

Review

Pink or blue: sex determination in malaria gametocytes

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Sex determination in eukaryotes defines whether an organism develops as male or female and is shaped by genetic and environmental factors. In the malaria parasite *Plasmodium*, recent data suggest that sex determination is decided at two critical checkpoints. First, chromatin remodeling activates the master regulator AP2-G, triggering a transcriptional cascade that commits the parasites to sexual development and drives the expression of genes crucial for male and female gametocytes. Second, post-transcriptional processes – including the stabilization and suppression of transcript via RNA-binding proteins – guide the branching into male or female gametocytes through threshold-trait-dependent mechanisms. This review highlights recent advances in understanding the molecular mechanisms that regulate the two checkpoints of sex determination in malaria gametocytes.

Diverse mechanisms regulate sex determination across eukaryotes

Sexual development in eukaryotes is governed by complex molecular mechanisms that determine whether an organism will develop as male or female. Contrary to early notions of a singular master switch controlling this process, it is increasingly understood that **sex determination** (see [Glossary](#)) is a dynamic and nonlinear interplay of molecular events. These events cumulatively shift the balance toward one sexual fate until a threshold is crossed that commits the sex identity to a stable developmental pathway, while actively repressing the alternative path (reviewed in [1]).

Sex development has particularly been studied in animals and is generally classified as either genotypic sex determination (GSD) or environmental sex determination (ESD) (reviewed in [2,3]). In GSD systems, sex is determined by the inheritance of specific alleles or chromosomes at fertilization. In many species, GSD is linked to master switch genes – such as the male-determining *sry* gene on the Y chromosome (XX–XY systems) of mammals or female-determining factors on the W chromosome (WZ–ZZ systems) in birds. In ESD systems, environmental cues such as temperature or social environment direct sexual fate during a critical developmental window, as has been shown for some reptiles and fish. Although traditionally seen as distinct categories, GSD and ESD exist along a continuum. Some species, such as the bearded dragon *Pogona vitticeps*, have a genetic ZW system but experience temperature-induced sex reversal, in which ZZ males develop as phenotypic females under high temperatures. This continuum allows for evolutionary flexibility, enabling the organism to counteract shifts in selective pressures by changing its **sex ratio**. As a general rule, ESD is favored in variable environments to align the sex with optimal conditions to secure reproduction. In contrast, a shift to GSD is favored in unpredictable or non-variable environments, where ESD may cause skewed sex ratios (reviewed in [1,2,4]).

Despite diverse initiating cues, sex differentiation across animals often relies on conserved regulators, especially transcription factors (TFs) and histone demethylases mediating male development

Highlights

Plasmodium falciparum gametocytes pass through two checkpoints during their development: checkpoint 1 leads to entry into the sexual phase, while checkpoint 2 determines the sexual identity.

Chromatin remodeling acts as the molecular switchboard to orchestrate transcriptional activity in gametocytes, first to initiate sexual commitment and then to steer gametocytes in the early stalk phase.

Sexual fate is locked in during the late stalk phase, as gametocytes cross a transcriptional threshold during transition from stage II to stage III.

Sex determination is dependent on the ratio of available male- and female-specific transcripts, which is regulated by RNA-binding proteins acting as post-transcriptional modulators and repressors.

The gametocyte's individual epigenome and transcriptome shape its distinct male or female proteome landscapes required for successful gametogenesis.

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(reviewed in [2,4]). A key example is DMRT1 (doublesex and mab-3 related transcription factor 1), a TF that determines male fate in many vertebrates (reviewed in [5]). In mammals, the master switch SRY activates the TF SOX9, which, together with DMRT1, promotes testis development and suppresses ovarian pathways (reviewed in [6]). In many female vertebrates, the aromatase CYP19A1 plays a central role for ovarian development by converting androgens to estrogens (reviewed in [7,8]). These regulators are themselves epigenetically controlled. For instance, in mammals, the lysine demethylase KDM3A (JMJD1A) is essential for activating *sry* by removing the heterochromatin mark H3K9me2. In the red-eared slider turtle *Trachemys scripta*, KDM6B acts as an epigenetic thermosensor which, at male-producing temperatures, removes H3K27me3 repressive marks from the *dmrt1* promoter. DMRT1 in turn activates genes important for testis development and represses the CYP19A1-encoding gene. Notably, KDM6B is regulated via temperature-sensitive **alternative splicing** that can introduce intron retention and premature stop codons, rendering the enzyme inactive at female-producing temperatures (reviewed in [2,5]).

In the malaria parasite *Plasmodium falciparum*, the molecular mechanisms of sex determination are as yet not well understood and currently a focus of intense research. As a haploid protist, *Plasmodium* reproduces primarily asexually within host cells, particularly erythrocytes. However, the parasite undergoes a sexual phase during its transition from the human host to the mosquito vector (Box 1). During this phase, a small percentage of blood-stage parasites differentiate into sexual precursor cells called **gametocytes** (Figure 1). In the absence of sex chromosomes, sexual fate decision in gametocytes depends on polygenetic factors and appears to be decided at two critical checkpoints: first, the epigenetically regulated lineage decision between asexual and sexual development, and second, the decision whether a gametocyte will become male or female based on the balance of sex-specific transcripts. In the past couple of years, multiple publications have contributed to a better understanding of the process driving sex determination in malaria parasites, thereby uncovering many parallels to sex determination in animals. This review highlights recent advances in **epigenetic gene regulation** and **chromatin remodeling** during **sexual commitment** and discusses the role of key regulators and **post-transcriptional control** mechanisms during male and female gametocyte maturation.

Box 1. Sexual differentiation in *Plasmodium falciparum*

Gametocytes are the intraerythrocytic sexual precursor cells of malaria parasites and the only parasite stage capable of continuing the life cycle within the mosquito vector. In *P. falciparum*, gametocytogenesis begins when a small fraction of asexual blood-stage parasites commits to sexual differentiation, a process influenced by environmental cues and accompanied by extensive transcriptional reprogramming. *P. falciparum* typically follows a 'next cycle commitment' route, which occurs in the trophozoite stage resulting in sexually determined merozoites that initiate gametocyte differentiation after reinvasion (whereas rodent parasites like *P. berghei* follow a 'same cycle commitment' route, which originates in the ring stage, from which gametocytes differentiate directly). Gametocyte development in *P. falciparum* progresses through five morphologically distinct stages (I–V) over ~10 days (contrary to other human pathogenic species and rodent malaria parasites, which require 2–3 days for gametocyte development) (Figure 1). Immature gametocytes (stages I–IV) sequester in the human bone marrow, where they complete most of their maturation. Upon reaching stage V, they undergo characteristic morphological changes and are released into the peripheral bloodstream. The mature male and female gametocytes are ingested by blood-feeding female *Anopheles* mosquitoes. In the mosquito midgut, gametocytes become activated in response to environmental shifts such as a drop in temperature. Activation triggers the egress of the gametocytes from the red blood cell and their differentiation into gametes – a process that takes ~15 min. During this time, male gametocytes undergo three rounds of nuclear division to produce eight motile microgametes. The female macrogamete retains all the factors necessary for further development as translationally repressed transcripts, which are translated following fertilization. Fertilization results in a zygote that transforms into a motile ookinete within ~20 h. The ookinete penetrates the mosquito midgut epithelium and settles at the basal side, where it develops into an oocyst. This stage marks the beginning of the sporogonic cycle, ultimately leading to the formation of infectious sporozoites that can be transmitted to a new human host (reviewed in [13,95,114]).

Glossary

Alternative splicing: a process by which different exons and introns from the same transcripts are joined together to produce multiple mRNA variants. This allows one gene to generate different forms of a protein, thereby increasing protein diversity.

ApiAP2 (Apicomplexan Apetala2) proteins: a family of transcription factors of Apicomplexan parasites that contain AP2 DNA-binding domains. These proteins regulate stage-specific gene expression to control key processes of parasite development, sexual commitment, and transmission.

Barcoded screening: a technique in which unique DNA or RNA barcode sequences are assigned to different genes or mutants, which are pooled and analyzed together, enabling efficient, parallel screening of many targets in a single experiment. The barcodes are tracked by high-throughput sequencing.

Chromatin remodeling: the dynamic process of reorganizing chromatin structure to regulate DNA accessibility. It involves protein complexes that modify, add, slide, or remove histones, thereby controlling access to regulatory sites for transcription of genes.

Epigenetic gene regulation: the control of gene activity without changing the DNA sequence itself through chemical or structural modifications of histones and/or DNA, thereby changing the accessibility of a gene locus.

Gametocyte: the sexual precursor stage of the malaria parasite crucial for the continuation of the life cycle in the mosquito vector. Female and male gametocytes develop in erythrocytes and circulate in the human/mammalian peripheral blood, where they may be ingested by blood-feeding *Anopheles* mosquitoes and continue sexual development.

Gametocytogenesis: the biological process during which asexual blood stages develop into gametocytes.

Post-transcriptional control: the regulation of gene expression after the initial transcription of RNA from the DNA template. It involves mechanisms like RNA splicing, editing or methylation, transcript stability and translational repression.

RNA-binding protein (RBP): a protein that specifically binds to RNA molecules and regulates their processing, stability, transport, or translation, thereby

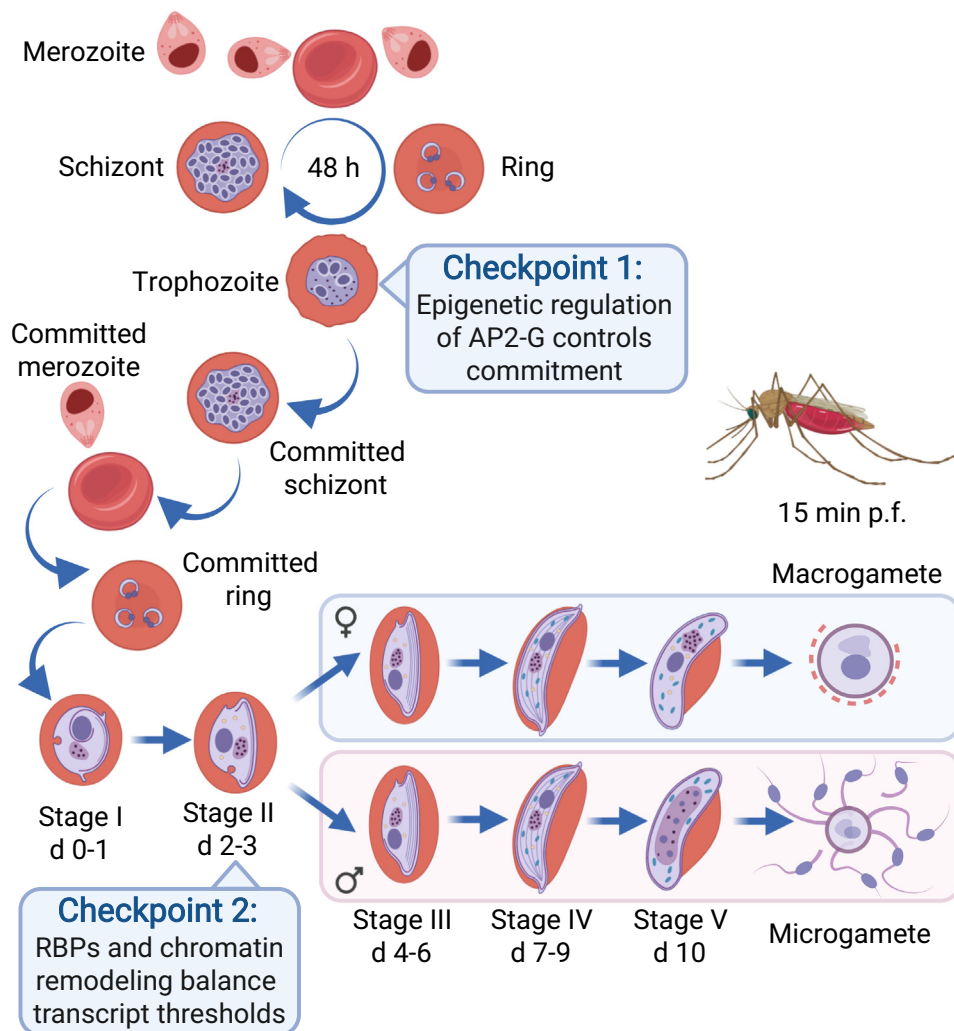


Figure 1. Sexual fate decisions involve two checkpoints in the human malaria parasite *Plasmodium falciparum*. *P. falciparum* parasites multiply in human red blood cells by asexual schizogony within 48 h. During the trophozoite-to-schizont transition, changes in the chromatin state of the *ap2-g* locus determine sexual commitment in a subpopulation of parasites (checkpoint 1). After reinvasion, sexually committed parasites enter a common early gametocyte development phase (stages I–II) lasting around 3 days (d), during which commitment to either male or female sex is determined (checkpoint 2). This process involves quantitative transcript thresholds, mediated by sex-specific chromatin profiles and a network of RNA-binding proteins (RBPs). After bifurcation, male and female gametocytes mature through three further morphologically distinct stages (stages III–V) within the next 6 days. Upon uptake of mature gametocytes by an *Anopheles* mosquito, fertilization-competent microgametes and macrogametes emerge within 15 min post-feeding (p.f.). Figure created using BioRender.

Checkpoint 1. Chromatin remodeling defines sexual commitment and early gametocyte development

The epigenetically regulated initiation of sexual commitment

Sexual commitment occurs when a proportion of parasites diverts from the asexual replication path and develops into non-replicative gametocytes. This developmental decision appears to happen stochastically in part of the population, but can be modulated by environmental factors,

controlling gene expression at the post-transcriptional level.

Sex determination: the biological process by which an organism develops into either a male or a female, based on genetic, environmental, or a combination of factors – here the ‘decision’ to become either a male or female gametocyte. Generally, genotypic sex determination (GSD) and environmental sex determination (ESD) are distinguished, dependent on the role of genetic versus environmental factors during the decision.

Sex ratio: the proportion of male to female organisms in a population, here the ratio of male and female gametocytes within the human/mammalian host.

Sexual commitment: the lineage decision during which asexual blood-stage parasites enter the sexual pathway and develop into gametocytes. This decision is regulated by the transcriptional activation of specific genes that are important for gametocyte formation. It marks the switch from asexual replication in the human host to sexual stage development in order to prepare for transmission to the mosquito vector (see gametocyte).

Single-cell transcriptomics: a technique used to detect and quantify RNA molecules from single cells (here the blood stages of *Plasmodium*) to identify individual transcriptomes and reveal transcript variations in subpopulation of cells (here gametocytes). When visualized as trajectories, cells can form a continuous developmental path, termed ‘stalk’, or may split into two distinct fates, termed ‘bifurcation’.

Translational repression: the process by which specific mRNAs are synthesized but kept in a silent state, preventing their translation into proteins until they are needed. This allows the parasite to store mRNAs during gametocyte development and activate them after transmission to the mosquito, ensuring proper timing of protein production for successful parasite development.

including parasitemia, drug exposure or nutrient deficiency (Box 1) [9–13]. In the rodent malaria parasite *Plasmodium berghei*, sexual development preferentially occurs in the hematopoietic niche within reticulocytes [14,15], suggesting that microenvironmental factors may provide metabolic cues that promote sexual development. Similarly, *P. falciparum* sexual stages sequester in the bone marrow during their differentiation [16–18]; however, *P. falciparum* gametocytes can also be induced under normal *in vitro* culture conditions.

One metabolite that has been linked to sexual commitment is the host-derived glycerophospholipid lysophosphatidylcholine (LysoPC). The parasites utilize serum LysoPC to generate phosphatidylcholine (PC) for membrane biosynthesis [19–23]. However, when LysoPC levels are depleted in culture medium, *P. falciparum* can activate an alternative pathway for PC synthesis, in which phosphoethanolamine N-methyltransferase (PMT) utilizes the methyl-donor S-adenosyl methionine (SAM) to generate PC *de novo* [19]. As a consequence, less SAM is available for other methylation reactions, such as histone methylation, which could limit H3K9me3 deposition by histone methyltransferases (HMTs). Indeed, conditions reducing SAM levels in an *in vitro* setting result in heterochromatin destabilization and promote the expression of heterochromatin-silenced genes, like the one encoding the TF AP2-G, which then initiates the path towards sexual development (Table 1) [24]. This metabolic competition for a limiting resource that is required for both asexual growth and the suppression of sexual differentiation represents an interesting concept on how microenvironmental changes influence the parasite's chromatin state and thereby regulate developmental decisions such as sexual commitment (Figure 1) (reviewed in [25]). It is noteworthy that these findings are based largely on *in vitro* investigations and therefore warrant cautious interpretation. Complementary evidence from *in vivo* human studies is required to provide a more comprehensive understanding of the role of the microenvironment in gametocyte induction.

AP2-G is a member of the **ApiAP2 (Apicomplexan Apetala2)** TF family and acts as a master switch by reprogramming asexual blood-stage parasites to initiate **gametocytogenesis** [26–29]. In asexual parasites, the *ap2-g* locus is silenced through chromatin-mediated repression (Figure 2). During silencing, heterochromatin protein 1 (HP1) binds to H3K9me3 at the *ap2-g* locus [30,31], a process that also appears to involve the histone deacetylase Hda2, as conditional depletion of Hda2 dramatically increases *ap2-g* transcription and sexual conversion (Table 1) [32]. The protein phosphatase PPM2 was recently suggested to dephosphorylate HP1 at serine 33, promoting sexual conversion by relieving the interaction of HP1 with chromatin, thus permitting *ap2-g* activation (Figure 2) [33]. Silencing of *ap2-g* in asexual blood stages further involves the ApiAP2 TF AP2-G5, which is enriched along the entire *ap2-g* locus [34]. Once synthesized, AP2-G can bind to its own promoter to increase its expression in an autoregulatory feedback loop [27,35] and AP2-G5 binding sites were detected in close proximity to AP2-G binding sites. Mutational analyses of the AP2-G5 binding motifs suggest that AP2-G5 may suppress *ap2-g* activation by blocking AP2-G binding to its own promoter [34]. AP2-G5 further interacts with the microorchidia protein MORC, a chromatin remodeler that is enriched in heterochromatic regions and critical for the global chromatin architecture in *P. falciparum*, thereby controlling chromatin accessibility at heterochromatic genes including *ap2-g* (Table 1) [36,37]. The histone acetyltransferase (HAT) GCN5 is also strongly enriched at the silent *ap2-g* locus in asexual parasites, and its deficiency results in significantly increased sexual commitment, indicating that GCN5 contributes to *ap2-g* repression, possibly by regulating the function of non-histone targets or by maintaining heterochromatin boundaries [38,39].

The earliest known positive regulator of gametocytogenesis is gametocyte development 1 (GDV1) (Table 1) [12,40]. GDV1 expression is upregulated in the trophozoite stage by as yet unknown mechanisms and acts as the initial transcriptional activator of the *ap2-g* locus during the

Table 1. Transcriptional and post-transcriptional regulators of gametocytogenesis

Name	GeneID <i>P. falciparum</i> <i>P. berghei</i>	Expression and function	Refs
Transcription factors (TFs) and associated regulators			
AP2-FG	PF3D7_1317200 PBANKA_1415700	TF that activates genes of the female lineage; interacts with PFG	[79,80]
AP2-FG2/AP2-O3	PF3D7_1429200 PBANKA_1015500	TF of female gametocytes that represses male-specific genes; forms in <i>P. berghei</i> a repressor complex with TF AP2R-2; activated by AP2-G	[78,83]
AP2-G	PF3D7_1222600 PBANKA_1437500	TF that initiates the expression of early gametocyte genes	[27,29]
AP2-G2	PF3D7_1408200 PBANKA_1034300	TF that activates early gametocyte genes and represses genes tied to asexual proliferation; acts downstream of AP2-G	[29,44–46,48]
AP2-G5	PF3D7_1139300 PBANKA_0909600	TF that silences the <i>ap2-g</i> locus after sexual commitment; activates male-specific genes	[34,49]
FD2/PFG	PF3D7_1146800 PBANKA_0902300	Regulator expressed in the female lineage; involved in female development; component of the RNF1/MD3 complex	[67,73]
FD3	PF3D7_1319600 PBANKA_1418100	ACDC protein expressed in the female lineage; involved in female development	[73]
GCN5	PF3D7_0823300 PBANKA_0707300	HAT involved in silencing the <i>ap2-g</i> locus; component of the SAGA complex	[38,39,65]
GDV1/ <i>gdv1-asRNA</i>	PF3D7_0935400 PF3D7_0935390	Regulator that binds HP1 and activates the <i>ap2-g</i> locus; regulated by <i>gdv1-asRNA</i> ; specific for <i>P. falciparum</i>	[40–42]
Hda2	PF3D7_1008000 PBANKA_1206200	HDAC involved in silencing the <i>ap2-g</i> locus	[32]
HDP1	PF3D7_1466200 PBANKA_1329600	TF that activates early gametocyte genes after AP2-G levels wane	[65]
HP1	PF3D7_1220900 PBANKA_1436100	Regulator that stabilizes heterochromatin by binding to H3K9me3; silences the <i>ap2-g</i> locus; is regulated in its activity by S33 phosphorylation through PPM2	[30,31,33]
(Putative) RNA-binding proteins (RBP)			
7-Helix-1	PF3D7_0525400 PBANKA_1240200	RBP expressed in the female lineage; interacts with translational repressors; involved in the release of repressed transcripts	[106]
CITH	PF3D7_1474900 PBANKA_1301300	RBP that represses female gametocyte transcripts; component of the RNF1/MD3 complex	[67,99]
DOZI/DZ50	PF3D7_0320800 PBANKA_1217700	DEAD-box RNA helicase that binds transcripts for morphogenesis in early gametocytes and represses transcripts in mature female gametocytes	[98,99,105]
FD1	PBANKA_1454800 PF3D7_1241400	RBP expressed in the female lineage; involved in female development; component of the RNF1/MD3 complex	[67,73]
FD4	PF3D7_1220000 PBANKA_1435200	Regulator expressed in the female lineage; involved in female development; component of the RNF1/MD3 complex	[67,73]
GD1	PF3D7_0927200 PBANKA_0828000	C3H1 ZFP expressed in the stalk and branching phase; involved in female development	[73]
MD1	PF3D7_1438800 PBANKA_1302700	RBP with dual function in male fate determination and male development; expressed in the stalk phase and male lineage	[71,73]
MD2	PF3D7_1233200 PBANKA_1447900	RBP expressed in the stalk phase and male lineage; involved in sex ratio determination and male development	[73]
MD3	PF3D7_0315600 PBANKA_0413400	C3H1-ZFP expressed in the stalk phase and male lineage; involved in sex ratio determination and male development; interactor of RNF1	[67,68,73]
MD4/ARID	PF3D7_0603600 PBANKA_0102400	Transcriptional regulator of male and female development	[73,88,89]
MD5/RBPm1	PF3D7_0414500 PBANKA_0716500	RBP involved in alternative splicing linked to male fertility	[73,85]
NSUN2	PF3D7_1111000	Methyl transferase that mediates m ⁵ C-modification of mRNA; stabilizes sex-specific	[91]

Table 1. (continued)

Name	GeneID <i>P. falciparum</i> <i>P. berghei</i>	Expression and function	Refs
	PBANKA_0936600	transcript	
PUF1	PF3D7_0518700 PBANKA_1233500	RBP expressed in the male lineage, involved in sex ratio determination and late gametocyte development; component of the RNF1/MD3 complex	[67,70]
PUF2	PF3D7_0417100 PBANKA_0719200	RBP that represses female gametocyte transcripts	[100,101]
RNF1	PF3D7_0314700 PBANKA_0412400	RING finger protein expressed in the stalk and branching phase; interacts with RBPs involved in sex determination; <i>mfi</i> expression is regulated by AP2-G5	[49,54,64,67]
SR-MG	PF3D7_1126800 PBANKA_0921600	RBP involved in alternative splicing linked to male fertility	[90]
ZNF4	PF3D7_1134600	C3H1-ZFP expressed in male lineage; regulates expression of fertility genes; component of the RNF1/MD3 complex; specific to <i>P. falciparum</i>	[69]

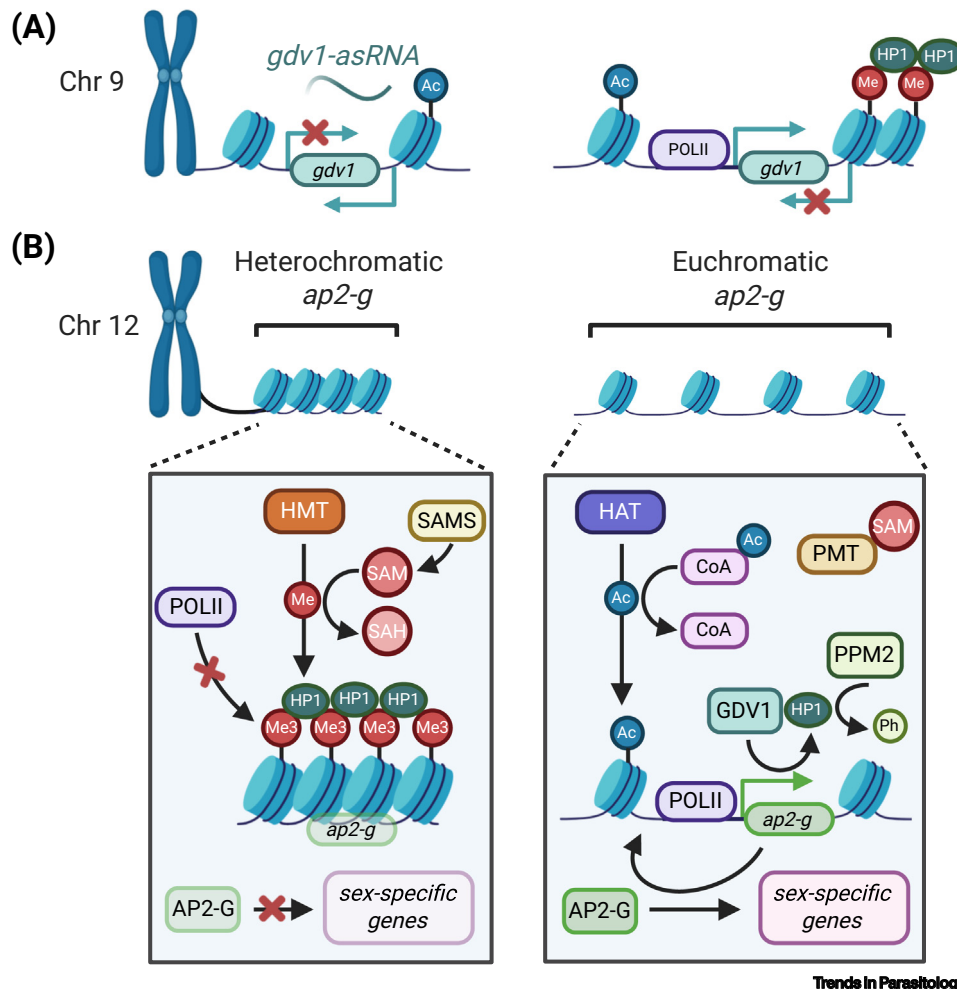
trophozoite-to-schizont transition, a process which involves its interaction with HP1 (Figure 2) [40–42]. *GDV1* expression anticorrelates with the levels of an antisense (as) long non-coding RNA (lncRNA), *gdv1-asRNA*, that originates downstream of the *gdv1* locus [41,43]. While it remains to be shown whether *gdv1-asRNA* acts as a direct repressor of *gdv1*, its own expression also appears to depend on heterochromatin dynamics, as the levels of H3K9me3 deposition at the *gdv1-asRNA* promoter correlate with gametocyte conversion rates in different parasite clones [43].

Chromatin remodeling following sexual commitment

In the committed schizont and the subsequent early gametocyte phase following reinvasion, AP2-G controls the activation of early gametocyte genes and also binds to promoters of genes linked to erythrocyte invasion [35,42]. Moreover, AP2-G induces the expression of AP2-G2, which co-regulates gametocytogenesis in conjunction with AP2-G by repressing genes of asexual blood stages and promoting those of early gametocytes (Table 1) [44–48]. While AP2-G expression is transient and ceases within the first 48 h after invasion [26], it triggers gametocytogenesis by initiating a cascade of downstream transcriptional activators and repressors ([34,46,49]; reviewed in [50,51]).

After the sexually committed merozoites reinvade red blood cells, they are irreversibly determined to follow the sexual development path (Figure 1). This early phase of gametocyte development is characterized by transiently accumulating levels of H3K36me2 and H3K36me3 in chromatin [52]. In stage II gametocytes, both repressive marks are enriched in the promoter regions of genes typically expressed during asexual development, as well as of those active at the initiation of sexual commitment (including *ap2-g*, *gdv1*, and *hda2*) [53]. Transcriptome analyses of asexual and sexual ring-stage parasites identified chromatin-related factors as signature genes typically elevated in the early sexual lineage, for example the lysine-specific histone demethylase 2 (LSD2) and the histone deacetylase (HDA1) [54–56]. Although their physiological role in gametocytogenesis remains to be shown, the high sensitivity of early gametocytes to inhibitors modulating histone methylation levels underlines the critical role of epigenetic regulation during early gametocyte development [53,57].

An expansion of subtelomeric H3K9me3-associated and HP1-bound heterochromatin domains is a typical feature during maturation of gametocytes [58–61]. This phenomenon, though, only begins after stage II [58], when H3K36me2/3 levels are declining again. Moreover, the gain of heterochromatin appears to be a consequence rather than a cause of repression at many gene loci [58],



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Figure 2. Epigenetic mechanisms determine the commitment toward sexual differentiation. (A) In asexual parasites, the promoter of *gdv1* (gametocyte development 1) is inactive, while *gdv1-asRNA* is expressed. During sexual commitment, the promoter of *gdv1-asRNA* assumes a heterochromatic structure by gaining H3K9me3 and HP1 (heterochromatin protein 1). This results in silencing of *gdv1-asRNA* and promotes *gdv1* transcription. (B) The *ap2-g* locus is repressed by heterochromatin in asexual parasites, limiting RNA polymerase II (POLII) access to the promoter. S-adenosyl-methionine (SAM)-synthase (SAMS) produces SAM as a precursor for histone methylation. SAM is converted into S-adenosyl-homocysteine (SAH) in the methylation reaction of H3K9me3 catalyzed by an unknown histone methyltransferase (HMT). Activation of *ap2-g* is modulated by metabolic competition of phosphoethanolamine N-methyltransferase (PMT) for the methyl-donor SAM, resulting in reduced H3K9me3 deposition and, presumably, increased histone acetylation via an unknown histone acetyltransferase (HAT) at the promoter. Dephosphorylation of HP1 by protein phosphatase PPM2 at serine 33 reduces HP1 affinity to chromatin resulting in its eviction through a process involving GDV1. AP2-G binds to its own promoter in a positive feedback loop and activates early sex-specific target genes, resulting in sexual commitment. Figure created using BioRender.

possibly suggesting that early H3K36me2/3-mediated repression of subtelomeric virulence genes and adjacent, asexual stage-specific genes may precondition heterochromatin expansion.

Collectively, the data indicate that the first checkpoint determining the lineage decision between asexual replication and sexual differentiation critically depends on chromatin remodeling events at two important genetic loci, *gdv1* and *ap2-g* (Figure 2), as well as the subsequent silencing of these sexual commitment initiators in early gametocyte development.

Checkpoint 2. Transcriptomic thresholds determine sexual fate

Transcriptional regulation in the late stalk phase

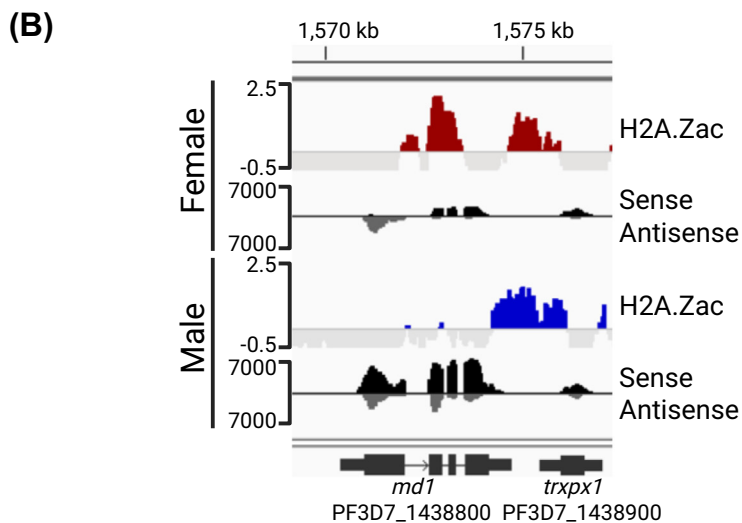
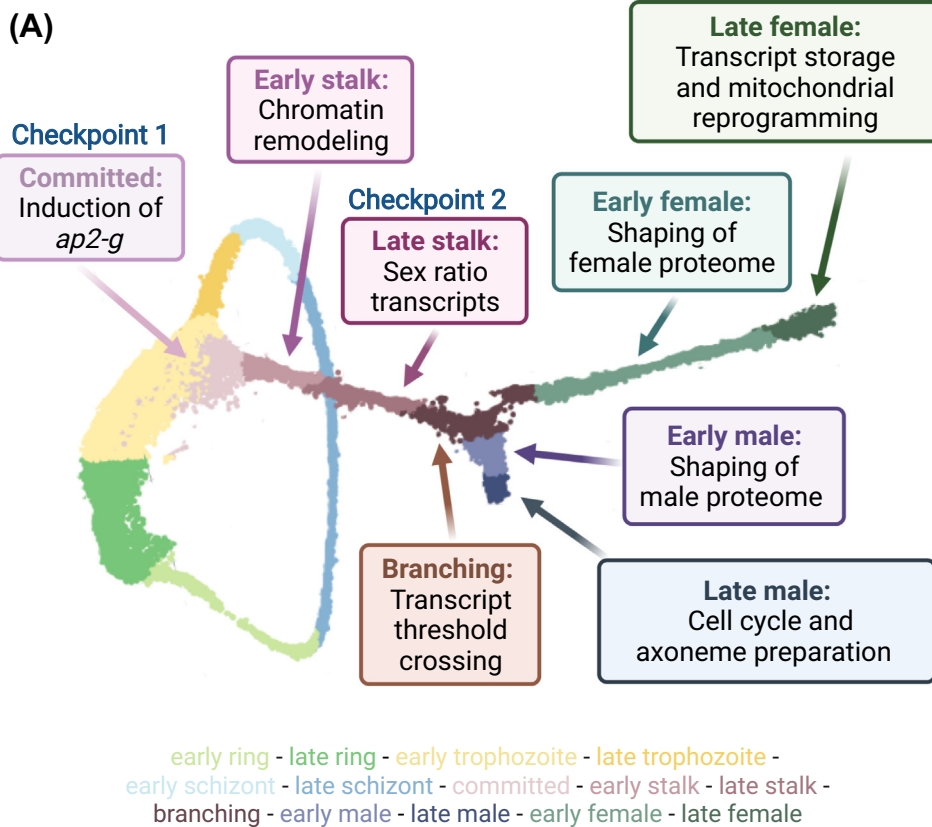
Early studies investigating gametocytogenesis supported a model in which male or female sex was determined concurrently with sexual commitment (reviewed in [62,63]). However, recent technological advances provide novel evidence suggesting that, after sexual commitment, a second checkpoint defines whether malaria parasites will develop into male or female gametocytes (Figure 1). New insights from **single-cell transcriptomics** indicate that, in *P. falciparum*, the committed parasites enter a common stalk phase of early gametocyte development (corresponding to stage I to stage II), before bifurcation of the male and female lineage occurs [54]. The study assigned a total of 179 genes to the stalk phase of *P. falciparum*, distributed among three modules – S1 to S3. Modules S1 and S2 mainly feature the transient expression of exported proteins such as ETRAMPs or MESA, proving a potential link to gametocyte sequestration. The majority of genes were identified in module S3, including genes related to chromatin remodeling like histone deacetylases (HDACs) and HMTs like the SET proteins, as well as histone variants [54]. The S3-mediated chromatin remodeling events in the stalk phase represent a first step towards sex determination (Figure 3A). This is reflected by the sex-specific distribution of the heterochromatin mark H3K9me3 and the histone variants H2A.Z and H2B.Z in male and female gametocytes, as discussed later [61]. Studies using epigenetic inhibitors further confirmed pivotal roles of HDACs and HMTs in directing gene expression profiles during *P. falciparum* gametocyte development [57,64].

Also found in S3 are TF-encoding genes, for example, the ones encoding MYCBP, HDP1, AP2-LT, and AP2-O2 (Table 1). The DNA-binding protein HDP1 (homeodomain-like protein 1) is important for early gametocyte formation with peak expression when AP2-G levels wane [65]. HDP1 regulates the expression of various early gametocyte genes, including the ones encoding ApiAP2-LT, a component of a histone acetyltransferase GCN5-associated SAGA complex and AP2-O2, crucial for parasite development in the mosquito midgut [38,44]. HDP1 also mediates the expression of proteins constituting the gametocytes' inner membrane complex (IMC), which is crucial for subsequent formation of male and female gametocytes, as discussed further.

RNA-binding proteins of the late stalk phase

A group of proteins with increased expression in the late stalk and branching phase are zinc finger proteins (ZFPs) – a large diverse family of proteins with one or more zinc finger domains involved in DNA- and RNA-binding – that function in cellular processes like transcriptional regulation and mRNA decay and stability (reviewed in [66]). One of the first ZFPs upregulated in maturing gametocytes is RNF1, a RING finger domain-containing RNA-binding E3 ubiquitin ligase (RBUL) that has originally been identified during the screening for Trichostatin A-deregulated genes of *P. falciparum* gametocytes [54,66]. RNF1 is part of a protein network containing various **RNA-binding proteins (RBPs)** with a predicted role in balancing threshold traits of gene products required for sex identity like PUF1 (Pumilio and Fem-3 binding factor 1), MD3 (male development 3), and ZNF4 (zinc finger protein 4) (Table 1) [67–70]. A recent gene-regulatory network analysis suggested that *rnf1* expression in early gametocytes is regulated by AP2-G5 and the ApiAP2 protein PF3D7_0420300 [49,67].

RNF1 engages with multiple regulators of sex determination, including the male lineage proteins ZNF4 and MD3 (Table 1) [68,69]. MD3 and ZNF4 are ZFPs with C3H1 motifs critical for RNA-binding (reviewed in [66]). Both proteins are highly abundant in immature gametocytes. Loss of MD3 delays male gametocyte maturation and shifts the sex ratio toward females. By contrast, ZNF4-deficiency does not affect gametocyte development, but blocks male gametogenesis by reducing male-enriched genes [68,69]. Noteworthy, MD3 shares most of its interactors with



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Figure 3. Epigenetic and post-transcriptional mechanisms govern the phases of sex determination in *Plasmodium falciparum* gametocytes. (A) Gene expression modules measured by single-cell transcriptomics support the discovery of sex determination mechanisms [54,74]. Sexually committed trophozoites are characterized by transcription of early gametocyte markers, prominently *ap2-g*. During the sexual stalk phase, at first chromatin remodeling

(Figure legend continued at the bottom of the next page.)

RNF1, which include various sex-specific proteins, like the members of the recently identified family of male development (MD) and female development (FD) regulators [67].

The key male development regulator is MD1, a dual-function protein that localizes to cytoplasmic granules of *P. falciparum* gametocytes (Table 1) [71]. Functional assays revealed that the N-terminal domain of MD1 alone is sufficient to specify male fate, while the C-terminal OST/HTH/LOTUS domain is essential for male development [71]. Male-specific expression of the N-terminal MD1 domain is linked to a bistable transcriptional pattern, which manifests as full-length *md1* transcript in male gametocytes, whereas in females two shorter transcripts representing *md1* exon1 asRNA and *md1* exon 2-4 sense RNA are generated (Figure 3B). Interestingly, chromatin profiling of male and female gametocytes indicates that the sex-specific expression pattern of *md1* sense and antisense transcripts is driven by differential chromatin remodeling of a bidirectional promoter in the first intron [61]. In female gametocytes, this intron promoter and the adjacent exon 2 are strongly enriched for the activating histone modification H2A.Zac [72], possibly forming a chromatin barrier for expression of the full-length *md1* transcript and promoting bidirectional transcription of the exon 1 asRNA and exon 2-4 sense RNA in females (Figure 3B). In male gametocytes, the intron promoter is devoid of H2A.Zac, facilitating uninterrupted transcription of the full-length *md1* mRNA from the upstream promoter.

Both MD1 and MD3 were also identified in *P. berghei* gametocytes during a **barcoded screening** for sex regulators [73]. Altogether, ten putative sex regulators were identified during this screen and categorized as GD1 (gametocyte development 1), male development regulators MD1 to MD5, and female development regulators FD1 to FD4 (Table 1). While GD1 and MD1 to MD3 are expressed during the stalk phase, the other regulators are upregulated during the bifurcation phase (see later). Like MD1 and MD3, MD2 is crucial for sex ratio determination, and *P. berghei* mutants lacking MD2 fail to produce male gametocytes. In contrast, GD1 promotes female gametocyte differentiation; its absence results in a sex ratio shift toward males [73]. Notably, GD1 shares multiple interactors with RNF1 and MD3, including the RBPs CITH, PUF1, NOT1G, PABP1, PDG2, and MKT1DP (MKT1 domain-containing protein), as well as FD1, FD2, FD4, the RNA helicase DBP1, and the adapter protein 14-3-3I [67,71]. Of particular interest, PUF1 is assigned to the male branch, but its deficiency leads to a marked reduction in late-stage *P. falciparum* gametocytes and a male-biased sex ratio, prompting speculation that it may act by repressing male transcripts [67,70,74].

The RNF1/MD3-based protein network likely has a pivotal regulatory role during the transition from the stalk to the branching phase – effectively the junction of sex determination. Supporting this, differential expression analyses comparing male-like and female-like *P. falciparum* gametocytes identified 44 genes with sex-biased expression emerging during the stalk phase [59].

factors and then genes important for modulating the sex ratio are upregulated. Branching marks the phase during which transcript thresholds lock in male or female sex. Male and female gametocyte development is affirmed by sex-specific proteomes driving the transcription of genes related to axoneme formation and DNA replication in male gametocytes, as well as transcript storage and mitochondrial metabolism in females. The UMAP Chromium 10x cell view of *P. falciparum* blood-stage parasites was extracted from the Malaria Cell Atlas database [74]. (B) Differential enrichment of H2A.Zac in male and female gametocytes suggests epigenetic regulation of sex-specific transcripts at the *md1* locus. ChIP-seq profile (log2 ChIP/input) of H2A.Zac at the *md1* locus and matched sense and antisense RNA levels [reads per kilobase million (rpkm)] in stage III female (red) and male (blue) gametocytes [61]. In female gametocytes, H2A.Zac peaks at the boundaries of intron 1 correlate with promoter activity driving the expression of sense transcripts covering exons 2-4, as well as antisense transcripts of exon 1. In male gametocytes, the absence of intronic H2A.Zac enrichment correlates with full-length sense and antisense transcripts spanning exon 1-4. The H2A.Zac peak localized downstream of the *md1* locus is present in the promoter region of the neighboring thioredoxin peroxidase 1 gene (*trpx1*). Data are visualized in IGV 2.16.0 (Integrative Genomics Viewer). Figure created using BioRender.

This suggests that transcriptional dimorphism begins prior to bifurcation, likely around stage II of gametocyte development [49,54]. Considering the involvement of numerous RBPs in sex determination, current data support a model in which transcript-level threshold effects govern sexual fate, during which male development is promoted by regulators such as MD3 and ZNF4, whereas female development is favored by factors like PUF1 and GD1.

The finale: sex-specific proteomes drive the maturation of male and female gametocytes

Gene expression following branching

During branching, transcript thresholds guide the *P. falciparum* gametocytes towards male and female fate (Figure 3A). Four early sex modules, E1 to E4, are assigned to bifurcation, comprising together 932 genes [49,54]. In E1, which features female-specific transcripts, more RBP and ZFP-encoding genes as well as the genes coding for the LCCL-domain protein family are located. In *P. falciparum*, the six LCCL-domain proteins are highly expressed during gametocyte maturation and form adhesion protein complexes associated with the gametocyte plasma membrane. The proteins are also found later in the crystalloids of ookinetes, as has been shown in *P. berghei*, and hence are considered female markers (reviewed in [75]). E1 further contains various genes encoding IMC components. Module E2 is particularly composed of male-specific transcripts including genes for DNA replication and flagellar function or the gamete fusogen HAP2 [76]. E3 contains genes assigned to both sexes, for example, the ones encoding the gametocyte surface protein P230, GEST (gamete egress protein), MDV1, or MGET (male gametocyte-enriched protein), while module E4 includes transiently expressed exported proteins likely involved in erythrocyte remodeling [54,77]. As gametocytes mature, five late sex modules with 529 genes, L1 to L5, can be distinguished [54]. Modules L1 and L2 exhibit sustained sex-specific gene expression that begins early and increases through maturation. Modules L3 to L5 contain genes that continue expression in only one lineage – male or female – after bifurcation.

Several ApiAP2 proteins regulate branching and sexual fate in *Plasmodium* (reviewed in [50,51]). For example, AP2-FG and AP2-FG2/AP2-O3 activate female-specific genes while repressing male-associated transcripts in rodent malaria parasites (Table 1) [78,79]. In *P. berghei*, AP2-FG associates with FD2 (here called PFG) to control genes required for female gametocyte maturation, gametogenesis, and ookinete development [80]. AP2-FG2/AP2-O3 act together with AP2R-2 and MORC as a repressor complex in early *P. berghei* gametocytes to promote female development [81]. ApiAP2-encoding genes – important for the zygote and ookinete development, such as *ap2-z*, *ap2-o*, and *ap2-o2* (regulated by HDP1, see above) – are also transcribed in female *P. berghei* gametocytes to prepare for post-fertilization development (Table 1) [48,82–84]. Male development in *P. falciparum* is driven by AP2-G5 (see earlier), which activates male-specific genes and may suppress female regulator genes like *ap2-o3* [49]. AP2-Tel (AP2-SP/EXP) indirectly represses female-specific genes in *P. berghei* [44], and uncharacterized ApiAP2 factors PF3D7_0420300 and PF3D7_1222400 may also regulate male gene expression (Table 1) [49].

Sex-specific transcript expression and regulation of sexual fate

A recent transcriptomic screen in *P. yoelii* identified 2847 genes differentially expressed between male and female gametocytes [85]. Among others, female-specific genes encode components of the IMC and the LCCL-domain protein complex as well as proteins important for meiotic activity, mitochondrial function and ookinete development, while genes of the male lineage are particularly involved in DNA replication and motility. These data are in accord with another recent barcoded screen for fertility genes in *P. berghei* [86]. The identified female fertility genes could primarily be

assigned to meiosis and mitochondrial metabolic reprogramming, while male fertility genes have roles in mitosis, axoneme assembly, and flagellar activity (Figure 3A) [86]. This is consistent with sex-specific transcriptomes from *P. falciparum* [61,87].

Among the sex-specific genes found in *P. yoelii* are a high number of genes encoding RBPs like ZNF4, PUF1, and the FD and MD proteins [85,86]. While some of the sex development regulators (i.e., MD1 to MD3, and GD1) are important for sex ratio determination (see earlier), other members of the group function after branching. Loss of *P. berghei* FD1 to FD4 leads to infertile female gametocytes lacking transcripts for subsets of core female markers (Table 1) [73]. In addition, deficiency of MD4 in *P. berghei* results in infertile males. However, another study assigned MD4, here termed ARID, to chromatin remodeling events important for male and female gametocyte development, and in accord with these findings, MD4/ARID in *P. falciparum* is a transcriptional regulator important for exflagellation and macrogamete fertility (Box 1) [73,88,89]. Furthermore, MD5 is linked to male fertility in *P. berghei* and its ortholog in *P. yoelii*, termed RBPm1, has crucial functions during alternative splicing of male-specific transcripts, in particular ones encoding axonemal proteins [73,85]. This study provided one of the first lines of evidence for alternative splicing events guiding sex determination in malaria parasites, which were confirmed by RNA velocity studies that showed increased splicing kinetics upon bifurcation of *P. falciparum* gametocytes [49,54]. In support of these findings, the alternative splicing factor SR-MG is crucial for male gametocyte development in *P. berghei* [90]. Another mechanism contributing to transcript diversity in gametocytes is the use of alternative promoters [54]. In addition to the *md1* gene discussed above (Figure 3B), one example of note is the Jumonji histone demethylase JmjC3, for which full-length transcripts are detected only in asexual parasites, whereas both male and female gametocytes express shorter versions corresponding to the terminal four and two exons, respectively. This results in the absence of the enzymatically active domain and could potentially be mechanistically relevant for the differential histone methylation patterns observed between asexual and sexual parasites [53,58,61].

Epitranscriptomic modification of mRNA also appears to be relevant for sexual differentiation. A recent study demonstrated that the methyl transferase NSUN2 mediates m⁵C-modification of mRNA, which is significantly increased in mature gametocytes [91]. This may contribute to the enhanced stability of sex-specific transcripts. In addition to alternative splicing and RNA methylation, lncRNAs play a role in sex determination. A recent screen identified 460 lncRNAs in *P. falciparum* which are exclusively expressed at the gametocyte stage and partially specific to males or females [92]. Among others, the female-enriched lncRNA-ch14 was identified. Deletion of lncRNA-ch14 changes the sex ratio towards males and leads to a downregulation of genes assigned to females, like the osmiophilic body protein G377, the LCCL-domain protein PfCCp3, and the ookinete surface protein P25.

Interestingly, the lncRNA-ch14 locus is heterochromatic in asexual parasites and male gametocytes but is devoid of heterochromatin in female gametocytes [61]. Several other genes are also differentially packaged by heterochromatin between stage III/IV male and female gametocytes, including GEEO (gametocyte erythrocyte cytosolic protein) and some exported proteins of the PHIST family that are also present on chromosome 14 [61]. This is consistent with similar observations regarding sex-specific heterochromatin patterns along genes encoding exported proteins in *P. berghei* [93]. Noteworthy, in stage III/IV female *P. falciparum* gametocytes, active gene expression is associated with a chromatin signature comprising acetylation of H3K27 and H2A.Z. By contrast, H3K4me3 and H3R17me2, which are enriched in active gene promoters during the asexual phase, are globally depleted from regulatory regions [61,94]. Further, H3K9ac correlates with transcript levels in male *P. berghei* gametocytes only, consistent with

the significant proportion of the female transcriptome being sequestered and stabilized by RBPs, as discussed later [93]. Together, these data corroborate the notion that sex-specific histone modifications continue to be relevant following branching.

A surprising finding of recent screens for sex-determining genes is the role of IMC components during sexual development. Gametocytes have an IMC, underneath their plasma membrane, consisting of flat membranous sacs, the alveoli, and supporting structural elements required for cell stability. During gametogenesis, the IMC disintegrates, and the membranes appear to be recycled as part of the macrogamete surface [95,96]. Single-cell transcriptomics followed by expression activity scoring unveiled higher transcript levels of IMC components in females compared to the male lineage, in particular PhIL1 (photosensitized INA-labelled protein 1) and members of the IMC1 family [49].

Translational repression in female gametocytes

Once gametocytes reach maturity, translational control becomes a key mechanism for determining the sexual fate (reviewed in [97]). Prior to gametogenesis, male gametocytes synthesize proteins essential for mitosis and axoneme assembly, whereas female gametocytes store transcripts that encode proteins required for ookinete development. Central to transcript storage are RBPs that function during **translational repression** (Figure 3). The repressed transcripts are packaged into messenger ribonucleoprotein particles (mRNPs), which aggregate into cytosolic granules. The most prominent repressors are DOZI (development of zygote inhibited) and its partner CITH (the homolog of worm CAR-1 and fly Trailer Hitch) (Table 1) [98,99]. Another translational repressor in gametocytes is PUF2, which represses numerous female gametocyte transcripts, including those encoding the ookinete surface proteins P25 and P28 [100,101]. Additional RBPs implicated in female gametocyte maturation and human-to-mosquito transmission include ALBA4 (acetylation lowers binding affinity 4), PABP (poly A-binding protein), and members of the CCR4-NOT complex, including CAF1 (CCR4-associated factor 1) [102–104]. Notably, most of these translational repressors are components of the RNF1/MD3-based protein network [67].

Recent functional studies in *P. falciparum* have revealed multiple roles for DOZI in gametocytes, since its absence disrupts both sexual commitment and gametocyte maturation [105]. In immature gametocytes, DOZI-containing mRNPs primarily bind transcripts essential for gametocyte morphogenesis, whereas in mature gametocytes, they store transcripts required for ookinete development (Box 1). Another crucial regulator of translational control is 7-Helix-1 [106]. This protein is also a component of cytosolic granules of female gametocytes and interacts with DOZI, CITH, and PUF2. Because 7-Helix-1 deficiency reduces P25 synthesis in activated female gametocytes, it is thought to facilitate the re-initiation of translation of previously repressed transcripts once gametogenesis begins. In this context, a recent study in *P. yoelii* demonstrated that approximately 200 transcripts – including those encoding P25, P28, IMC components, and mitochondrial proteins – are released from translational repression shortly after fertilization [107].

The combined findings suggest that maturation of male versus female gametocytes begins with the epigenetically controlled presence of sex-specific transcripts in the two lineages, followed by sex-specific proteomic landscapes and the storage of transcripts in female gametocytes that need to be transported from the macrogamete to the zygote and are required for the early mosquito phase of the malaria parasite.

Concluding remarks

Current research aims to uncover the molecular mechanisms of sex determination in animals, challenging and reshaping previous paradigms. The long-standing concept of a single 'master

Outstanding questions

Which chromatin modulators and epigenetic regulators drive the changes in chromatin composition during sexual commitment and early stalk phase development of *Plasmodium*?

Which transcriptional regulators govern the choice between male and female fates, and is one sex the default state?

What environmental cues influence sex determination during the stalk phase of gametocyte development?

Why is sexual fate determined so late in *P. falciparum* gametocyte development, specifically during the late stalk phase?

Why does the maturation time of gametocytes differ between *Plasmodium* species, and how is this variation reflected in transcriptional regulation of sexual fate?

Would transcriptional and post-transcriptional regulators involved in sex determination represent promising targets for transmission-blocking strategies?

regulator' dictating sex has been replaced by an understanding that sex determination is influenced by a diverse range of genetic and environmental factors. Increasing evidence shows that many animals rely on a combination of environmental cues, such as temperature, and genetic elements, including sex chromosomes. Even in species where genetic factors are predominant, sex determination operates along a continuum, ranging from monofactorial to polygenic systems (reviewed in [4]). Only when genetic and/or environmental factors surpass a critical threshold does an individual commit to a male or female developmental pathway.

Due to many parallels with animal systems, molecular parasitology often draws on findings from animal models. This is also true for the study of sex determination in malaria parasites. The discovery of GDV1 in 2012 [40] and AP2-G in 2014 [27] in the human malaria parasite *P. falciparum* initially raised hopes of identifying a master regulator of both sexual commitment and sex determination (reviewed in [62,63]). However, modern single-cell transcriptomics and proteomics analyses support the view that sexual fate in malaria parasites is determined later in gametocyte development than previously thought. Recent studies highlight the critical role of transcript regulation in sex determination and lineage development. Processes such as RNA synthesis, modification, suppression, and degradation appear to be central in balancing the transcriptome of each gametocyte. This regulation may ultimately enable the parasite to cross a transcriptional threshold that determines male or female fate. Current data suggest that, in *P. falciparum*, this threshold occurs during the late stalk phase of gametocyte development – specifically, in stage II. At this stage, governed by distinct epigenetic signatures, the individual transcriptome shapes the distinct male or female proteome landscapes required for successful gametogenesis and subsequent ookinete development in the mosquito.

The findings discussed here raise the question of why sexual fate is determined so late in gametocyte development. If we consider that GSD typically occurs in stable environments while ESD evolves under fluctuating conditions, then malaria parasites like *P. falciparum* appear to follow an environmentally influenced system of sex determination. But which environmental factors drive the parasite to delay its sexual decision until such a late stage? And why do *P. falciparum* gametocytes exhibit a prolonged maturation time compared to other human malaria species (see [Outstanding questions](#))?

Several factors have been proposed to influence the sex ratio in malaria parasites (reviewed in [108]). Previously, *in silico* modeling and transmission studies using rodent and human malaria parasites suggested that gametocyte density affects sex ratio and, consequently, transmission success. In low-density infections, producing more male gametocytes could be advantageous to ensure that all available females will be fertilized. In contrast, at high gametocyte densities, a male-biased sex ratio becomes inefficient because the parasite risks producing more males than there are females to mate with [109–112]. Further, coinfections are predicted to influence the sex ratio: female-biased ratios are favored when genetically related males compete for females, whereas high genetic diversity promotes a male-biased sex ratio to ensure fertilization success [113].

In conclusion, current data support a model whereby the sex ratio of malaria gametocytes appears to follow an epigenetically driven quantitative transcript threshold trait principle involving multiple gene products. This mechanism would allow lineage commitment to occur midway through gametocyte development, enabling parasites to adjust their sex ratio in response to environmental changes within the human host. This adaptive flexibility is likely a key factor in the parasite's evolutionary success. Future research is expected to identify additional molecules involved in sex determination, uncover the environmental factors and molecular sensors that influence sexual fate in malaria gametocytes, and evaluate their potential as targets for transmission-blocking measures (see [Outstanding questions](#)).

Acknowledgments

This work was supported by the German Research Foundation (DFG), project grants PR905/19-1 (within the priority programme SPP 2225) and PR905/20-1 to GP and project grant B1 (within the RTG2740 'Immunomicrotope') to MP. We apologize to all authors whose work could not be acknowledged due to space limitations.

Declaration of interests

The authors declare no competing interests.

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