Original Article

Influence of Pre-Feeding Starvation Duration on the Blood-Feeding Success of *Anopheles cracens* Under Laboratory Conditions

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Abstract

Background: Malaria, caused by *Plasmodium* parasites, is transmitted to humans through the bites of infected *Anopheles* mosquitoes. Laboratory-reared *Anopheles* mosquitoes play a crucial role in advancing research aimed at eliminating malaria transmission, particularly zoonotic malaria caused by *Plasmodium knowlesi* in Malaysia. *Anopheles cracens*, an incriminated vector for *P. knowlesi*, remains relatively underexplored in laboratory-based vector research and still lacks a clearly defined optimal starvation duration for its blood feeding. This study assessed the length of starvation duration against the female mosquito feeding rate, in order to determine the optimal starvation duration.

Methods: The mosquitoes were subjected to various starvation durations (6, 8, 10, 12, 14 and 16 hours) before blood feeding. All other key parameters were kept constant. For each starvation duration, 25 female *An. cracens* mosquitoes were exposed to the blood meal, and the experiment was repeated a total of three times (n= 75 for each starvation duration).

Results: The results showed a positive association between starvation duration and feeding rate, with longer starvation durations leading to higher feeding rates. The 16-hour starvation group achieved the highest feeding rate at 68% (68–72%). However, no statistically significant differences were observed among the groups due to high variability in the data.

Conclusion: In conclusion, a positive trend between starvation duration and feeding success was observed. Further studies with larger sample sizes and controlled physiological states will enable more precise determination of the optimal starvation threshold and its influence on *P. knowlesi* infection rates under laboratory conditions.

Keywords: *Plasmodium knowlesi*; *Anopheles cracens*; Starvation duration; Blood feeding rate; Laboratory-reared mosquitoes

Introduction

Malaria is a parasitic infection caused by protozoa of the genus *Plasmodium* and transmitted to humans through the bites of infected *Anopheles* mosquitoes. Globally, malaria continues to impose a substantial public health burden, with an estimated 282 million cases and 610,000 deaths reported in 2024 alone (1). Five major *Plasmodium* species are known to naturally infect humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* (*P. ovale curtisi* and *P. ovale wallikeri*) and *P. knowlesi.* Of these, *P. knowlesi* has emerged as a growing concern in Southeast Asia, where rising cases of zoonotic malaria threaten the regional malaria elimination goals. In Malaysia, *P. knowlesi* is now the sole cause of indigenous malaria in humans, making it a significant barrier to achieving malaria elimination (1).

Most individuals get malaria when they are bitten by an infective mosquito carrying the malaria parasites. For this disease, mosquitoes from the *Anopheles* genus serve as the vectors of the *Plasmodium* species. There are around 460 known species of *Anopheles* mosquitoes, and more than 100 of them may be human malaria vectors; so far, only 30–40 common species can transmit *Plasmodium* parasites in humans in endemic areas (2). The transmission of zo-

onotic malaria involves a critical stage in the parasite's life cycle: transfer from humans or macaques to *Anopheles* mosquitoes, where the parasite undergoes sporogonic development. The principal vectors of *P. knowlesi* and other simian malarias in Asia belong to the *Anopheles* leucosphyrus group, which consists largely of forest-dwelling species. Within Malaysia, important simian malaria vectors include *An. latens*, *An. introlatus* and *An. balabacensis* (Leucosphyrus complex), *An. cracens* (Dirus complex) and *An. hackeri* (Hackeri subgroup) (3). A study by Vytihilingam et al. (4) has established *An. cracens* as the vector of *P. knowlesi* in Kuala Lipis, Pahang and notably, this species is highly anthropophilic, preferring to feed on humans rather than animals.

Sugar deprivation (starving) in laboratory-reared mosquitoes before blood feeding is a common practice in vector biology and infection studies to enhance their feeding motivation. In experimental settings, mosquito blood-feeding success is strongly influenced by pre-feeding sugar starvation. Starving female mosquitoes increases their hunger level, thereby improving the feeding rates and ensuring consistent parasite transmission in laboratory studies. Standard mosquito rearing protocols recommend sugar starvation for 5–12 hours to enhance responsiveness to blood meals (5). However, optimal starvation durations vary by species and experimental context. For example, Kumpitak et al. (6) achieved a 90% engorgement rate in *An. dirus* after only 6 hours of starvation, while Timinao et al. (7) reported significantly higher feeding success (60%) in *An. farauti* following overnight (~21 hours) starvation compared to shorter durations (27–45%). Similarly, Amir et al. (8) described starving *An. cracens* for 24 hours prior to blood feeding to support colonization and maintenance. However, this duration may not represent an optimal starvation period for *An. cracens*, as no studies have systematically evaluated starvation length in this species. Although Amir et al. (8) reported starving *An. cracens* for 24 hours, their work applied this duration solely as part of colony establishment procedures rather than as an experimental assessment of the species' starvation–feeding response. These findings collectively highlight the critical role of optimising starvation duration in experimental infections. Since different *Anopheles* species respond differently to starvation, the ideal duration must be species-specific. Yet, for *An. cracens*, one of the vectors of *P. knowlesi* in Malaysia, there is still limited laboratory-based evidence to guide optimal starvation practices. Addressing this gap is essential to improving experimental transmission studies such as infection experiments and ultimately, advancing understanding of zoonotic malaria dynamics in Malaysia.

Materials and Methods

Mosquito colony maintenance

Anopheles cracens mosquitoes were reared in the insectary under standard laboratory conditions, maintained on a 12-hour light dark cycle at a temperature of 24–26 °C and 60–80% relative humidity (8). The larvae were fed ground fish food (Tetra Bits Complete Fish Food, Malaysia). Adult mosquitoes were provided with a 10% sucrose solution, prepared by dissolving 10 g of sugar (CSR Gula Kasar, Malaysia) in 100 ml of water along with two vitamin B-complex tablets, available in a container containing a tissue-wrapped glass slide soaked in the solution and placed within the cage. To enable oviposition and maintain the colony, adult female mosquitoes aged 7–10 days were fed fresh blood collected from donors after informed consent was obtained.

Optimisation of starvation period

The pre-feeding starvation duration was evaluated to identify the best starvation duration with the highest feeding rate. Each replicate consisted of 25 mosquitoes, and three biological replicates were performed for each starvation duration, resulting in a total of 75 mos-

quitoes. For the feeding experiment, a paper cup covered with tulle mesh fabric was prepared, and 25 mosquitoes were aspirated from the cage into the cup (Fig. 1). To maintain humidity, a cotton pad soaked with distilled water was placed on top of the mesh. The mosquitoes were then starved for the designated durations (6, 8, 10, 12, 14 and 16 hours) before blood feeding.

Blood feeding after starvation

Following the starvation duration, mosquitoes (7–10 days after emergence, blood-feeding naive) were exposed to approximately 2 mL of fresh blood (collected from donors) for 1 hour in the dark, using a Hemotek blood-feeding system (Hemotek Ltd., UK) (Fig. 2), with a black plastic sheet draped over the feeder to maintain darkness during feeding. The blood temperature was maintained at 37 °C using the Hemotek system to mimic human body temperature, while all other environmental conditions were identical to those used during colony maintenance in the insectary. A Parafilm membrane (cut to 3× 3 cm and stretched to ~5× ~5 cm) was used as the membrane through which mosquitoes fed. Before exposure, the membrane was pre-conditioned with human odour by rubbing the Parafilm onto a human body part to deposit natural human odour compounds onto its surface, thus enhancing its attractiveness to mosquitoes. Each starvation duration was evaluated in three independent experiments to ensure result validity and reproducibility. To determine the blood-feeding rate, the number of visually identified engorged mosquitoes was recorded out of the total of 25 mosquitoes in each replicate. The blood-feeding rate was then calculated as the percentage of engorged mosquitoes over total mosquitoes.

Data analysis

Data were analysed using GraphPad Prism version 10.4.0, with normality assessed via the Shapiro-Wilk test, group comparisons conducted using the Kruskal-Wallis test, Pearson correlation applied to evaluate the relationship between starvation duration and feeding rate, and regression analysis performed to identify the best-fitting model.

Results

Before blood feeding, the mosquitoes were starved for different starvation periods (6, 8, 10, 12, 14 and 16 hours), and the respective feeding rate was observed. Table 1 and Fig. 3 demonstrate the results of the feeding rate for different starving duration. Based on the data, normality testing by groups using the Shapiro-Wilk test showed that the data in the 6- and 16-hour starvation groups were not normally distributed. Thus, data were reported in median [interquartile range]. No statistically significant difference was observed in feeding rates across the different starvation period groups of mosquitoes, as indicated by a p-value of 0.414 using the Kruskal-Wallis test ($H(5)= 5.015$, $p= 0.414$). On the other hand, a Shapiro-Wilk test was performed on the entire dataset (regardless of group) to determine the appropriate correlation test. The results indicated that the data were normally distributed. Therefore, a Pearson correlation analysis was conducted, showing a statistically significant moderate positive correlation, with a correlation coefficient (r) of 0.495 ($p= 0.0369$) and a 95% confidence interval ranging from 0.0361 to 0.781. The data also exhibited a simple linear trend, as demonstrated by the regression analysis shown in Fig. 4. Both statistical tests indicate that the feeding rate increases with the starvation period, suggesting a linear relationship between the two parameters.

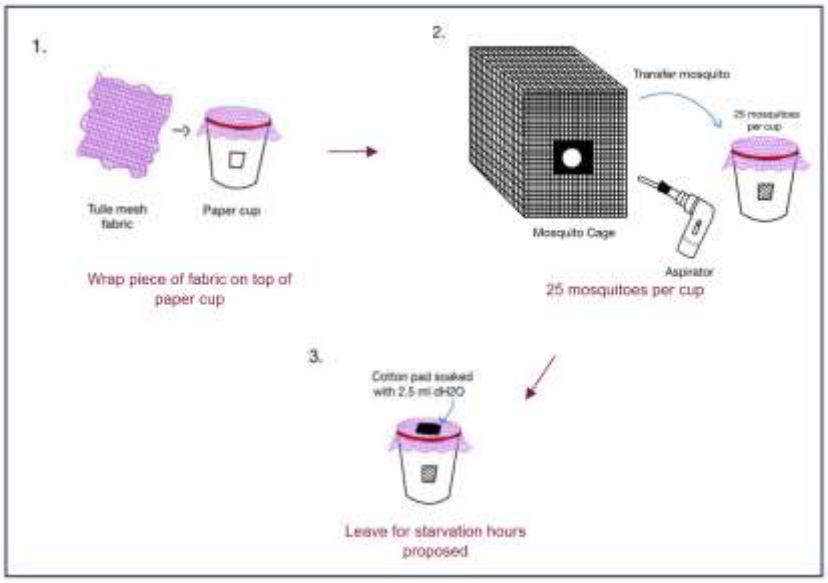

Fig. 1. Illustration of the *Anopheles cracens* starvation procedure prior to blood feeding under laboratory conditions, Malaysia, 2025

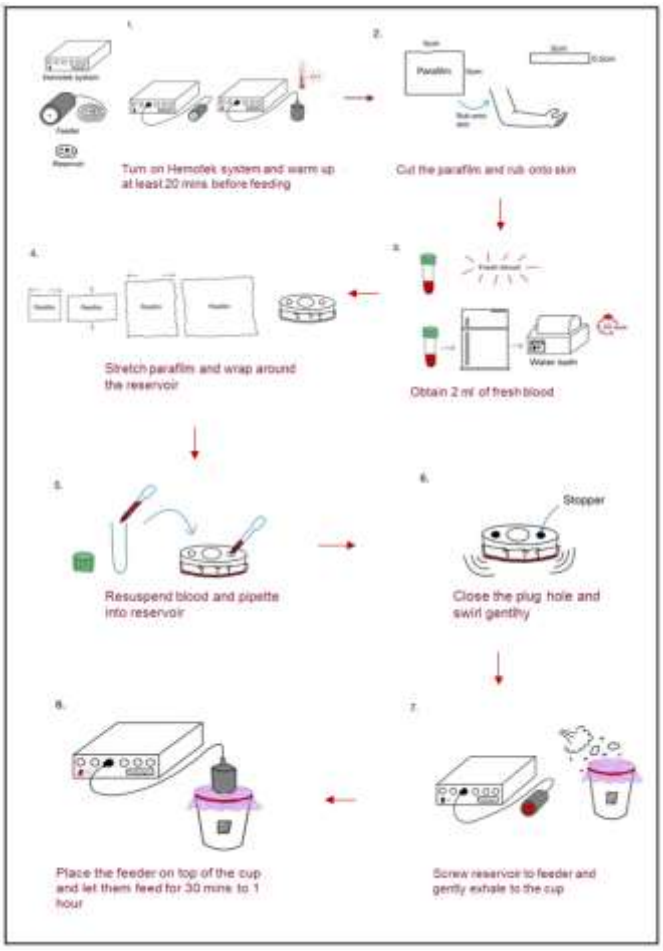

Fig. 2. Illustration of the blood-feeding procedure for *Anopheles cracens* under laboratory conditions, Malaysia, 2025

Table 1. *Anopheles cracens* feeding rates across the starvation durations, presented as median and interquartile range (IQR), Malaysia, 2025

Starvation durations (hours)	Blood feeding rate (%)			Median	Interquartile range (IQR)
	Replicate 1	Replicate 2	Replicate 3		
6	19/25 (76%)	3/25 (12%)	3/25 (12%)	12%	12–76%
8	12/25 (48%)	5/25 (20%)	9/25 (36%)	36%	20–48%
10	7/25 (28%)	13/25 (52%)	4/25 (16%)	28%	16–52%
12	11/25 (44%)	13/25 (52%)	6/25 (24%)	44%	24–52%
14	6/25 (24%)	10/25 (40%)	20/25 (80%)	40%	24–80%
16	17/25 (68%)	17/25 (68%)	18/25 (72%)	68%	68–72%

Fig. 3. Feeding rate of *Anopheles cracens* mosquitoes following different starvation periods, Malaysia, 2025. The dots indicate the median, whereas the error bars denote the interquartile range

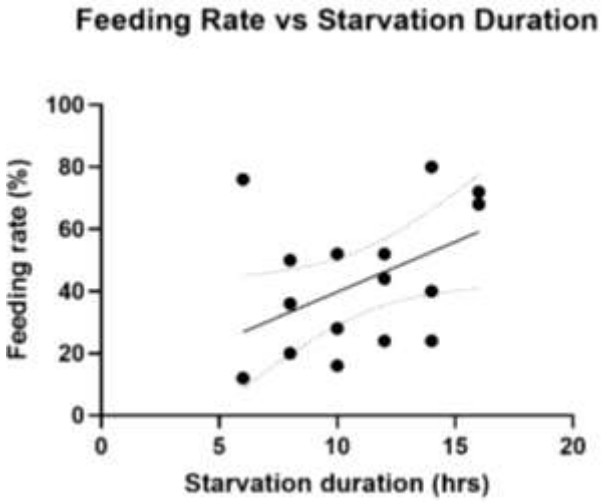

Fig. 4. Simple linear regression analysis of feeding rate and starvation period for rearing *Anopheles cracens* in the laboratory, Malaysia, 2025. The line indicates the regression trend across the observed data. Dotted lines indicate 95% confidence intervals

Discussion

This study represents a systematic attempt to optimise the pre-feeding starvation duration in *An. cracens*, an important vector of *P. knowlesi* in Malaysia. Although statistical analysis did not reveal significant differences among the groups, the overall trend demonstrated a clear positive association between starvation duration and blood-feeding success. These findings indicate that prolonged sugar deprivation enhances host-seeking motivation and feeding response, consistent with observations reported in other *Anopheles* species.

Increased blood-feeding success following prolonged starvation has also been reported in other mosquito species. Sugar deprivation reduces available energy reserves, thereby increasing host-seeking behaviour and the likelihood of accepting a blood meal. Timinao et al. (7) investigated optimal membrane-feeding conditions for *An. farauti*, including the effect of pre-feeding starvation duration. Mosquitoes were subjected to starvation periods of 2, 4, 6 and approximately 21 hours (overnight) before blood feeding. The authors reported that overnight starvation resulted in the highest blood-feeding rate (approximately 60%) compared to those observed after 2, 4 or 6 hours of starvation, despite a prolonged starvation period also being associated with increased mosquito mortality. These findings are consistent with the results of the present study, in which *An. cracens* exhibited a higher blood-feeding rate over the course of starvation, suggesting that prolonged sugar deprivation enhances feeding motivation across different *Anopheles* species.

Similarly, Aswat et al. (9) reported comparable findings while optimising membrane-feeding conditions for *An. funestus*. The authors evaluated starvation durations of 2, 6 and 18 hours and found that mosquitoes starved for 18 hours achieved the highest blood-feeding rate while maintaining a mortality rate of less than 1%. Their results demonstrated that increasing the duration of starvation improved blood-feeding success, which is consistent with the findings of the present study in *An. cracens.*

The increasing blood-feeding rate observed with prolonged starvation may be explained by starvation-induced physiological changes in mosquitoes. Ouédraogo et al. (10) demonstrated that sugar deprivation alters insulin signalling and reduces juvenile hormone synthesis, thereby modifying nutrient allocation and increasing the physiological responsiveness of mosquitoes to nutrient acquisition. Although their study did not directly evaluate blood-feeding behaviour, these physiological changes may increase the motivation of mosquitoes to seek and accept a blood meal once a host becomes available, providing a plausible explanation for the increased feeding rates observed in the present study. Besides, starvation could also enhance the mosquitoes' host-seeking activity by increasing their responsiveness to host-associated cues such as heat (increased temperature) and odours, thereby increasing the likelihood of blood feeding.

One of the limitations of the present study is the small number of both biological and technical replicates due to the availability of mosquitoes with good health conditions. Hence, future studies should increase the number of biological and technical replicates and screen the mosquitoes' health and conditions before the experiment in order to improve the reproducibility and statistical power of the findings. Furthermore, longer starvation periods (for example, 18–24 hours) and additional factors such as membrane type, blood source and environmental conditions during feeding (including humidity, environmental temperature, lighting, and method to include human odour), should also be evaluated to further optimise the membrane-feeding efficiency. Additional outcomes, including engorgement level, blood meal volume and post-feeding survival, should also be assessed to support the establishment of a standardised feeding protocol for *An. cracens*.

Conclusion

Despite inherent limitations, this study provides valuable insights into the feeding behaviour of *An. cracens* under laboratory conditions. The findings demonstrated a clear positive trend indicating that longer pre-feeding starvation periods were associated with higher blood-feeding rates, although the results are not statistically significant. By refining laboratory handling procedures and environmental controls, future research can build on this foundation to further enhance feeding consistency, ensuring that *An. cracens* remains as a reliable model for zoonotic malaria studies in Malaysia. Future investigations incorporating larger sample sizes, prolonged starvation duration and controlled physiological states will enable more precise determination of the optimal starvation threshold and its influence on *P. knowlesi* infection rates under laboratory conditions.

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Ethical considerations

This project was approved by the Institutional Biosafety and Biosecurity Committee of Universiti Malaya (UMIBBC/NOI/R/FOM/PARA-003/2019-04102021). The protocol for the collection and use of blood samples from volunteer donors was approved by the Medical Research Ethics Committee of Universiti of Malaya Medical Center (MRECID No: 2022822-11491).

Conflict of interest statement

The authors declare that there is no conflict of interest.

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