Irrespective of the tremendous suffering caused by malaria, a Plasmodium infection by pathogenic blood stages is only transient and an obligate step toward the Anopheles vector where sexual reproduction and genetic recombination of the unicellular parasite takes place. Recent expression profiling studies identified the molecular make-up of female and male gametocytes. Differential promoters and translational repression through mRNA binding by a female-specific helicase help to fine-tune the expression of these sexual stage-specific genes. However, we are only just beginning to discover the triggers that initiate gametocytogenesis and the developmental programs that drive sexual development.

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Introduction
Malaria is the most important vector-borne disease and is caused by unicellular Plasmodium parasites that have the capacity to specifically invade and replicate inside erythrocytes. Following invasion, some merozoites develop into gametocytes rather than a new generation of invasive stages. Eventually, these sexual stages form gametes and combine to form a diploid zygote inside the Anopheles midgut (Figure 1). Physiological sexual reproduction is an obligate phase during Plasmodium life cycle progression, but does it also result in genetic recombination?

Infected individuals frequently harbor multiple and consecutive infections of genetically distinct parasite strains potentially resulting in extensive genetic recombination and continuous transmission to the mosquito vector [1]. Although still controversial, nearly all population genetics studies indicate a ‘sexual’ population structure of the most pathogenic malaria parasite, Plasmodium falciparum [2,3]. The observed high rates of meiotic recombination through outcrossing in natural P. falciparum populations result in persistence of a gene pool rather than clonal lineages. Understanding parasite population genetics and sexual reproduction remain research priorities in times where clinical case management is complicated by the rapid worldwide spread of drug-resistant parasite strains. Genetic variability in combination with rapid asexual reproduction appears to be a winning strategy and makes Plasmodium and other apicomplexan parasites, such as Toxoplasma, some of the most successful pathogens.

In general, apicomplexan parasites are haploid organisms that follow a meiotic program typical for other single cells such as yeasts, where the first meiotic divisions occur only after fertilization and zygote formation, a process termed zygotic meiosis. What sets these parasites apart from most other single cell eukaryotes are fundamental morphological changes that clearly distinguish large, nonmotile female from small, flagellar male gametes (Figure 1), a strategy reminiscent of sexual reproduction in multicellular organisms.

Sexual development and reproduction are arguably the most dramatic and significant events in any animal’s life and none so true for malaria parasites. Outburst of the male gametes (exflagellation) led to the discovery of Plasmodium as the causative organism of malaria by Alphonse Laveran and remains one of the most spectacular views on malaria parasites through a normal light microscope. Gametocytes and gametes form distinctive life cycle stages (Figure 1) that are relatively easy to obtain from whole blood (rodent malaria model species) or blood cultures (P. falciparum). The latter have the unique capacity to sequester in the human bone marrow, possibly mediated through a subset of the var family of P. falciparum-specific antigens [4], and hence, avoid clearance in the spleen (reviewed in [5,6]).

Here, we focus on the molecular events during the remarkable developmental program that drives gamete formation and maturation. The recent availability of the complete genome sequence of P. falciparum [7] as well as an ever increasing amount of partial genome sequences for a variety of rodent malaria model species, that is, Plasmodium yoelii yoelii [8], Plasmodium berghei, and Plasmodium chabaudi chabaudi [9*], has facilitated a range of postgenomic studies (reviewed in [10]). These have identified the repertoire of sexual stage-specific genes providing an expanded portfolio for transmission-blocking vaccine strategies.

Expression profiling of the sexual stages
The first systematic analyses of gene transcription in the Plasmodium sexual stages using prepublication releases of
the ongoing sequencing projects employed standard approaches, for example, shotgun DNA microarrays [11] and differential display analysis [12], for transcript comparisons between *P. falciparum* gametocytes and asexual blood stages. Global expression profiling studies, including proteome datasets [13,14] and gene transcription analysis [15] in *P. falciparum* and a combined transcriptome and proteome analysis in *P. berghei* [9,10], identified large subsets of sexual stage-specific genes and genes that are shared with other life cycle stages (Table 1). Comparison of gametocyte-producing and nonproducing lines solved the problem of confounding contaminations with asexual blood stages [16,17]. Extrapolating from these studies it appears that roughly 5% of all genes may be truly sexual stage-specific, though proteome studies suggest a significantly larger level of sexual stage-specific proteins (Table 1). However, so far it has not been possible to perform an accurate study of all life cycle stages since several, such as oocysts and liver stages, remain elusive.

Important insights into the molecular determinants of female and male gametocytes came from a proteome study in *P. berghei* [18]. Using transgenic parasites expressing green fluorescent protein in a sex-specific manner, male and female *P. berghei* parasites were separated by flow cytometry and analyzed by mass-spectrometry (Table 1). These data confirmed the presence of significant amounts of mitochondrial and ribosomal proteins in female gametocytes, whereas proteins associated with the axoneme and flagella and those involved in DNA metabolism were predominantly found in males. Only 69 sexual stage-specific proteins were shared including many hypothetical proteins for which a function has yet to be established [18].

**Control of sexual stage-specific gene expression**

The multitude of distinct life cycle stages and biological strategies associated with any of these require a highly
coordinated and probably complex regulation of gene transcription and protein expression. Over the past years a number of studies shed light on the unique regulatory mechanisms that drive formation and maturation of the sexual stages (Figure 2).

*P. falciparum* encodes four ribosomal RNA gene units, two each active during blood stage development and in mosquito stages. Their expression is temperature regulated as demonstrated by the cold-shock response of a luciferase transgene under the control of a mosquito-stage rRNA promoter [19]. By contrast, other genes are differentially transcribed in both asexual and sexual stages. The first indication of such transcriptional control came from B7 set [20]. SET is a chromatin assembly factor, and it plays a dual role in asexual (replicating) blood stages and male gametocytes, which undergo three rapid rounds of genome replications upon entry of the mosquito midgut. Alternative transcription start sites and an intron in the 5′ untranslated region (UTR) of the gametocyte transcript distinguish the two activities. Similarly, different-sized transcripts with a considerably smaller intron were later described for α-tubulin II, which is highly upregulated in male gametocytes [21]. Transcriptional control of set was functionally confirmed using GFP-fused reporter constructs in *P. berghei* and *P. falciparum* [22], though the exact mechanism through which such promoters operate remain obscure. Microarray analysis of gametocyte development also helped to identify a cis-regulatory element driving sexual stage-specific transcription [16].

Similar to other eukaryotes, *Plasmodium* gametocytes also store maternal transcripts for subsequent use in gametes [23] or ookinetes [9]. Previously, two gametocyte-specific genes encoding RNA-binding proteins of the pumilio family (PUF) were identified in *P. falciparum* and PfPUF1 was shown to bind specifically to the Nanos response element (NRE), a signature found in the 3′ UTR of translationally repressed transcripts [24]. However, out of 95 *P. falciparum* genes with an NRE-like motif in their 3′ UTR sequences only two appear to undergo translational repression during sexual stage development [23]. It seems likely that translational repression through this motif may also occur in other stages of the life cycle, for example in sporozoites or merozoites. The identification of additional nine translationally repressed genes permitted the classification of a conserved 3′UTR region that contains an NRE-like submotif [9], which in turn

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**Table 1**

*Plasmodium* gene expression studies

<table>
<thead>
<tr>
<th>Species</th>
<th>Total expressed</th>
<th>Gametocyte-expressed</th>
<th>Gametocyte-specific</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. falciparum</em></td>
<td>4,557</td>
<td>3,128 (67%)</td>
<td>204 (4%)</td>
<td>[15]</td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>4,294</td>
<td>3,363 (78%)</td>
<td>-</td>
<td>[23]</td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>Differential</td>
<td>-</td>
<td>246</td>
<td>[16]</td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>Differential</td>
<td>-</td>
<td>117</td>
<td>[17•]</td>
</tr>
<tr>
<td><em>P. berghei</em></td>
<td>Differential</td>
<td>-</td>
<td>106</td>
<td>[9•]</td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>2,415</td>
<td>1,147 (47%)</td>
<td>376 (16%)</td>
<td>[13]</td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>1,289</td>
<td>931 (72%)</td>
<td>315 (24%)</td>
<td>[14]</td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td></td>
<td>645 (50%)</td>
<td>97 (8%)</td>
<td></td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td></td>
<td></td>
<td>575 (45%)</td>
<td></td>
</tr>
<tr>
<td><em>P. berghei</em></td>
<td>2,904</td>
<td>1,197 (41%)</td>
<td>-</td>
<td>[23]</td>
</tr>
<tr>
<td><em>P. berghei</em></td>
<td>1,836</td>
<td>733 (40%)</td>
<td>127 (7%)</td>
<td>[9•]</td>
</tr>
<tr>
<td><em>P. berghei</em></td>
<td>1,293</td>
<td>802 (62%)</td>
<td>406 (31%)</td>
<td>[18•]</td>
</tr>
</tbody>
</table>

※These studies compared gametocyte-producing and nonproducing lines to identify gametocyte-specific transcripts. ※Gamete-expressed and gamete-specific proteins, respectively. ※Total number of sexual stage-specific proteins, including 163 proteins shared between gametocytes and gametes. ※As little as 69 sexual stage-specific proteins are shared between male and female gametocytes.
identified 20 more translationally repressed genes in *P. berghei*. A distinct but functionally related regulatory motif was also found involved in the translational repression of the gamete/ookinete surface antigens P25 and P28 [25]. The sex-specific proteome analysis revealed an RNA helicase that is restricted to female gametocytes and shares homologies with the DDX6 family of DEAD-box RNA helicases [18]. These helicases are an integral component of maternal messenger ribonucleoprotein (mRNP) particles and involved in the storage and silencing of mRNAs encoding meiosis-associated proteins [26]. A detailed biochemical and genetic analysis in *P. berghei* demonstrated that the *Plasmodium* gene, termed dozi (development of zygote inhibited) has a similar activity [27]. DOZI was found tightly bound to multiple target mRNAs and was localized to cytoplasmic bodies in the female gametocytes. Importantly, these structures were absent in parasites where *pbdozi* was genetically disrupted. Microarray analysis comparing the knockout and wild-type parasites revealed 370 transcripts to be significantly reduced including seven of the nine reported previously [9].

Although these studies have provided us with refreshing insights into the transcriptional and post-transcriptional control of gene expression during the development of the sexual stages of the parasite’s life cycle, additional regulatory mechanisms are probably operational: first, antisense transcripts have been detected in all blood stages, asexual and sexual, and have been suggested to regulate transcription [28], though a mechanistic understanding and indeed proof of functionality are still lacking; second, alternative splicing has been associated with a gene-dense cluster enriched in gametocyte-specific genes and may represent yet another level of transcriptional control [29]; third, although next to nothing is known about post-translational modifications and control that take place in *Plasmodium*, it is likely that these mechanisms, including degradation by the ubiquitin–proteasome system, further fine-tune a rapid response to a changing environment and developmental program.

**Triggering sexual differentiation**

There are two important questions that can be asked concerning the onset of sexual development in malaria parasites. First, at an individual parasite’s level, when does a parasite ‘decide’ to become a gametocyte and what triggers this decision? Second, from a parasite population’s viewpoint, what triggers the development of the first gametocytes?
Early experiments suggested that merozoites from a single schizont are likely to produce either asexual or sexual stages parasites [30,31]. Using female-specific and male-specific markers, pfg377 and α-tubulin II, respectively, it was discovered that siblings from a single schizont develop into gametocytes of the same sex confirming that the parasites are committed to sexual differentiation before schizontogony [32]. This early commitment is exemplified by the targeted disruption of an asexual stage-specific gene, termed P. falciparum gene implicated in gametocytogenesis (pfg27), resulting in a faltering gametocyte production [33]. Transcription analysis of early gametocytes [17] and the discovery of a P. falciparum-specific subtelomeric gene family of which 6 genes (out of 36) are coexpressed with pfs16 and pfg27 [34*], provided additional proof for this theory and a considerable increase in early development markers. One of these markers, termed male development gene 1 (pfmdv-1), has been independently identified and characterized by targeted gene disruption [35]. The resulting knockout P. falciparum parasites produce fewer gametocytes and have a defect in exflagellation.

The increased knowledge of early gametocytogenesis markers will hopefully provide us with the tools we need to address an even more interesting question, what triggers this switch causing parasites to develop into schizonts producing ‘asexual’-committed, ‘male’-committed, or ‘female’-committed merozoites? A number of factors have been linked to gametocytogenesis including (i) parasite factors (e.g. genetic variations and parasite density); (ii) host factors (e.g. immunity); and (iii) other environmental factors (e.g. drug treatment) (reviewed in [5,36]). Several aspects have been and are continuing to be linked to gametocyte levels in patients; however, the data are fragmented and even conflicting at times. Recently, studies linking fever and gametocyte levels in human malaria infections demonstrated that in Plasmodium vivax parasite counts and fever were higher after emergence of the first gametocytes, while the opposite was true for P. falciparum [37,38]. As a general trend, stress appears to trigger an increased commitment to sexual development, something that had been recognized almost a century ago [39].

**Triggering fertilization**

By contrast to our limited understanding of the molecular, cellular, and environmental triggers that induce gametocytogenesis, our understanding of the cascades involved in the further development of the sexual stage parasites in the mosquito midgut have improved considerably over the past years. After complete development of the male and female gametocytes in the blood, these parasites enter a state of cell cycle arrest, awaiting the ingestion through a mosquito blood meal. The extreme change in environmental conditions, most notably a sudden drop in temperature of 10–15 °C, but also a decrease in pH, and the sudden presence of mosquito-derived factors, such as xanthuric acid, kick off the emergence of gametes [40,41].

A plant-like calcium-dependent protein kinase (CDPK4), though present in all blood stages, was shown to respond to a xanthuric acid-induced increase of cytosolic calcium in gametocytes [42]. This response is essential for the development of gametes by regulating progression of the cell cycle in the male gamocyte. The sex-specific proteome analysis [18**] detected 10 sexual stage-specific protein kinases and protein phosphatases. Of these, six were male-specific and two female-specific, strongly suggesting that distinct sex-specific signaling cascades regulate gamete maturation. P. berghei with a disrupted male-specific putative mitogen-activated protein (MAP) kinase 2 gene fail to produce male gametes with female gamete formation and fertility unaffected [18**,43,44]. Surprisingly, disruption of the P. falciparum ortholog proved impossible indicating an additional essential role for PfMAP2 in asexual stages [45]. Disruption of a female-specific putative NIMA-related kinase did not affect gamete formation or fertilization but abolished meiotic replication and, hence, ookinete formation [18**,46].

The actual process of fertilization requires sensing and recognition of the female gametes followed by cell fusion. Characterization of knockout parasites of the known gamete surface antigens established a requirement of Pf48/45 and Pf230 for completion of fertilization [47,48], whereas Pf47 is not essential [49]. A fundamental question that remains is whether and how the subsequent meiotic division differs from other eukaryotes.

**Outlook**

Genome-scale transcription and expression analyses have provided a large scope of candidate genes that perform specific functions in the sexual stages of Plasmodium parasites. Inevitably, functional gene analysis lags behind, but some crucial progress has been made, in particular, at the level of identifying stage-specific protein kinases important for cell cycle progression of male and female parasites. Yet, data are still fragmented and for most of the protein kinases the stimuli that activate them and their downstream targets remain elusive.

Important progress has been made in understanding the mechanism that drive stage-specific transcription and expression, particularly through the discovery of translational repression [9*] and the role of the RNA helicase DOZI in this process [27**]. Proteome analysis also suggests that ready-made proteins may be stored, mainly in female gametes, for later use in her development [18**]. It will be interesting to identify the mechanisms and binding partner(s) used for stockpiling individual proteins.
Finally, despite the characterization of an increasing number of early gametocytogenesis markers, their cellular functions and the corresponding environmental signals that trigger their production remain elusive. A thorough understanding of these processes might eventually lead to the discovery of drug and vaccine targets that could help prevent the production of sexual-stage parasites and transmission to the mosquito vector, thus blocking the life cycle and spread of malaria.

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References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


