

Unlike the synchronous *Plasmodium falciparum* and *P. chabaudi* infection, the *P. berghei* and *P. yoelii* asynchronous infections are not affected by melatonin

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Abstract: We have previously reported that *Plasmodium chabaudi* and *P. falciparum* sense the hormone melatonin and this could be responsible for the synchrony of malaria infection. In *P. chabaudi* and *P. falciparum*, melatonin induces calcium release from internal stores, and this response is abolished by U73122, a phospholipase C inhibitor, and luzindole, a melatonin-receptor competitive antagonist. Here we show that, *in vitro*, melatonin is not able to modulate cell cycle, nor to elicit an elevation in intracellular calcium concentration of the intraerythrocytic forms of *P. berghei* or *P. yoelii*, two rodent parasites that show an asynchronous development *in vivo*. Interestingly, melatonin and its receptor do not seem to play a role during hepatic infection by *P. berghei* sporozoites either. These data strengthen the hypothesis that host-derived melatonin does not synchronize malaria infection caused by *P. berghei* and *P. yoelii*. Moreover, these data explain why infections by these parasites are asynchronous, contrary to what is observed in *P. falciparum* and *P. chabaudi* infections.

Keywords: malaria, calcium, melatonin, cell cycle, rhythm, sporozoite

Introduction

Malaria, caused by the parasite of genus *Plasmodium*, represents a major public health issue due to the growing resistance to current anti-malarial drugs.¹ The World Health Organization (WHO) estimates that 300 to 500 million people are infected annually, and the number of deaths exceeds one million. The periodical fever peaks, which occur generally in 24-hour multiple intervals are the most striking trait of the malarial infection. This observation suggests that the erythrocyte rupture and reinvasion process is an extremely synchronized event. When a red blood cell (RBC) is infected by *Plasmodium*, their spectrin network is changed by the parasite.² This RBC modification is only one of many operated by the parasite during its life cycle, due to, for example, intercellular protein trafficking.³

In 1929, Boyd⁴ demonstrated that the life cycle of the chicken parasite *P. cathemerium* changed in accordance with changes in the light/dark cycles to which the host was submitted. In 1934, Taliaferro and Taliaferro⁵ delayed the schizogony of the monkey parasite *P. brazilianum* in 12 hours only by altering the host's photoperiod. This evidence stressed the importance of the photoperiod on the parasite's life cycle. In 1939, Stauber⁶ reported that incident light on the host's body surface or shone directly over the parasites does not alter the development of the infection. The light has to be perceived by the host for the synchronization signal to be delivered to the parasites in the bloodstream.

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In 1970, Hawking,⁷ who was studying *P. vivax*-infected patients, suggested that fever results from a burst in the number of merozoites in the host's bloodstream, and concluded that the parasites display a synchronous development to produce this population increase.

In 1976, Trager and Jensen⁸ successfully maintained *P. falciparum* in culture, and observed that the parasites lost their synchrony. This observation suggested an involvement of the host's physiology on the maintenance of the infection rhythm. The host's temperature was suggested to perform a role in this phenomenon, as there is a conspicuous period between the fever peaks.⁹ However, this possibility was rejected based on several lines of evidence, such as the variation of the schizogony times between different *Plasmodium* species.^{10,11}

A periodicity is also observed during the sexual stage of *Plasmodium*'s life cycle. This observation was initially made by Shah in 1934, who encountered a peak in *P. cathemeruim* gametocyte number at 1800 hrs. Similar results were reported by Gambrell¹² and Hawking and collaborators.¹³ In 1970, Hawking showed that production of gametocytes of the monkey parasite *P. knowlesi* depends on the host's circadian rhythm, increasing significantly during the night. This phenomenon has since been reported for several *Plasmodium* species.¹⁴ The appearance of the invertebrate-infective forms in the bloodstream at a time close to the feeding period the vector is a very important adaptive feature, which ensures the propagation of the infection. This cyclic and precise temporization of the appearance of gametocytes, coinciding with the vector's feeding pattern was called the "Hawking Phenomenon" by Garnham and Powers.¹⁵

Melatonin, a hormone secreted in a rhythmic fashion by the pineal gland, is a highly conserved molecule, as its presence can be observed in organisms ranging from archaeobacteria to vertebrates.^{16,17}

We have shown that the hormone melatonin is able to synchronize the life cycle of *P. chabaudi* and *P. falciparum* *in vitro* and that this effect is abolished by luzindole, a melatonin receptor antagonist.¹⁸ The synchronism is also lost *in vivo* in pinealectomized mice and upon injection of luzindole. Furthermore, synchronism in pinealectomized mice can be restored by melatonin administration.

The question then arises as to the evolutionary advantage for the parasites of cell cycle synchronization by host-produced melatonin. A hypothesis is that synchronous maturation might be a strategy to evade the host's immune system.¹⁸⁻²⁰

As to the molecular mechanism of melatonin action in the parasites we have shown that melatonin can elicit

an increase in intracellular calcium concentration in *Plasmodium* trophozoites. In addition, in *P. falciparum*, we have demonstrated that the melatonin-signaling pathway involves a complex crosstalk between Ca²⁺ and cAMP,²¹ and further activation of protein kinase A (PKA). Protein kinases are key components in *Plasmodium*-signaling pathways.²²⁻²⁴ Plasmodia genomes encode handling machinery for the both second messengers.^{25,26} Additionally, several reports support the importance of calcium signaling in parasites.²⁷⁻³⁶ Calcium also plays a crucial role on invasion events, inducing proper apical alignment of the merozoite.³⁷

In addition, *Anopheles* mosquito feeding habits occur during darkness,³⁸⁻⁴¹ a period during which the levels of melatonin are the highest.

In this report we addressed the problem of the evolutionary role of synchronicity by comparing *in vitro* the effects of melatonin on cell cycle and Ca²⁺ levels in parasites that *in vivo* have a highly synchronous development (*P. chabaudi*) with the strains *P. berghei* and *P. yoelii* that develop asynchronously *in vivo*.⁴²⁻⁴⁵

The data demonstrate that *in vitro* the asynchronous strain of *P. berghei* and *P. yoelii* melatonin is devoid of any effect on either Ca²⁺ signaling or cell cycle control on the blood stage and that melatonin does not produce any effect in *P. berghei* infection of mice and infected primary hepatocytes.

Here we present evidence that *P. berghei* and *P. yoelii*, both of which lead to asynchronous infections, do not respond to melatonin, strengthening the evidence that melatonin plays a major role in determining the rhythm of *Plasmodium* infection.

Materials and methods

Parasites

P. berghei NK65 and *P. yoelii* were maintained in BALB/c mice by infection passaging. The procedure for collecting blood and removing platelets has been described previously by Hotta and colleagues.⁴⁶

P. berghei ANKA sporozoites were obtained from the salivary glands of infected *Anopheles stenphensi* mosquitoes and used to perform *ex vivo* and *in vivo* liver stage infection experiments.

In vivo experiment with *P. berghei*

Wistar rats, with a body weight of approximately 300 g, where inoculated with 10⁷ erythrocytes infected with *P. berghei* NK65 parasites. The rats were maintained with food and water *ad libitum*, in a 12 hours light/12 hours dark

photoperiodic regime. Every day, at ZT11, blood samples were collected from tail blood, to measure parasitemia by counting no less than 1000 cells in Giemsa-stained blood smears.

In hepatic stage assays 6–10 weeks old, male C3H mice, which have a high physiological concentration of melatonin,⁴⁷ were maintained in a 12 hours light/12 hours dark photoperiodic regime.

Luzindole treatment was performed by i.v. injection of 15 mg/kg of luzindole (Sigma-Aldrich, St Louis, MO) at midnight on three consecutive days. Mice were infected with 20,000 sporozoites at day 2 of luzindole treatment.

Fluorescent Ca²⁺ determinations

To obtain isolated parasites, 10⁸ infected RBC per ml were briefly treated with saponin (10 mg/ml) and washed twice in buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM D-Glucose and 50 mM MOPS, pH 7.2) and resuspended in the same buffer supplemented with 2.8 mM probenecid (Sigma-Aldrich), an organic anion transport inhibitor.⁴⁸ The cell suspension was then incubated for 50 min at 37 °C with 6 μM Fluo-3 AM (Molecular Probes, Carlsbad, CA) and washed three times with buffer A, for removal of extracellular probe. All the experiments and incubations were carried out in the presence of protease inhibitors: leupeptin, pepstatin A, antipain, chymostatin (20 μg/ml) and 0.5 mM benzamidin.

Fluorescence was measured at 37 °C with a Shimadzu RF-5301PC Spectrofluorimeter (Shimadzu, Kyoto, Japan), with an excitation wavelength of 505 ± 5 nm and an emission wavelength of 530 ± 5 nm. Calcium concentration was assessed as described in Hotta and colleagues.⁴⁶

In vitro experiments

Parasites were maintained in RPMI 1640 with 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), supplemented with 50% FCS. Melatonin was added at different concentrations, and incubated for 18 hours. Analyses of Giemsa-stained smears were performed, counting no less than 1000 cells per smear for parasitemia and life form distribution assessment. Triplicate smears were prepared for each experimental condition. Results are presented as the mean of three independent experiments.

Infection quantification ex vivo

Mouse primary hepatocytes were isolated by perfusion of mouse liver lobules with liver perfusion medium (Gibco/Invitrogen, Carlsbad, CA) and purified using a 1.12 g/ml;

1.08 g/ml and 1.06 g/ml Percoll gradient. Cells (5 × 10⁴ per well) were cultured in William's E medium containing 4% FCS, 1% penicillin/streptomycin, in Lab-Tek™ chamber slides (Nunc™). Hepatocytes were maintained in culture at 37 °C and 5% CO₂.

Primary hepatocytes were incubated with 400 nM melatonin for 30 minutes and infected with 20,000 *P. berghei* sporozoites.

Infection was determined 45 hours after sporozoite addition by counting the number of exoerythrocytic forms (EEFs) on cells fixed with ice-cold methanol for 10 minutes and stained with the mouse monoclonal antibody 2E6⁴⁹ and an AlexaFluor488 labeled goat anti-mouse secondary antibody (Molecular Probes/Invitrogen).

Infection quantification by qRT-PCR

The determination of liver parasite load *in vivo*, was performed according to Bruna-Romero and colleagues.⁵⁰ Livers were collected and homogenized in denaturing solution (4 M guanidine thiocyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl and 0.7% β Mercaptoethanol in diethylpyrocarbonate [DEPC]-treated water), 40 h after sporozoite injection. Total RNA was extracted using Qiagen's RNeasy Mini kit, following the manufacturer's instructions. RNA for infection measurements was converted into cDNA using Roche's Transcriptor First Strand cDNA Synthesis kit, according to the manufacturer's protocol. The quantitative reverse transcription polymerase chain reactions (qRT-PCRs) were carried out using Applied Biosystems' Power SYBR Green PCR Master Mix and were performed according to the manufacturer's instructions on an ABI Prism 7000 system (Applied Biosystems Inc., Foster City, CA). PbA-specific primer sequences were 5'-AAG CAT TAA ATA AAG CGA ATA CAT CCTTAC - 3' and 5'-GGA GAT TGG TTT TGA CGT TTA TGT G - 3'.

Results

[Ca²⁺]_i pools in *P. berghei* and *P. yoelii*

A large number of cellular events in both low and high eukaryotes employ Ca²⁺-based signaling pathways. Extensive work on Ca²⁺ homeostasis and signaling has provided evidence for the major role of the endoplasmic reticulum in these processes as well of the participation of other organelles such as mitochondria, lysosomes and Golgi in Ca²⁺ storage in mammalian cells.⁵¹

P. berghei possesses calcium-handling mechanisms, such as the Ca²⁺-ATPases of endoplasmic reticulum (SERCA), sensitive endoplasmic reticulum (ER)-like pool and acidic pools.

To investigate the role of intracellular Ca^{2+} pools in these parasites we have isolated *P. berghei* from red blood cells and loaded the parasites with Fluo-3 AM calcium dye. Figure 1a shows the effect of addition of 5 μM thapsigargin (THG),

a SERCA inhibitor,^{52,53} on isolated *P. berghei* parasites. THG promotes an increase in the cytosolic calcium concentration, (205 ± 32.85 nM) thus confirming that these cells can store the Ca^{2+} ion in the ER. We further investigate whether acidic

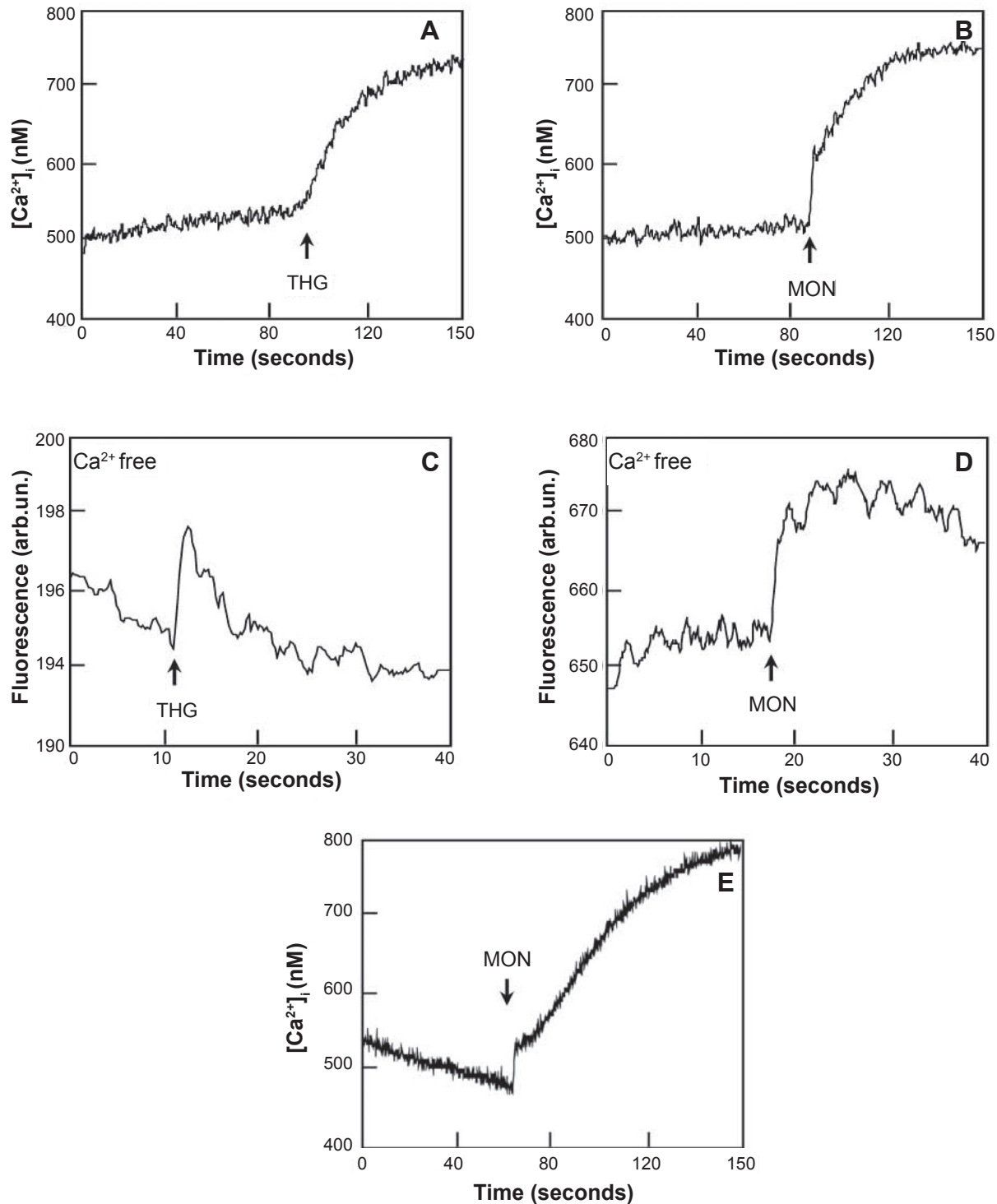


Figure 1 Calcium mobilization in Fluo-3 labeled *P. berghei*-isolated parasites. **A)** Addition of thapsigargin (THG, 5 μM) in 1 mM Calcium medium. **B)** Addition of 25 μM monensin (MON) to medium containing 1 mM Calcium. **C)** Addition of 5 μM THG to calcium-free medium. **D)** Addition of 25 μM MON to calcium-free medium. **E)** Addition of 20 μM MON to *P. yoelii* parasites, in 1 mM calcium medium.

pools could also play a role on Ca^{2+} homeostasis in *P. berghei*. Figure 1b shows that monensin, a Na^+/H^+ ionophore (25 μM) elicits an 265 ± 30.96 nM increase in $[\text{Ca}^{2+}]_i$ of *P. berghei*.

P. yoelii also possesses mechanisms to handle calcium. Addition of THG to isolated parasites results in a 248 ± 52.61 nM ($n=8$) $[\text{Ca}^{2+}]_i$ elevation (data not shown), showing that the ER is able to participate in calcium homeostasis. Acidic pools are also present and can be mobilized, as addition of monensin (20 μM) promotes a 291.4 ± 65.70 nM $[\text{Ca}^{2+}]_i$ increase (Figure 1E).

Melatonin does not elicit an increase in Ca^{2+} concentration in *P. berghei* and *P. yoelii*

In order to further analyze the importance of melatonin in the control of Plasmodia cell cycle, we sought to investigate its effects on *P. berghei*, a species of *Plasmodium* that, unlike the vast majority of mammalian *Plasmodium* species, has an asynchronous development in the live mouse.

The simplest *in vitro* test to address the sensitivity of *P. berghei* and *P. yoelii* to melatonin is to determine whether the hormone can increase the cytoplasmic Ca^{2+} concentration, a well established early event caused by melatonin in *P. chabaudi* and *P. falciparum*. Figure 2 shows that addition of up to 20 μM melatonin did not lead to an increase in Ca^{2+} concentration in *P. berghei* or *P. yoelii*, regardless of whether calcium was present or not in the medium. In addition, the effect of melatonin on *P. yoelii* is shown Figures 2C and D.

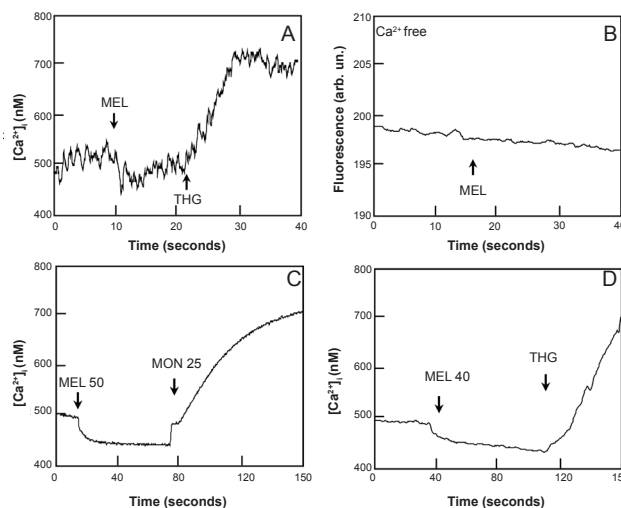


Figure 2 Effects of melatonin addition in isolated, Fluo-3 labeled, *P. berghei* and *P. yoelii* parasites. **A)** Addition of 20 μM melatonin (MEL) to *P. berghei* in medium containing 1mM calcium. **B)** Addition of 20 μM MEL to *P. berghei* in calcium-free medium. **C)** Addition of 40 μM melatonin (MEL 40) followed by 25 μM monensin to *P. yoelii* in medium containing 1mM calcium. **D)** Addition of 50 μM melatonin (MEL 50) followed by THG (5 μM) to *P. yoelii* in medium containing 1 mM calcium. These experiments show that melatonin was not able to elicit a calcium response on these parasites.

The experiment shown in Figure 2 (panel a) shows that, similarly to what found in other Plasmodia, if after melatonin *P. berghei* are treated with thapsigargin (THG), an inhibitor of the sarco-endoplasmic reticulum ATPase,^{52,53} a clear increase in $[\text{Ca}^{2+}]_i$, (205 ± 32.85 nM, $n=3$) is observed. In accordance with these results, panel b shows that monensin, a Na^+/H^+ ionophore, that can induce the release of Ca^{2+} from an acidic pool in other Plasmodia strains, also elicits a strong increase in $[\text{Ca}^{2+}]_i$, 265 ± 30.96 nM $n=3$ in *P. berghei*. *P. yoelii* also lacks response for melatonin, while still have calcium pools that are capable of mobilization (Figures 2C and 2D). Taken together, these results most likely reflect the absence of a melatonin receptor coupled to Ca^{2+} mobilization rather than a unique characteristic of *P. berghei* or *P. yoelii* Ca^{2+} homeostasis.

Melatonin does not interfere with *P. berghei* and *P. yoelii* life cycle

Melatonin receptors might couple to other signaling pathways (eg, cAMP). In order to test whether in *P. berghei* melatonin could affect the cell cycle through a Ca^{2+} -independent mechanism, we tested whether the hormone could synchronize *in vitro* *P. berghei* life cycle as is the case with *P. chabaudi* and *P. falciparum*.^{18,20,21} Again, hormone concentrations up to 100-fold higher than those capable of strongly affecting Plasmodia development in the other strains were totally ineffective in synchronizing either *P. berghei*'s or *P. yoelii*'s life cycle (Figure 4).

P. berghei can infect both rats and mice. In C57BL/6 mice, *P. berghei* primarily causes a severe syndrome known as cerebral malaria.⁵⁴ We thus checked whether the lack of *in vitro* sensitivity of *P. berghei* may be due to an artifact of these artificial culture conditions or of the specificity of the disease in mice. Wistar rats were inoculated with 10^7 *P. berghei*-infected erythrocytes and the distribution of life forms on the fifth day after infection was investigated in blood smears. Figure 5 shows that also in rats *P. berghei* does not display a synchronous development. Indeed the percentage of rings and trophozoites are very similar (unlike in the mouse infected with *P. chabaudi* where trophozoites largely predominate at this time). Schizonts are hardly observed in infected rats as already reported by Desowitz and Barnwell presumably due to sequestration of the RBC-containing parasites at this stage in the microvasculature.⁴²

Melatonin does not modify the *P. berghei* liver infection load

The inhibition of melatonin receptor does not affect the parasite load in the livers of mice infected with *P. berghei*

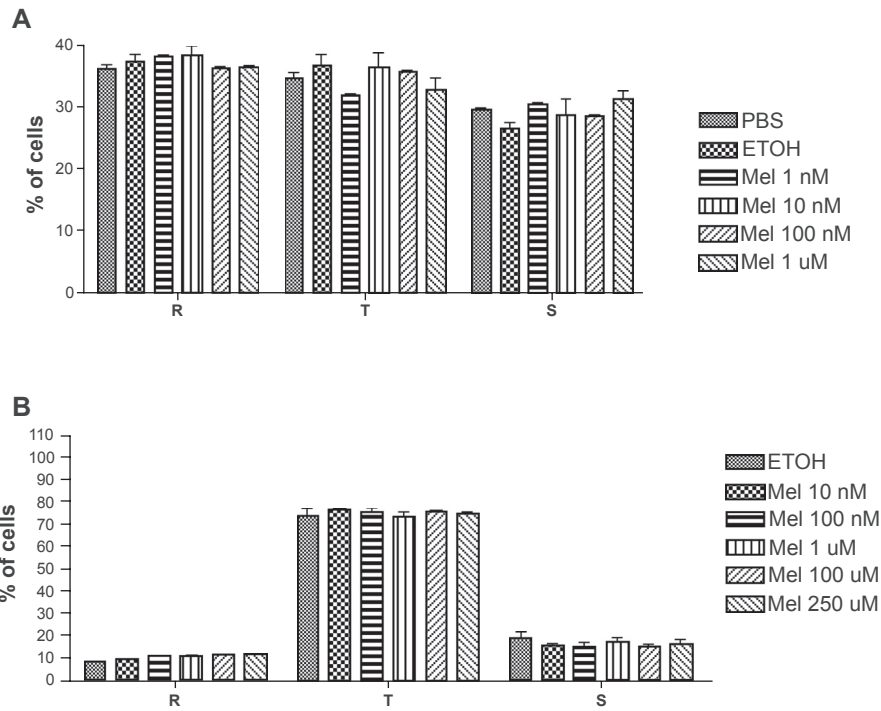


Figure 3 *In vitro* culture of *P. berghei* **A**) and *P. yoelii* **B**) incubated with different melatonin concentrations. The figure shows the distribution of *P. berghei* and *P. yoelii* life forms after 18 or 13 hours incubation, respectively, with different melatonin concentrations (1 nM, 10 nM, 100 nM e 1 μM). There are no statistical differences in the distribution. The results are presented as the mean of three independent experiments.

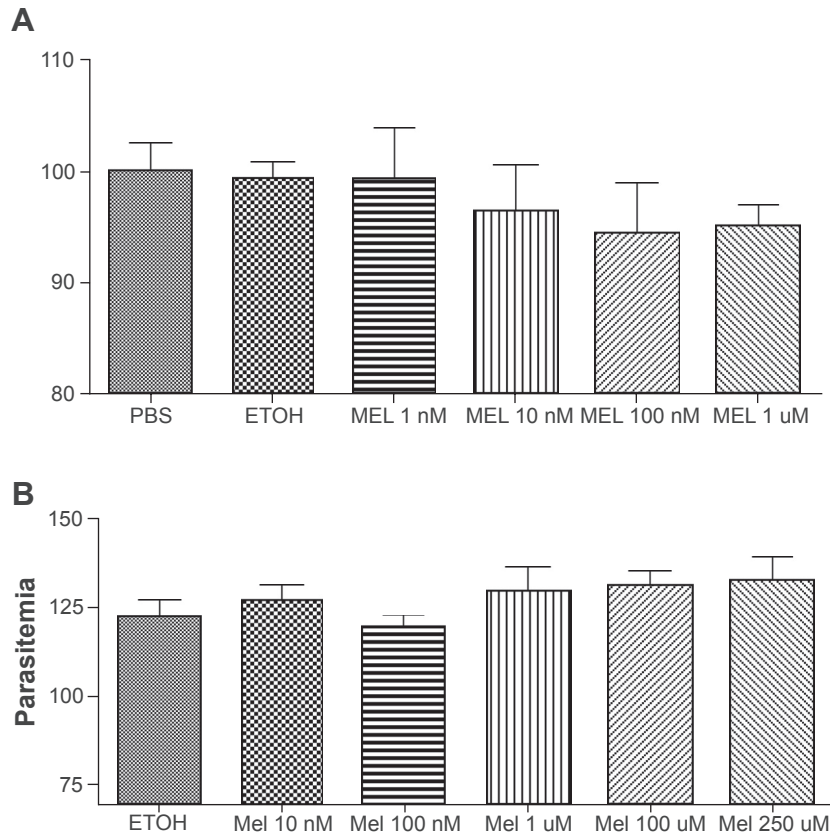


Figure 4 *In vitro* culture of *P. berghei* **A**) and *P. yoelii* **B**) incubated with different melatonin concentrations. The figure shows *P. berghei*- and *P. yoelii*-infected red blood cells (iRBC) after 18 or 13 hours incubation, respectively, melatonin concentrations (1 nM, 10 nM, 100 nM and 1 μM). There are no statistical differences in the number of iRBC. The results are presented as the mean of three independent experiments.

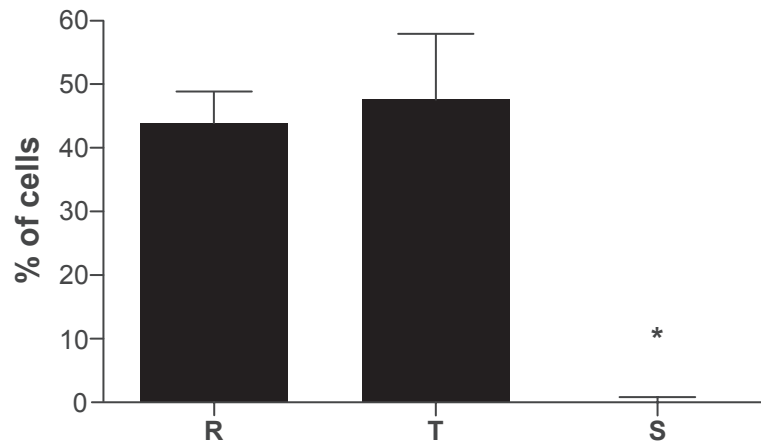


Figure 5 Distribution of *P. berghei* in infected Wistar rats, five days after inoculation of 10^7 infected erythrocytes.

Notes: To assess life forms distributions, no less than 1000 cells were counted in Giemsa-stained smears. No statistical differences were observed between the percentage of rings and trophozoites. Schizonts were not present in peripheral bloodstream due to microvasculature sequestration.

Abbreviations: R, rings; T, trophozoites; S, schizonts.

ANKA sporozoites. In addition, we did not observe a significant difference between the *P. berghei* infection levels of melatonin-treated mouse primary hepatocytes and that of control hepatocytes.

We have also considered the possibility that other stages of *P. berghei* might sense melatonin. Addition of melatonin to *P. berghei* gametocyte is not able to elicit an increase of calcium levels (Bilker O, personal communication) as is the case when xanthurenic acid is added.⁵⁵

Discussion

The spectrofluorimetry results obtained show that *P. berghei* and *P. yoelii* display mechanisms that sustain the $[Ca^{2+}]_i$ against an extracellular calcium concentration in the millimolar range. By using isolated parasites loaded with fluorescent dyes we showed here that the endoplasmic reticulum plays a role in the $[Ca^{2+}]_i$ maintenance. The storage of the calcium ion is mediated by a SERCA, since thapsigargin inhibits this

enzyme, and promotes and $[Ca^{2+}]_i$ increase. The experiments involving the Na^+/H^+ ionophore monensin have shown that an acidic pool also participates in calcium homeostasis, as the ionophore also elicits an $[Ca^{2+}]_i$ increase. The presence of intracellular calcium pools in *P. berghei* was previously demonstrated by Marchesini and colleagues.⁵⁶

In 2000, Hotta and colleagues showed that melatonin could mobilize calcium from internal stores in isolated *P. chabaudi* and that this hormone is responsible for synchronization of the infection. The use of the phospholipase C inhibitor U73122 or of the melatonin receptor competitive antagonist luzindole abolished the melatonin-mediated calcium response, suggesting that a calcium pathway is involved in the transduction of the hormone signal. This pattern is also observed in *P. falciparum*, as reported by Beraldo and colleagues²¹ using isolated parasites and infected RBCs.

Strikingly, melatonin does not induce an elevation in $[Ca^{2+}]_c$ of *P. berghei* or *P. yoelii*, both of which lead to an

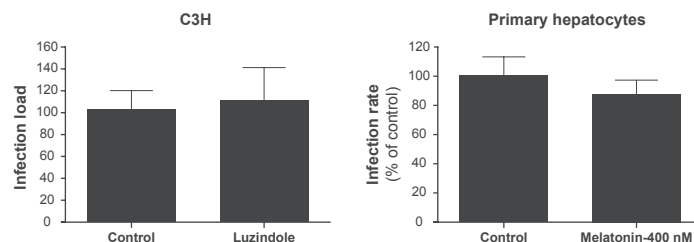


Figure 6 A) Effect of inhibition of melatonin receptor in C3H mice infected by *P. berghei* sporozoites. Liver infection load was measured by qRT-PCR analysis of *P. berghei* 18 S rRNA in liver extracts taken 40 h after sporozoite i.v. injection, and plotted as a percentage of the mean of negative control samples. The plot represents three independent experiments ($n = 18$). No statistical significances were observed. **B)** Effect of 400 nM melatonin on infection of mouse primary hepatocytes by *P. berghei* sporozoites. Infection rates were calculated for each sample well as the number of EEFs, plotted as a percentage relative to the mean of negative control samples. Results are expressed as the mean \pm SD of triplicate in three independent experiments.

Abbreviations: EEFs, exoerythrocytic forms; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; SD, standard deviation.

unsynchronized infection. This observation prompted the question of whether melatonin was able to synchronize these infections, as we previously reported to be the case with both *P. chabaudi* and the human malaria parasite *P. falciparum*. To evaluate whether, despite not promoting calcium mobilization, melatonin was able to synchronize *P. berghei* and *P. yoelii*, we incubated parasites with various melatonin concentrations. The analysis of giemsa-stained smears has shown that the hormone was not able to synchronize the infection of these rodent parasites at a maximum concentration of 1 μM for *P. berghei*, and 250 μM for *P. yoelii*, in contrast to that is found in *P. chabaudi*, whose cell cycle can be modulated by 10 nM of melatonin.¹⁸

Here we show that melatonin does not elicit a calcium response nor does it affect the distribution of *P. berghei* and *P. yoelii* life forms, which display an unsynchronized infection *in vivo*. These data strengthen the hypothesis that *Plasmodium* utilizes melatonin to synchronize its life cycle,^{18,19,21} and, in the case of *P. berghei* and *P. yoelii*, we suggest that the non-response to melatonin is one of the reasons that this infection is unsynchronized.

While the physiology of malaria parasites seems similar, all displaying maturation stages such as ring, trophozoite, and schizont, the molecular machinery is distinct for different parasite strains. It is known that 80% of rodent malaria genes do possess an ortholog in *P. falciparum*.⁵⁷

According to Guha and colleagues⁵⁸ melatonin inhibits hepatocyte apoptosis and liver damage induced during malarial infection. In addition, *P. berghei*-infected hepatocytes are protected against apoptosis and this protection seems to be triggered by both host and parasite molecules.⁵⁹ However, our results suggest that melatonin is not involved in hepatic infection by *Plasmodium berghei* ANKA sporozoites.

The molecular nature of the melatonin receptor in Plasmodia is currently under investigation. However, the complete absence of any functional effect of melatonin on *P. berghei* *in vitro* and its *in vivo* asynchronous development even in rats (that have a strong circadian melatonin production rhythm) suggests that this strain of *Plasmodium* does not express melatonin receptors.

Taken together these data add important novel support to the hypothesis that melatonin is responsible for the *in vivo* synchronicity of other Plasmodia species^{16,18–20} and suggest that the lack of response to melatonin is one of the reasons why the *in vivo* cell cycle of *P. berghei* and *P. yoelii* is unsynchronized.

Finally, the work presented here provides a clear link between the importance of host melatonin and synchronization

of malaria parasites thus showing that the distribution of *P. berghei* and *P. yoelii* life forms is not affected by melatonin whereas, in contrast, it exerts a marked biological effect on *P. chabaudi* and *P. falciparum*.

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