP-dependent and Protein Kinase C-dependent pathways involved in the induction of gametocytogenesis [70,98-104]. Yet the exact pathway for induction of gametocytogenesis is still to be determined.

Parasite factors

Mixed-genotype infections have been associated with a higher gametocytaemia in *P. chabaudi*, indicating that the parasite responds in terms of transmission to the presence of a competitor [105]. This has, however, not been investigated in *P. falciparum*. There also is a widely recognized correlation between asexual parasitaemia and commitment to sexual development. It is commonly accepted that, when the levels of asexual parasitaemia decrease, the rate of conversion to gametocytogenesis will increase [79,106]. An autocrine factor may be responsible for the link between asexual parasitaemia and stimulation of sexual conversion. This was first demonstrated by Williams [107], who observed higher conversion rates in cultures supplemented with conditioned media and grown in co-culture. This was also observed by Dyer & Day [108], who hypothesized that the sexual pathway is the default pathway and that its inhibition by an autocrine factor would favour asexual growth in the first instance, the sexual route being then favoured in a density-dependent manner. The fact that lysed parasitized erythrocytes stimulate gametocytogenesis [109] could indicate that this factor is liberated at schizogony with the rupture of the erythrocyte. However, the actual factors responsible and the way in which they can modify the level of sexual conversion are still to be determined.

The parasite appears to have the ability to modulate the proportion of the parasite population within an infection that undergoes sexual development. In 1908, Stephens and Christophers concluded that gametocytogenesis increases when conditions are unfavourable to the parasite [110]. Indeed "stress" seems to be the general enhancer of sexual conversion whether from the host immunity, drug-pressure and competing parasites in the host. This fast adaptive system seems to be regulated by a signal transduction pathway and allows *P. falciparum* to maximise its transmission success.

The sex of gametocytes

Plasmodium species are hermaphrodite. An asexual parasite can develop after several rounds of multiplication into either a male or a female gametocyte [111]. In *P. falciparum*, gametocytes are produced from asexual stages. All the gametocytes produced from one sexually committed schizont are of the same sex, suggesting that sex is determined at the very beginning of sexual development [14]. However, gametocyte sex can only be microscopically differentiated from stage III onwards.

Sex-ratio in malaria parasite

The sex ratio has been shown to be generally female-biased in *P. falciparum* and related protozoan species [94,112-117]. In *P. falciparum*, ordinarily one male gametocyte is observed for three or four females. This is mainly explained by the fact that one male gametocyte can give rise to up to eight male gametes, thus establishing an approximate 1:1 ratio in the mosquito midgut. However, large fluctuations in gametocyte sex ratio are observed [94,113,118].

The work of Burkot and colleagues [111] suggests that the sex ratio is clone specific and would, therefore, have a genetic component. This is widely accepted in the literature given the role of natural selection in determining sex ratios [112,114].

Sex allocation theory

The way by which *Plasmodium* modulates its gametocyte production and gametocyte sex ratio has recently been reappraised within an evolutionary framework wherein gametocyte allocation is considered as an adaptive phenotype. The framework proposes two complementary cues to explain the observed gametocyte sex ratio variation: (a) the average complexity of infection and thus the level of in-breeding and (b) that the sex ratio is optimized to ensure successful fertilization in the short term.

Complexity of infection

In-breeding is thought to play a role in the observed variation in sex ratio [112]. If having an equal number of male and female (in the mosquito) is the optimal sex allocation strategy in a random mating population [119], a bias in the sex ratio is expected where this condition is not fulfilled. In a monoclonal population (100% inbred), the sex ratio that maximizes the success of transmission, i.e. the one producing the greatest number of zygotes, will be favoured, hence creating a very female-biased sex ratio. Whereas in the case of outcrossing, natural selection will favour a less female biased sex ratio, because a clone pro-

ducing more males will have a higher genetic representation in the progeny than one producing a more female biased sex ratio. This corresponds in fact to a shift in the level of selection from the clone level to the individual parasite level.

Malaria parasites do not mate randomly and can exhibit elevated levels of in-breeding [120], which can contribute to the observation of a female biased sex ratio. A few studies bring support to these predictions in *P. falciparum* [112,113,121]. But this alone is insufficient to explain all the fluctuations observed [122].

Fertility insurance

The parasites also have fast adaptive mechanisms to respond to the environment in order to maximize fertility. It has been shown that they respond to at least two factors. Anaemia appears to favour a less female-biased sex ratio [118,123]. An increase in the human hormone erythropoietin, induced by anaemia, directly or indirectly triggers this higher commitment to the production of male parasites [123]. As infection progresses, malaria-induced anaemia coincides with an increase in immune pressure against gametocytes; such a pressure would disadvantage the male gamete in the mosquito gut, given their motile status and the urgency to find a female to mate with (within 30 minutes for a successful fertilization). A more male-biased sex ratio would, therefore, be more favourable for a successful transmission [122]. Another possible explanation is that anaemia reduces the chance of encounter between male and female gametes, because of the lower gametocyte content in the bloodmeal [124]: a higher proportion of male would, thus, maximize the chances of fertilization [5]. A recent study by Reece *et al.* [125] demonstrates a higher longevity of male gametocytes in *P. chabaudi*. This could account for some of the variation in sex ratio observed in the course of an infection. However, this question needs to be further investigated in *P. falciparum*.

The second related factor is the temporal fluctuations of gametocytaemia. A gametocytaemia peak was associated with a higher proportion of females from this peak during the two following weeks. This would be favourable in that the encounter of a male and female is guaranteed by the large number of gametocytes, a balanced gamete sex ratio, therefore, giving the greatest reproductive success [118]. Other factors which have been shown to induce gametocytogenesis do not appear to influence sex ratios, such as the vertebrate host testosterone [126] and drug-treatment [91]. This also suggests that sexual development and sexual differentiation operate through two distinct induction pathways. It is vital to understand the mechanism of sex determination and of sex ratio control given the importance that sex ratios have on the infectivity to mosquitoes [94,122].

Evolutionary considerations

Sex in the Apicomplexa

Sexual reproduction is a common phenomenon in natural populations; all apicomplexan have a sexual stage in the course of their life cycle [127]. The advantages conferred by sex have intrigued evolutionary biologists for many years. The various theories [128-132] fall into one of two types of models: the environmental model, which suggests that sex has evolved to allow adaptation to a changing environment by favouring recombination, and the mutation-based models, which assume that sex is advantageous to suppress deleterious mutations more rapidly. However, the explanation might be in a pluralistic approach [133].

Aside from the nucleus, *P. falciparum* has two organelles containing DNA: the mitochondria (linear, 8 kb) and the apicoplast (circular, 35 kb). The latter seems to be evolutionarily related to a structure present in red algae and chloroplast of plants. This plastid is essential to the parasite, but its comprehensive role is still uncertain [134]. The exclusive maternal inheritance of this structure suggests the existence of a cytoplasmic incompatibility between male and female gametes, as previously demonstrated in plants [135]. Such incompatibility had been strongly suspected in outcrossing experiments and could be linked to reproductive isolation of the parasite from different geographical areas [136]. In the same way, research into evolutionary maintenance of sex can benefit from the large amount of work already done in plants and algae, of which the sex life seems quite related or may even be homologous to that of *P. falciparum*.

Apicomplexan parasites supposedly benefit from genetic recombination following sexual achievement during meiosis. Recombination has been shown to occur in *P. falciparum* [137-140], *Plasmodium vivax* [141], *P. chabaudi* [105], and *Toxoplasma gondii* [142].

Mixed infections

Where there is recombination, there has to be at least two genetically distinct parasites; the interaction of several populations of different genotypic types remains elusive. The adaptive sex ratio function of complexity of the parasite population in the long term has already been discussed (see Sex allocation theory). It was postulated that the parasite could also have a fast adaptive system responding to the complexity of the infection [143]. This was borne out by the fact that multiple-genotype infections are often more successful in terms of transmission than single-genotype infections [105]. This might not reflect co-operation between conspecific clones, but it

could be that competition between parasites increases the chances of transmission by creating an advantageous effect on the competiting clones. This may be confirmed by the work of Arez and colleagues [144], who observed a lower proportion of mixed-genotype infections in the mosquito than in the human host. This may, however, only be due to the fact that mixed-infections are not necessarily synchronous, which lowers the chances of recombination. The dynamics of genotype interaction needs to be further characterized, to reveal how it might affect both the disease and the transmission.

Commitment to gametocytogenesis has also been shown to be altered when another species of human malaria parasite is present. For instance, the presence of *Plasmodium malariae* may boost the gametocyte production of *P. falciparum* [145].

Why so few gametocytes?

Not only are gametocytes the first step to sexual genetic recombination, but they also are the transmission stage. A great selection pressure is, therefore, upon them. The transmission phenotypes observed in natural infections must be ones that optimize the greater chances of a successful shift of host. A positive correlation has been found between gametocyte density in the blood and infectiousness to mosquitoes [94,146-148]. However, this correlation must be qualified as loose and may be hampered by the low sensitivity of microscopy [149] and by transmission-blocking immunity [19].

Plasmodium and closely related apicomplexan have evolved an asexual erythrocytic cycle. This character is thought to have evolved several times in the Apicomplexa [127]; an acceptable explanation is that erythrocytic proliferation allows them to generate more transmission stages than with tissue merogony alone. Furthermore, Dyer and Day [79] proposed that indefinitive rounds of asexual proliferation would augment the length of time of circulation of gametocytes and therefore increase the chances for successful transfer to the mosquito. One cannot forget, however, that various related parasites, such as *Leucocytozoa* and *Haemoproteus*, release only sexual stages in the blood and are very successfully transmitted [150].

More intriguing is the fact that very few of the erythrocytic stages actually commit to a differentiation to transmission stages in the blood [reviewed in [151]]. It is paradoxical that, having the possibility to generate more gametocytes through asexual proliferation, the parasite actually makes less, especially since a higher density would increase transmission [152]. Several hypotheses have been proposed to explain this restraint.

Taylor and Read [151] have postulated that immune pressure through transmission-blocking activity could account for the low density of gametocytes. Transmission-blocking immunity has been demonstrated for several surface antigens of *P. falciparum*. If this anti-gamete immunity is dependent on the density of gametocytes, it is obviously advantageous to reduce the number of gametocytes. Alternatively, it could be that the level of this immunity was dependent on the ratio of asexual: sexual parasites, the asexual parasites acting as a decoy for the immune system. Piper and colleagues [63] proposed a role for a cross-stage immunity against PfEMP-1. An increase of anti-PfEMP-1 immunity with age was observed, which was accompanied by a decline in prevalence and density of asexual and sexual blood stages of *P. falciparum*. PfEMP-1 being expressed on the surface of both asexual stages and young gametocytes (see Sequestration of gametocytes), regulation of the level of gametocytes could have evolved either by controlling asexual proliferation (the source of gametocytes) or directly by affecting maturing gametocytes.

It has also been proposed that frequencies of super-infection, modulated by cross-immunity between the phenotypes involved, may serve to constrain gametocyte production to levels less than would be optimal for transmission in a single-phenotype context [153].

Another possible explanation is that the gametocyte number is kept low in order to reduce the damage mosquito stages are likely to inflict on their host [151]. Evidence that natural selection drives the parasite towards lessening the number of mosquito stages in a mosquito to favour survival of the host and, therefore, successful completion of the life cycle, has only recently come to light. The ookinete, which is responsible for the penetration of the stomach wall, is the most harmful stage to the mosquito; this stage has been shown to be capable of apoptosis (programmed cell death) [154], which results in a reduction in the number of ookinetes and, therefore, lessens the damage due to penetration and eventual opportunistic bacterial infection.

It has been demonstrated that the parasite has evolved several strategies to maximize transmission in spite of the low number of gametocytes: (a) aggregation of gametocytes to favour encounter of males and females in the bloodmeal [155] (b) preferential localization of infectious rodent-malaria gametocytes in sub-dermal capillaries [156] and possible sequestration of mature gametocytes in the derma [157] and (c) active suppression of insect melanization by ookinetes [158].

Malaria parasites may have evolved restraint in the production of transmission stages in order to avoid harmful

consequences either directly for the parasite through the immune system of the definitive host or to spare the vector in order to maximize its success in transmitting the parasite. This restraint provides an evolutionary justification for the development of autocrine/exocrine mechanisms for means of communication. One could imagine that asexual parasites express a highly labile diffusible molecule [108] that prevents other asexual parasites from undergoing gametocytogenesis or encourages them to pursue asexual proliferation. When a high level of "stress" (immunity) arises, the parasite population would respond by restricting the expression of the diffusible molecule and, therefore, would stimulate a higher level of asexual parasites to develop sexually.

Conclusions

P. falciparum gametocytes exhibit adaptability and biological achievement. The gametocytogenesis of this parasite, comparable to a puberty process, obviously contributes to its present tropical-wide success. The present review addressed several aspects of gametocytes, focusing on sex differentiation and determination, transmission strategies and evolution; such a review endeavours to highlight the areas where the gametocyte might be targetted for control interventions.

To date, reported attempts to fight the parasite burden by targetting the gametocyte are non-existent. However, when one considers malaria control strategies as a whole, gametocytes are often secondary targets. Any anti-anopheline intervention, as it reduces vector density, also decreases the passage of gametocytes from man to mosquito. Newly developed drugs, such as combination therapy with artemisinin derivatives, have as their main purpose the efficient removal of asexual stages, but the gametocytocidal activity of these drugs is considered an important issue. There also is a recent "courant de pensée"among the scientific community that the management of anti-malarial resistance will necessarily involve gametocyte control.

Author contributions

AMT scanned the literature and wrote the draft. All authors contributed to the writing of the manuscript and approved the submitted version.

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