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Malaria parasites and circadian rhythm: New insights into an old puzzle ☆

Lucas Borges-Pereira [a], Bárbara K.M. Dias [a,b], Maneesh Kumar Singh [a], Celia R.S. Garcia [a,*]

[a] Department of Clinical and Toxicological Analyses, University of São Paulo, São Paulo, Brazil;
[b] Department of Parasitology, University of São Paulo, São Paulo, Brazil

The hallmark of malaria, an infectious disease caused by parasites from the *Plasmodium* genus, is cyclic fever. Fevers and chills generally occur within 48 h (h) for *Plasmodium falciparum* (Fig. 1A) and *Plasmodium vivax* and every 72 h for *Plasmodium malariae*. This periodic fever has been known for almost a century to be a consequence of synchronous maturation of the parasites inside the host erythrocytes followed by the rupture of infected cells and the massive release of parasites into the bloodstream (Garcia et al., 2001; Stauber, 1939; Taliaferro, 1925). In the 1970s, Hawking observed that many *Plasmodium* species follow multiple 24-h life cycles in the vertebrate host, suggesting that the malaria parasite has a circadian rhythm (Hawking, 19701975). The circadian rhythm functions as an internal oscillator that repeats approximately every 24 h inside an organism; this rhythm is controlled by an external stimulus, such as a photoperiod, and is responsible for synchronizing behavioral and physiological rhythms (Rusak and Zucker, 1975).

Several explanations for *Plasmodium* infection coinciding with the host's circadian clock have been proposed. The first evidence of rhythm in host-parasite interactions was proposed as Hawking's hypothesis, which suggested that parasites have evolved to match the availability of vector timing to maximize their transmission. In one such study, Hawking showed that transmissible microfilaria of *Wuchereria bancrofti* migrate in the host's circulation during the evening period when mosquito bites occur frequently, thereby facilitating the transmission of parasites. Based on this observation, Hawking proposed a similar theory for *Plasmodium* parasites in which mature schizonts evolved to rupture at a specific time to ensure that the maturation of gametocytes coincided with the nocturnal activity of mosquitoes.

However, this hypothesis lacked experimental support until recently, when it was shown that gametocytes of the rodent malaria parasite *P. chabaudi* coincide with mosquito rhythm and become more infective, despite exhibiting a lower blood count (Schneider et al., 2018). Similarly, another study demonstrated that in an avian model, *Plasmodium* transmission was more frequent during the evening period (Pigeault et al., 2018). This study was further proof of a classic experiment in which inverting the host light-dark cycle also caused the inversion of the intraerythrocytic cycle of avian (*P. cathemerium*) malaria parasites, and erythrocyte rupture shifted to the daytime from night (Boyd, 1929). The recent findings revitalized the idea of the Hawking hypothesis of host-parasite interaction and suggested the relevance of circadian rhythm.

In 1978, Gore and Noblet exposed *Leucocytozoon smithi*-infected turkeys to normal, continuous, or inverted light and found that the turkey body temperature and gametocyte numbers coincided with the midpoint of the light period in natural and reversed-light exposure. However, turkeys subjected to continuous light exposure exhibited constant temperature and asynchronous gametocytes (Gore and Noblet, 1978).

Gore et al. (1982) also investigated the effect of L. *smithi* gametocyte rhythmicity in pinealectomized and ocular enucleated turkeys after exposing them to either 14 h light:10 h dark or to darkness with intermittent 10–20-min periods of red light. These researchers did not observe the pineal gland to play a direct role in gametocyte periodicity; however, an indirect role in the regulation of parasite rhythmicity was evident (Gore et al., 1982).

A similar method was employed to isolate the merozoites of *P. chabaudi*. The authors observed that schizogony shifted to daytime after artificially lighting the mouse chambers from 6 p.m. to 6 a.m. This procedure allows the diurnal rupture of erythrocytes (David et al., 1978). Additionally, the establishment of the *in vitro* culture of *P. falciparum* by Trager and Jensen reported that parasite synchronicity was lost outside the vertebrate host (Trager and Jensen, 1976). These findings indicate that the host's circadian rhythm controls parasite development inside red blood cells.

In the vertebrate system, biological clocks have been identified as transcription–translation-based feedback loops and have been directly linked with metabolism, cell growth, immunity and signaling (Takahashi, 2017). In recent years, efforts have been made to identify the molecular cues that mediate the circadian rhythm in malaria parasites while they proliferate inside the vertebrate host.

Searching for the host-derived signal, Hotta et al. considered melatonin to be a strong candidate for controlling asexual parasite development. The asexual growth of *P. falciparum* is responsible for the clinical symptoms of malaria and can cause cerebral malaria, which can be lethal if left untreated. The first evidence that melatonin may modulate the intraerythrocytic cycle or asexual development, promoting parasite

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* Corresponding author.
E-mail address: cgarcia@usp.br (C.R.S. Garcia).

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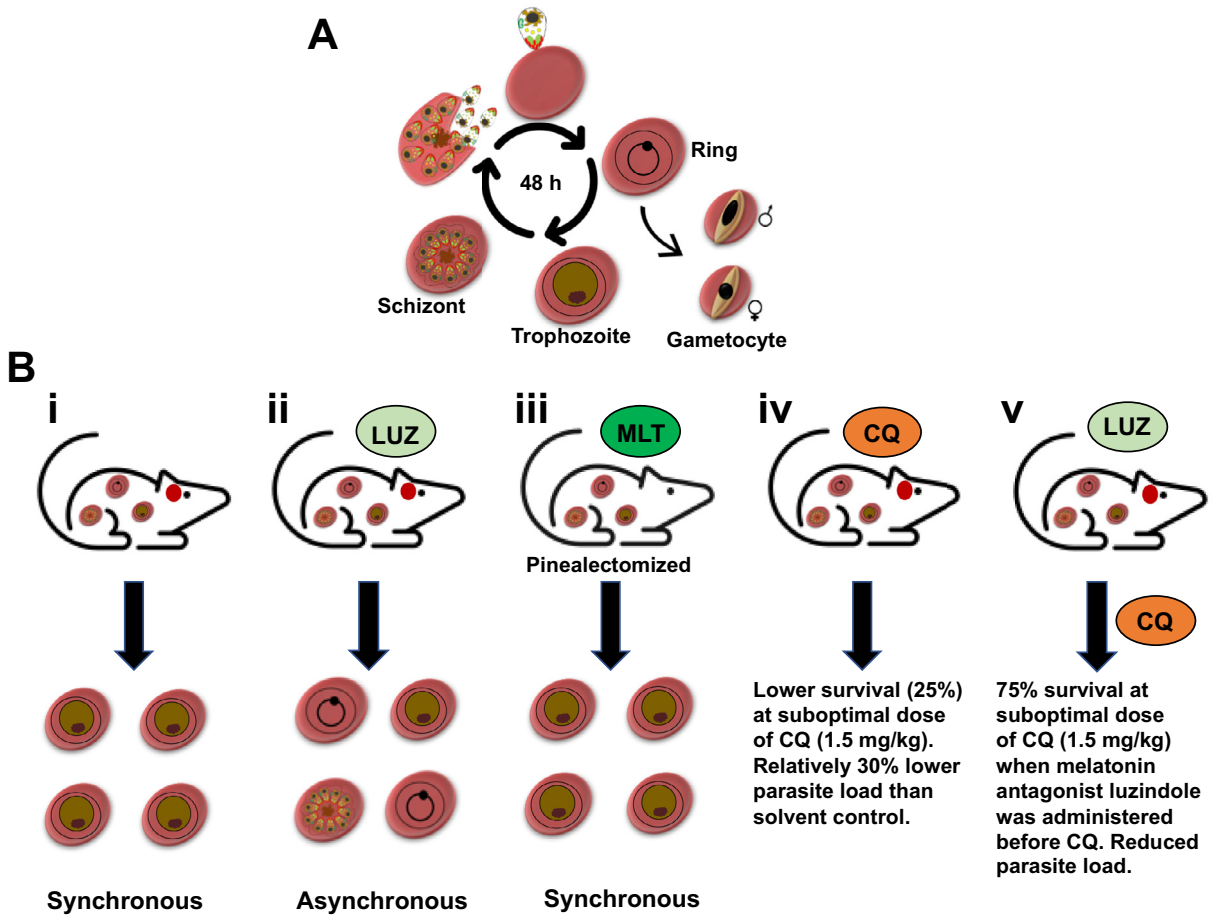

Fig. 1. **(A)** Asexual replication inside the host's erythrocyte. Once merozoite invade the red blood cells, it undergoes through distinct morphological stages – ring, trophozoite and schizont. This whole process takes 48 h in *P. falciparum*. Very few parasites deviate to sexual replication and form gametocytes which is taken up by mosquito during blood feed. **(B)** Different conditions where **(i)** Normal mice infected with *P. chabaudi* quickly returned to synchronous state within few days; **(ii)** Melatonin receptor blocker luzindole administration to mice disrupt the synchrony of *P. chabaudi* parasites; **(iii)** Pinealectomized mice also exhibit asynchronous growth but melatonin administration modulate asexual replication back to synchronous state. Red dot representing pineal gland. **(iv)** Administration of suboptimal dose of chloroquine (1.5 mg/kg) has little effect on survival rate of *P. chabaudi* infected mice but; **(v)** Suboptimal dose of melatonin in combination of luzindole increase the mice survival rate to 75% and reduces the parasite load to normal chloroquine dose (3 mg/kg) which has maximal effect on mice survival.

maturation (from trophozoites to schizonts), was obtained through both *in vitro* and *in vivo* experiments, where melatonin was added to *P. falciparum* culture and injected into pinealectomized mice infected with *P. chabaudi* (Fig. 1B) (Hotta et al., 2000). Next, we proposed that bursting in a coordinated manner could avoid exposing new merozoites to host immune effectors and splenic clearance.

The signaling cascade underlying melatonin-dependent regulation of the *Plasmodium* life cycle was also investigated and determined to be associated with cAMP, phospholipase C (PLC), production of IP_3, and Ca^{2+} release from the endoplasmic reticulum (Hotta et al., 2000; Alves et al., 2011; Beraldo et al., 2005). Taken together, these intracellular signals function collectively to promote a temporal upregulation of gene expression related to a subset of genes from the ubiquitin/proteasome system (UPS) (Koyama et al., 2013; Lima et al., 2013a, 2013b). Interestingly, the *P. falciparum* protein kinase 7 (PK7) knockout strain fails to respond to maturation promoted by melatonin treatment and the activation of the UPS genes (Koyama et al., 2012). Importance of parasite rhythm and drug effect has been studied and it was shown that *P. chabaudi* infected mice have higher survival rate even at suboptimal chloroquine (1.5 mg/kg) when administered in combination with melatonin antagonist luzindole (Fig. 1B) (Bagnaresi et al., 2008). This study implicates the effective malaria treatment strategy by altering parasite's rhythm and melatonin related indole compound might be a promising approach. To this end, Dias et al. has pointed the participation of a *P. falciparum* protein kinase PfeIK1 in the melatonin signaling cascade, since melatonin presented no effect in parasitemia in parasites lacking this protein (PfeIK1$^-$). In addition, the authors identified indole compounds able to abrogate the hormone effect in the parasitemia of *P. falciparum* 3D7 *in vitro* and with antimalarial activity. Triptiofen, one of the compounds tested, abolished the effect of melatonin in parasitemia and presented the lowest IC_{50} value *in vitro*. This compound was implicated to act at the mature forms of the intraerythrocytic cycle of *P. falciparum* 3D7, significantly decreasing parasitemia after 36 h treatment with 1 μM and 10 μM (Dias et al., 2020).

RNA-seq data of *P. falciparum*-infected RBCs treated with melatonin display 38 genes that are differentially expressed in wild-type parasites. The same experiment was performed in the PK7 knockout parasites, resulting in a distinct gene expression pattern that suggests the participation of PK7 in the melatonin signaling cascade (Lima et al., 2016). Nevertheless, melatonin was also able to modulate the expression of three genes (FIS1, DYN1, and DYN2) that encode protein homologs of mammalian FIS1 and DRP1 (Dynamin Related Protein 1) involved

in mitochondrial fission. These findings suggest that melatonin may also play a role in the maturation of the *P. falciparum* mitochondrion (Scarpelli et al., 2019).

Beraldo and Garcia have shown that melatonin precursors, N-acetyl serotonin, serotonin, and tryptamine are equally able to modulate the intraerythrocytic cycle of *P. falciparum* (Beraldo and Garcia, 2005). Interestingly, we also observed that unlike synchronous *P. falciparum* and *P. chabaudi* infection, *P. berghei* and *P. yoelli* asynchronous infections were not affected by melatonin addition *in vitro*, with no elevation in cytosolic Ca^{2+} concentration being observed in the parasite (Bagnaresi et al., 2009).

The role of melatonin in parasite biology has been discussed elsewhere (Koyama et al., 2013; Bagnaresi et al., 2012; Srinivasan et al., 2014). Two independent laboratories have recently published new insights regarding daily rhythms associated with malarial infection. Exposing *P. chabaudi*-infected mice to either complete darkness or light-dark cycles, Rijo-Ferreira et al. evaluated the effect of the light-dark period on the gene expression profile. Interestingly, more than 4000 genes from a total of 5400 genes in the *Plasmodium* genome were cycling, and the peak of gene expression did not change significantly from one condition to another, indicating that parasite rhythms persist in their rhythmic host in the absence of light. Additionally, the authors showed that parasites were able to synchronize their rhythms to the host rhythms using mutant mice (Fbxl3 mutant) with a prolonged daily cycle subjected to complete darkness. The time of host food intake was investigated, and the authors concluded that the host food intake pattern does not drive parasite rhythms. Arrhythmic mice mutant for Cry1/Cry2 were subjected to complete darkness and used to test whether parasites maintain their rhythms even in an arrhythmic host. The rhythm of gene expression was similar in both conditions, and parasites were able to maintain their 24 h cell cycle rhythmicity for approximately five days. However, parasites in arrhythmic mice experienced decay and loss of synchrony in the following 16 days. These findings suggest that *P. chabaudi* possesses an intrinsic clock that drives parasite rhythms, although host cues are essential to synchronize one parasite to another in a population (Rijo-Ferreira et al., 2020).

In 2020, Smith et al. performed a time-series transcriptomic analysis in four geographically distinct *P. falciparum* parasites to investigate the molecular signature of periodic genes when host-derived extrinsic factors were absent. The authors observed an abundance of conserved periodic genes; among them, approximately 92% of the genes have signature rhythmic oscillation. In this model, the researchers observed that the variation in cycle period length of parasites is very similar to that of known circadian cell lines (Smith et al., 2020). This study notes that the host's cue is involved in the synchrony of the intraerythrocytic cycle. Nonetheless, this cue increases the complexity of host-parasite periodicity owing to not only the parasite's intrinsic rhythm but also the external factors affecting the host's role in cell-cycle rhythm.

Finally, this issue is an old and fascinating problem for *Plasmodium* biology, and it requires further investigation. Understanding the open question of how the parasite senses and transduces the environmental cues that induce the progression of its cell cycle may lead us to the development of new antimalarials.

Author contributions

Benedito M. dos Santos: Conceptualization, Writing – Original Draft, Writing – Review & Editing; **Pedro H.S. Pereira**: Writing – Original Draft, Writing – Review & Editing; **Ce9~lia R.S. Garcia**: Conceptualization, Writing, Review & Editing and Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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