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Malaria Infection Suppresses Proestrus and Estrus While Prolonging Diestrus in Mice

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ABSTRACT

Background: Malaria remains a significant global health concern. Beyond its well-known pathogenesis and effects, the impact of malaria on female reproductive health, particularly estrous cycle dynamics, is poorly understood. This study investigated the influence of *Plasmodium berghei* (NK65 strain) infection on estrus cycle phases in female Swiss mice.

Methods: Eighteen female Swiss mice (8–12 weeks old) were assigned to control (uninfected) and infected (*P. berghei* NK65 strain) groups (9 per group). Estrus cycles were monitored through daily vaginal cytology for 30 days (15 days pre-inoculation and 15 days post-inoculation). The number of days spent in each estrus phase (proestrus, estrus, metestrus, and diestrus) was recorded for each group. The differences before and after inoculation in the two groups were analyzed using a Chi Squared test for proportions., with statistical significance set at a P value less that 0.05 (two-sided).

Results: Parasitemia in infected mice reached an average of 24% by Day 14, confirming successful infection. Control mice maintained normal estrus cycles, with no significant post-inoculation disruptions. However, infected mice, exhibited severe estrus cycle disturbances, including suppression of proestrus and estrus phases post-inoculation ($p < 0.05$) and a significant increase in diestrus duration ($p < 0.05$).

Conclusion: *Plasmodium berghei* infection significantly disrupted estrus cycle dynamics in female mice, primarily suppressing the reproductive phases essential for ovulation while prolonging diestrus. These findings underscore the potential impact of malaria on female reproductive health. Further research is needed to elucidate the underlying mechanisms driving these disruptions.

Introduction

Malaria, caused by *Plasmodium* species and transmitted to humans by female *Anopheles* mosquitoes, remains a major global health concern, affecting millions annually1. While its impact on immune function and organ systems is well characterized, the systemic effects of malaria on reproductive health remain poorly understood.

The estrus cycle is a complex physiological process in female mammals, moderating hormonal balance and reproductive health. Systemic infections and inflammatory conditions can disrupt the hypothalamic-pituitary-ovarian (HPO) axis, leading to hormonal imbalances and reproductive dysfunction. For instance, sepsis has been shown to decrease estrogen and progesterone concentrations in women, likely due to interactions between the hypothalamic-pituitary-adrenal (HPA) and HPO axes. Corticotropin-releasing hormone (CRH) from the hypothalamus can inhibit gonadotropin-releasing hormone (GnRH) release, and glucocorticoids released during sepsis can suppress luteinizing hormone (LH) secretion from the pituitary, resulting in reduced estrogen and progesterone levels[2]. These disruptions in ovulatory cycle can adversely impact fertility. Although extensive research has focused on the pathophysiology of malaria, its influence on estrus cycle dynamics remains underexplored.

Estrus

The estrus cycle in female mice is a cyclical pattern of ovarian activity transitioning between reproductive

receptivity and non-receptivity[3]. This cycle, lasting approximately 4–5 days, consists of four distinct phases: proestrus, estrus, metestrus, and diestrus[4]. Each phase is characterized by specific changes in the vaginal smear, which reflects variations in hormone levels that regulate reproductive functions as shown in table 1[5,6].

The short length and regularity of the estrus cycle make it a valuable model for studying reproductive function and assessing the integrity of the hypothalamic-pituitary-ovarian axis that governs female reproduction. Given its relevance and consistency, the estrus cycle is widely used in research[5].

Table 1: Characteristic features of Phases of Estrous Cycle in Mice

Phase	*Duration*	*Vaginal Smear Characteristics*	*Hormonal Dynamics*	*Implications*
Proestrus	< 24 hours	Predominantly nucleated epithelial cells	Rising estradiol due to follicle-stimulating hormone (FSH) stimulation	Prepares for ovulation, behavioral receptivity begins
Estrus	12–48 hours	Predominantly cornified epithelial cells	Luteinizing hormone (LH) surge triggers ovulation, declining estradiol	Fertile window, peak sexual receptivity
Metestrus	8–24 hours	Mix of cornified epithelial cells and leukocytes	Increasing progesterone, decreasing estradiol	Transition phase, corpus luteum formation
Diestrus	48–72 hours	Predominantly leukocytes with few epithelial cells	High progesterone if corpus luteum is active, low estradiol	Reproductive quiescence, non-receptivity

Similar to several other diseases, malaria pathology is characterized by inflammation. While inflammation may contribute to malaria parasite elimination, excessive and prolonged inflammation can lead to severe malaria. Recent studies suggest that infections and inflammatory conditions can interfere with the HPO axis, leading to altered reproductive hormone levels and reproductive dysfunctions such as amenorrhea and anovulation[2,7]. Infections such as endocrine gland infections, sexually transmitted infections, and chronic stress are known to cause hormonal imbalances[8]. However, there is limited research on the effects of malaria on estrus cycle dynamics. A previous study reported that *Plasmodium berghei* caused a delay at the proestrus phase in rodents, suggesting a lack of ovulation during infection[9].

This study investigates the effects of malaria infection on the estrus cycle in mice, focusing on phase-specific disruptions. We hypothesize that malaria-induced systemic changes may disrupt normal hormonal cycling, leading to the suppression of reproductive phases and an extended diestrus phase. By identifying these disruptions, our research provides critical insights into the physiological consequences of malaria, highlighting its potential to impair female reproductive function.

Materials and methods

Animals and housing conditions

Eighteen female Swiss mice, aged 8–12 weeks, were used in this study. The animals were housed under standard laboratory conditions, including a controlled temperature (22–24°C), a 12-hour light/dark cycle, and access to food and water *ad libitum*. All experimental protocols were approved by the University of Ibadan Animal Care and Use Research Ethics Committee (UI ACUREC), protocol number NHREC/UIACUREC/05/12/2022A.

Experimental design

The mice were randomly divided into two groups (n = 9 per group):

1. **Control Group:** Uninfected mice.
2. **Infected Group:** Mice infected with *Plasmodium berghei* (NK65 strain).

The estrus cycle dynamics were monitored for a total of 30 days, which included 15 days before infection and 15 days post-infection.

Malaria infection protocol

The *Plasmodium berghei* (NK65 strain) parasite, obtained from the Malaria Reference Reagent Resource (MR4) center and maintained at the Drug Research Unit laboratory of the Institute for Advanced Medical Research and Training (IAMRAT), was used. Parasites were passaged in Swiss mice to ensure viability and consistency. Blood was collected from donor mice exhibiting high parasitemia into heparinized tubes containing Acid Citrate Dextrose (ACD) as an anticoagulant. The blood was diluted in phosphate-buffered saline (PBS) to achieve a final concentration of 1×10^7 parasites/mL. Each mouse in the infected group received an intraperitoneal injection of 0.2 mL of the parasitized blood suspension (equivalent to 2×10^6 parasites approximately), while each control mouse received 0.2 mL of PBS.

Parasitemia was allowed to develop over 72 hours, after which parasite density was monitored by preparing Giemsa-stained thin blood smears from tail vein blood on Days 0, 1, 2, 3, and 7 post-inoculation. Parasitemia was quantified as the percentage of infected red blood cells in a total count of 1,000 erythrocytes observed under 1000X magnification using a light microscope.

Estrus cycle monitoring

Identification of characteristic features different estrus cycle phases (Table 1, and Table 2) was done in accordance to published work by Ajayi and Akhigbe[5]. Estrus cycle phases were determined by daily vaginal cytology to assess cycle dynamics and detect potential disruptions. Vaginal smears were collected at the same time each day (8:00 am–11:00 am) using disposable 3 mL graduated plastic pipettes. Serially, a pipette was gently inserted into the vaginal canal, and a small volume of normal saline was carefully injected and aspirated to collect cells. The collected specimen was transferred onto a glass slide from which a smeas was prepared for cytological examination.

The smears were air-dried and stained using Giemsa stain (1:10 dilution) for 45 minutes to enhance the visualization of vaginal cell types. After staining, slides were gently rinsed, air-dried, and stored. Cytological evaluation was performed under a light microscope at 100x, 400x, and 1000x magnifications to identify the estrus phases based on the relative proportions of nucleated epithelial cells, cornified epithelial cells, and leukocytes.

Table 2: Characteristic Counts of Cell Types During Different Estrous Phases

Estrous Phase	Nucleated Epithelial Cells (%)	Cornified Epithelial Cells (%)	Leukocytes (%)	Description
Proestrus	70–90	0–10	10–20	Predominantly round nucleated epithelial cells w ith minimal leukocytes and cornified cells. Prepares for ovulation.
Estrus	0–10	90–100	0	Large, flat, keratinized cells without nuclei, indicating peak fertility.
Metestrus	20–40	20–40	30–50	Mix of nucleated and cornified epithelial cells, with increasin g leukocytes.
Diestrus	5–20	5–10	70–90	Dominated by leukocytes, indicating a quiescent reproductive phase after ovulation.

The mice were monitored for 15 days before inoculation and 15 days after inoculation to assess changes in estrus cycle dynamics. The total number of days spent in each estrusphase (proestrus, estrus, metestrus, and diestrus) was counted and recorded for each mouse.

1. Count of Days in Each Phase
    - The number of days each mouse spent in each phase was conuted separately for the pre-inoculation and post-inoculation periods.
2. Group Counts
    - The total days spent in each phase was counted across all mice within each group (control and infected).
    - These values were compared between the pre- and post-inoculation periods to assess intra-group differences.
    - the comparisons were used to evaluate the impact of malaria infection on estrus cycle patterns, particularly disruptions such as the suppression of proestrus and / or prolonged diestrus.

The counts were done for each mouse in the group with each group of nine mice expected to have a total of 270 days, 9 x 15 = 135 days pre-innoculation, and 9 x 15 = 135 days post innoculation.

Statistical Analysis

A 2×2 contingency table was constructed to compare the proportions. Statistical significance was determined using Chi Squared test for proportions. The analysis was performed using EpiCalc Version 2000, with statistical significance set at $p < 0.05$ (two-sided).

Results

Experimental design overview

A total of 18 mice aged 8–12 weeks were divided into two groups: control (n = 9) and Infected (n = 9). Estrus cycle phases were monitored daily over a 30-day period, which included 15 days pre-inoculation and 15 days post-inoculation. Control mice received phosphate-buffered saline (PBS), while infected mice were inoculated intraperitoneally with *Plasmodium berghei*. Two mice in the infected group died before completing the 15-day post-inoculation period, reducing the total count to 105 after inoculation.

Parasitemia and infection monitoring

Parasitemia was assessed to confirm successful infection in the infected group. Monitoring began 72 hours post-inoculation and was conducted on Days 0, 1, 2, 3, and 7. On day 7, the average parasitemia in the infected group reached 24%, confirming effective establishment of infection. No parasitemia was observed in the control group at any time point.

Estrus cycle dynamics before and after infection

The estrus cycle phases were monitored in both control and infected mice before and after *Plasmodium berghei* inoculation. The results showed significant disruptions in estrus cycle dynamics following malaria infection in the infected group.

Estrus cycle changes in control mice

In the control group, the number of days spent in each estrus phase before and after inoculation remained relatively similar, with no significant differences across the cycle phases (Table 3). Proestrus, estrus, metestrus, and diestrus were all present in comparable proportions before and after inoculation, indicating that the estrus cycle remained largely unaffected.

Table 3: Number of days spent at different Estrus Cycle Phases of the nine Control Mice Before and After PBS administration

Estrus Phase	Before Inoculation (n 9 x 15 = 135)	After Inoculation (n = 9 x 15 = 135)	p-value (two-sided)
Proestrus	21 (15.56%)	27 (20.00%)	0.4261
Estrus	33 (24.44%)	42 (31.11%)	0.2770
Metestrus	33 (24.44%)	30 (22.22%)	0.7735
Diestrus	48 (35.56%)	36 (26.67%)	0.1482

Estrus cycle changes in infected mice

In contrast, infected mice exhibited profound alterations in their estrus cycle. Following inoculation, proestrus and estrus phases were absent, with all infected mice spending most of the post-innoculation period in diestrus (93.33%) (Table 4). A significant reduction in metestrus was also observed.

Table 4: Number of days spent at different Estrus Cycle Phases of the nine malaria infected Mice Before and After Inoculation

Estrus Phase	Before Inoculation (n = 9 x 15 = 135)	After Inoculation (n = 7 x 15 = 105)	p - value (two-sided)
Proestrus	24 (17.78%)	0 (0.00%)	0.000014
Estrus	30 (22.22%)	0 (0.00%)	0.000001
Metestrus	27 (20.00%)	7 (6.67%)	0.005924
Diestrus	54 (40.00%)	98 (93.33%)	0.000000

Comparison of control and infected groups after inoculation

Examination of the counts and the differences between the control and infected groups highlighted the impact of malaria infection on estrus cycle dynamics. The cycle remained unaffected in control mice whereas infected mice failed to enter proestrus and estrus. Metestrus was also significantly reduced, and diestrus was significantly prolonged.

DISCUSSION

This study investigated the impact of *Plasmodium berghei* (NK65 strain) infection on the estrus cycle dynamics of virgin female Swiss mice, focusing on changes in the number of days spent in each phase before and after inoculation. The findings revealed that malaria infection significantly disrupted the estrus cycle, leading to a marked suppression of proestrus and estrus and a significant increase in the duration of diestrus.

Estrus

Mice infected with *P. berghei* exhibited substantial alterations in their estrus cycles, with a pronounced shift toward prolonged diestrus. Diestrus, marked by the predominance of leukocytes and indicative of a quiescent reproductive state, accounted for 93.33% of the total cycle days post-inoculation in infected mice. In contrast, the control group maintained a regular estrus cycle with all phases present, including proestrus and estrus.

The disruptions observed in this study align with previous research demonstrating that systemic infections and inflammatory conditions significantly impair female reproductive function. For instance, malaria as a systemic disease, triggers an inflammatory response involving the release of cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), which, in combination with the activation of the hypothalamus-pituitary-adrenal (HPA) axis, disrupt the hypothalamic-pituitary-gonadal (HPG) axis[10,11]. Also, research indicates that malarial infection disrupts host metabolism and results in hormonal imbalances, with variations influenced by immune response, parasite species, infection severity, sex, age, nutritional status, and infection stage[10]. These findings suggest that inflammatory processes in malaria-induced hormonal imbalances can suppress ovulation, as evidenced by the absence of proestrus and estrus in infected mice.

Proestrus and estrus are critical phases for reproductive readiness and ovulation. Their suppression in malaria-infected mice underscores the severity of the reproductive disruptions caused by malaria infection. Proestrus, characterized by the proliferation of nucleated epithelial cells in preparation for ovulation, and estrus, the peak fertility phase dominated by cornified epithelial cells, were entirely absent post-inoculation in infected mice. While a previous study by Aina *et al.*[9] reported a delay in proestrus rather than its full suppression in *Plasmodium berghei*-

infected mice, our study demonstrates the complete absence of proestrus and estrus post-inoculation[9].

This suppression may represent an adaptive mechanism in which the body conserves energy for immune responses rather than reproduction. However, this adaptation may have potential fertility consequences, as prolonged suppression of proestrus and estrus limits ovulation and decreases reproductive capacity. Notably, the interplay between malaria and reproductive hormones is complex. Studies suggest that estrogen levels influence immune responses, with physiological levels enhancing immunity and potentially offering protection against severe malaria[10, 11, 12]. Conversely, the malaria parasite may actively alter estrogen levels, disrupting reproductive function while simultaneously affecting immune responses. This two-way interaction underscores the broader physiological impact of malaria on female reproductive health.

The findings of this study may suggest clinical relevance, particularly for women in malaria-endemic regions. Chronic or recurrent malaria infections may contribute to menstrual irregularities, subfertility, or even infertility by disrupting hormonal regulation and ovarian function. Other studies have suggested that a decrease in malaria prevalence corresponds to an increase in fertility rates, further emphasizing the impact of the disease disease's on reproductive health[13]. Additionally, malaria is a known contributor to adverse pregnancy outcomes, including miscarriages, preterm births, and low birth weight, due to its effects on the placenta and maternal physiology[14,15]. These findings underscore the importance of integrating reproductive health considerations into malaria management strategies, particularly in endemic regions where the disease burden is high.

The findings of this study align with previous researches demonstrating that systemic infections and inflammatory conditions disrupt estrus cycles in female mammals[16,17]. Similar disruptions, including prolonged luteal or diestrus phases have been observed in bacterial infections and stress-induced inflammation[17]. However, the extent of disruption caused by *Plasmodium* infection appears more pronounced, likely due to the combination of systemic inflammation, immune suppression, and endocrine disruption unique to malaria.

Conclusion

This study demonstrates that malaria infection significantly disrupts the estrus cycle in female mice, primarily through the suppression of proestrus and estrus and a marked prolongation of diestrus. These findings suggest broader physiological consequences of malaria, emphasizing its impact on reproductive health in addition to hematological and neurological effects. Given the clinical relevance of these ovulatory disruptions, there is a need for more research on the underlying hormonal mechanisms and potential interventions with the aim of mitigating potential adverse reproductive consequences of malaria, particularly in malaria endemic regions.

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