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RESEARCH ARTICLE

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Optimisation of adult *Anopheles funestus* blood-feeding on an artificial membrane feeding system

Ayesha S Aswat[1,2], Riann Christian[3] and Lizette L Koekemoer[1,2]

[1]*Wits Research Institute for Malaria, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa*
[2]*Centre for Emerging Zoonotic & Parasitic Diseases, National Institute for Communicable Diseases, a Division of the National Health Laboratory Service, Johannesburg, South Africa*
[3]*College of Agriculture and Environmental Sciences, University of South Africa, Pretoria, South Africa*

Malaria is one of the most severe vector-borne diseases caused by *Plasmodium* parasites and transmitted by *Anopheles* mosquitoes. Laboratory-reared anophelines are essential to advance research needed to reduce or eliminate malaria. The success of laboratory rearing as well as studies on parasite-mosquito transmission, is advanced by using an artificial membrane feeding systems. These require the optimisation of mosquito feeding to ensure that an optimal number of mosquitoes feed, thereby enabling successful reproduction or research sample sizes. In this study, various parameters such as the type of artificial membrane, density of adults in the feeding cup, age of the mosquito, duration of starvation, method of starvation, the volume of blood meal, duration of feeding, feeding in the light or dark and the effect of lactic acid were evaluated to determine their impact on the feeding rate of a main African malaria vector, *Anopheles funestus*. By optimising the artificial membrane feeding parameters, an increase in the feeding rate of the *An. funestus* mosquitoes was observed. The results obtained from these parameters increased the feeding rate of *An. funestus* above 50%. However, feeding rates were not significantly increased by the type of membrane, mosquito density, the volume of blood meal, duration of feeding and the addition of lactic acid to the cattle intestine membrane. Therefore, this study provides information on suitable conditions for adult mosquito feeding that allows for successful laboratory rearing and colony maintenance. Furthermore, it provides additional information for research studies that are dependent on blood-feeding, such as transmission blocking studies, endectocide studies etc.

CORRESPONDENCE
LL Koekemoer

EMAIL
Lizette.koekemoer@wits.ac.za

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INTRODUCTION

Malaria is caused by *Plasmodium* parasites. *Plasmodium falciparum* (*P. falciparum*) infections in humans are responsible for the most severe clinical symptoms and are the deadliest type of malaria. Malaria is a major global health problem with an estimated 247 million malaria cases and almost half of the world population being at risk (WHO 2022). *Anopheles funestus sensu stricto (An. funestus)* is one of the most proficient African malarial vectors (Gillies and De Meillon 1968; Gillies and Coetzee 1987; Kahamba et al. 2022).

This species, an afrotropical vector of human malaria, exhibits both endophilic and anthropophilic behaviour (Gillies and De Meillon 1968; Debrah et al. 2021). Additionally, it shows resistance to commonly used insecticides in vector control and boasts a higher survival probability compared to other *Anopheles* species (Coetzee and Koekemoer 2013; Zengenene et al. 2021). Colonising *An. funestus* is challenging and limits research on this species (Gillies and De Meillon 1968; Coetzee and Koekemoer 2013; Ngowo et al. 2021). Furthermore, current transmission-blocking studies have never been conducted using this important African malaria vector species (Delves et al. 2012; Eldring et al. 2017; Reader et al. 2021).

In addition, blood-feeding of female mosquitoes is crucial for normal *Anopheles* colony maintenance, but the feeding rate success varies depending on various factors, such as the species of the mosquito, whether the mosquito was reared in a colony or collected in the wild and the adaption level of the colony (Timinao et al. 2021). The rate of blood-feeding is dependent on the experimental conditions under which the feeding is carried out and this includes the starvation duration prior to exposure, the parameters of starvation (such as water access), the membrane type utilised, the feeding rate duration, the age of the mosquito, the amount of blood in the feeder, the temperature of the water bath as a proxy for blood temperature as well as other parameters that could impact the rate of blood-feeding (Timinao et al. 2021). Studies concerning membrane feeding have been conducted with a range of parameters and these resulted in variations in the success of feeding and might be species-specific (Timinao et al. 2021). A study conducted by Timinao et al. (2021) indicated that the optimum feeding conditions for *An. farauti s.s.* were 50 mosquitoes/cup, mosquitoes that were starved overnight and were given 350–500 µl of blood via a Baudruche membrane for a minimum of 10–20 minutes. However, a study conducted by Coulibaly et al. (2017) on *An. coluzzii* indicated that there was no significant difference in the feeding rate between the parafilm and the Baudruche membrane. The variation in blood-feeding habits is due to extrinsic and intrinsic factors such as genetics, behavioural characteristics, climatic factors etc. (Melgarejo-Colmenares et al. 2022). Therefore, it is crucial to optimise the feeding condition for each mosquito colony. Furthermore, mosquito density could also affect the feeding rate. Rutledge et al. (1964) indicated that the larger the sample size per cage, the lower the feeding rate and this makes the removal of the unfed mosquitoes difficult. In addition, lactic acid is a crucial component of human

sweat and it can act synergistically with particular compounds that are volatile to enhance mosquito attraction (Raji et al. 2019). Mosquitoes that are anthropophilic in nature are attracted to high concentrations of lactic acid (McMeniman et al. 2014). As a single stimulus, lactic acid is a weak attractant but it can be a strong attractant when combined with other compounds from human skin odours (Steib et al. 2001).

Since *An. funestus* is known to be almost exclusively anthropophilic in nature (feeding on humans), it is, therefore, essential to optimise the feeding conditions to ensure maximum feeding rate on an artificial membrane feeding system for research purposes (such as transmission-blocking studies) or to improve colony rearing. The feeding rate optimisation can also increase the precision of the research results. Felamboahangy et al. (2023) indicated that the laboratory rearing of *An. funestus* is challenging and the average artificial blood-feeding rate obtained using the Hemotek feeder was 48.78%. This further indicates that the artificial blood-feeding of *An. funestus* needs to be optimised to ensure a high feeding rate for colony rearing and downstream research analysis. Thus, certain parameters such as the type of artificial membrane, the sample size, age of the mosquito, starvation duration, volume of blood, feeding duration, and feeding in light or dark were tested to improve the feeding rate of *An. funestus* mosquitoes. Additionally, this study also evaluated the impact that lactic acid has on the feeding rate of the mosquito. An improved feeding rate will improve reproductive success (more females will be able to produce eggs). An increased feeding rate will also reduce the number of the starting material (females) needed for research purposes (e.g., transmission-blocking studies) and this will result in an increase in sample size (number of fed females) available for studies.

METHODS

Optimising the feeding parameters for *An. funestus* was conducted using defibrinated cattle blood since there are various safety and ethical issues when obtaining human blood and already used in the laboratory. Furthermore, there are limited human blood donors, and blood obtained that is stored in blood banks is used in medical emergencies instead of experimental insectary work (Gunathilaka et al. 2017). Additionally, human blood is not usually used for mosquito feeding due to the high risk of contamination with various pathogens that may not be detected in blood bank screening routines. However, cattle blood is easily available from a reputable commercial abattoir (registered facility to ensure health inspections by the meat inspection division of South Africa's national Department of Agriculture) and it is used as a standard blood meal for colony maintenance in insectaries (Maharaj et al. 2022). In addition, cattle-intestine membrane, which is also easily available from a commercial health and safety registered butcher, is already routinely used as a standard artificial membrane for colony maintenance in our insectary. Although cattle blood is routinely used, there is a risk of colony contamination with chemical residues, veterinary medications, or undesirable pathogens. To reduce the risk, insect rearing facilities have used irradiation to sterilise blood meals (Pritchard and Rogers 1987). Chemical residues or veterinary medications in livestock not used for human consumption were evaluated in the past and resulted in high mosquito mortalities (Koekemoer pers comm), while this effect has not been observed when using cattle blood from a reputable abattoir.

Mosquito samples and maintenance

Adult female *An. funestus* were used in this study from a colony called FUMOZ. The colony was established in the year 2000 from wild-caught samples from Mozambique (Hunt et al. 2005) and was housed at the Maureen Coetzee insectary (Wits Research Institute for Malaria). The mosquitoes were reared under standard insectary conditions (26–28 °C with ± 70–90% relative humidity and 12 h:12 h light/dark photoperiod) (Maharaj et al. 2022). Newly emerged male and female mosquitoes were placed in a Bugdorm cage (W30 × D30 × H30 cm, size and cat. no.: DP1000) and allowed to mate. Females were removed for experimental feeding as detailed below.

Artificial feeding set-up

A sample size of 40 unfed adult females *An. funestus* mosquitoes were placed in 350 ml plastic cups covered in nets. Glass feeders (Glastechniek, Peter Coelen B.V., The Netherlands, diameter 15 mm), were placed on top of the cups and were connected to a circulating water bath (Figure 1A). This allowed the water to be circulated through the glass feeders to ensure that the blood is kept at the desired temperature of 37 °C. The bottom bases of the glass feeders were covered with a cattle-intestine membrane. Defibrinated cattle blood (400 µl) was added to the feeder by means of a pipette and the mosquitoes were fed for 30–35 minutes in the dark. Once feeding was completed, the number of partially fed, unfed and fully fed mosquitoes were recorded (Figure 1B). Three biological replicates (the feeding rate assay

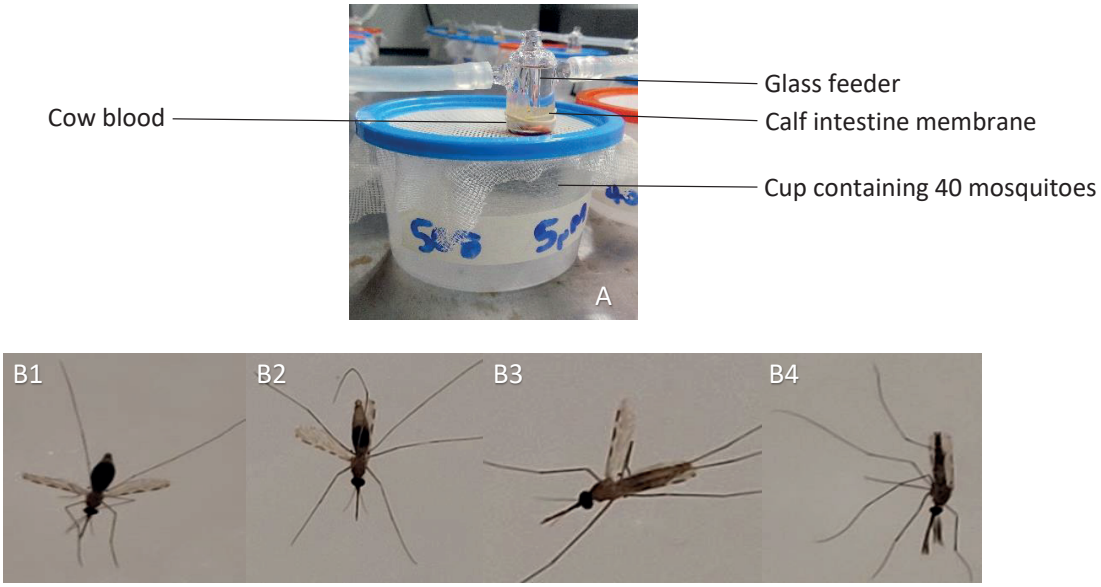

Figure 1: Setup of the artificial membrane-feeding assay (A); and separation of mosquitoes after imbibing a blood meal (B); Fully fed mosquito (B1); partially fed mosquito (B2); unfed mosquito (B3); and the male mosquito (B4).

for each replicate was conducted a week apart and used different biological cohort of females to reflect the biological variation in the mosquito) with two technical replicates (using the same biological cohort of material in duplicate to reflect the technical variation of the experiments) were carried out to test each feeding parameter and the age of the mosquito ranged from 7–10 days old (unless stated otherwise). The mosquitoes were starved for a minimum of four hours prior to the blood-feeding rate being evaluated (unless stated otherwise). This setup was used in all the assays conducted, but any changes to evaluate a specific parameter (e.g., age) were specified below.

Feeding rate success using different artificial membranes

To determine the ideal membrane that will improve the feeding rate of the *An. funestus* female mosquito, the feeding rate between mosquitoes that fed on untreated parafilm, cattle intestine membrane, and parafilm that was exposed to natural human odour (feet sweat) for 30 minutes (the parafilm was placed inside a shoe for approximately 30 minutes to expose the parafilm to human odour (sweat)) was compared. The parafilm was cut to 5 cm × 5 cm and the thickness of the membrane was reduced when stretched to 10 cm × 10 cm. Rubber elastic bands were used to attach both the cattle intestine membrane and the parafilm membrane to the feeders (Dias et al. 2020). The feeding procedure followed was the same as described in section *Artificial feeding set-up*.

Mosquito density per cup

To determine the ideal sample size that will improve the feeding rate of the *An. funestus* mosquitoes, the number of mosquitoes fed/total number of mosquitoes used between 0.07 female/cm^3 (n = 20), 0.11 female/cm^3 (n = 40), and 0.23 female/cm^3 (n = 80) mosquitoes per cup were compared. The density of the mosquitoes was determined by dividing the sample size of the mosquitoes by the volume of the cup (e.g., 20 mosquitoes/350 ml). The blood-feeding procedure followed was the same as described in section *Artificial feeding set-up*.

Age of the mosquito

To determine the ideal age of the *An. funestus* mosquitoes that will result in the highest feeding rate, the feeding rate between mosquitoes that were aged 3–5 days old, 7–10 days old, and 11–13 days old were compared. The procedure followed was the same as described in section *Artificial feeding set-up*.

The duration of starvation before exposure: 2 h, 6 h, overnight starvation of approximately 18 h

To determine how many hours before a blood meal the *An. funestus* female mosquitoes should be starved to improve the feeding rate, the mosquitoes were starved from sugar water for two hours, six hours, and overnight starvation of approximately 18 hours. The mosquitoes were also divided into two groups, those mosquitoes that had access to water or no access to water (dry-starved) prior to blood-feeding. The procedure followed was the same as described in section *Artificial feeding set-up*.

Volume of blood required

Anopheles funestus mosquitoes were provided with either 100 µl, 200 µl, 400 µl or 800 µl of a blood meal to determine the amount of blood that will improve the feeding rate. The volume of blood could not be increased due to the maximum capacity of the glass feeders being 800 µl. The procedure followed was the same as described in section *Artificial feeding set-up*.

Duration of taking a blood meal

Mosquitoes were fed for a duration of 30 minutes, 45 minutes, and 60 minutes in the dark to determine the duration of feeding to improve the feeding rate in the *An. funestus* adult female mosquitoes. The procedure followed was the same as described in section *Artificial feeding set-up*.

Impact of light on the blood-feeding

To determine if light exposure influences the feeding rate of the *An. funestus* female mosquito, they were allowed to feed either in the light or dark. Mosquitoes that were fed in the dark were covered with a dark material while mosquitoes that were fed in the light were fed under insectary conditions where the daytime cycle was switched on. The procedure followed was the same as described in section *Artificial feeding set-up*.

Effect of lactic acid on the feeding rate

To determine the effect that lactic acid has on the feeding rate of the *An. funestus* mosquitoes, the feeding rate between lactic acid-treated cattle intestine membrane and untreated cattle intestine membrane (routinely used as the control) were compared. The lactic acid-treated cattle intestine membrane was tested at a concentration range of 2.5%, 5%, 10%, 20%, 40% and 80%. The lactic acid was rubbed against the cow intestine membrane with cotton wool. The procedure followed was the same as described in section *Artificial feeding set-up*.

Statistical Analysis

To determine the percentage feeding rate, the number of fully fed mosquitoes were divided by the total number of mosquitoes (fully fed, partial fed and unfed mosquitoes) for each replicate and multiplied by 100. Furthermore, the results obtained from the following tested parameters: the effect of lactic acid on the feeding rate and feeding in the dark vs feeding in the light were analysed using the unpaired t-test statistical analysis. The results obtained from all the other parameters tested were analysed using the one-way ANOVA statistical analysis. GraphPad prism V9.3.3 was used to conduct the statistical analysis.

In addition, to determine the combined impact these eight parameters have on the feeding rate of the mosquito, a generalised linear mixed effects model (GLMM) statistical analysis was conducted using MATLAB R2023a. Feeding rate was the dependent variable while the parameters tested were the fixed variables and the replicates were the random effects.

RESULTS

A total of eight feeding parameters such as the mosquito density, type of artificial membrane etc. were evaluated to improve the feeding rate of the *An. funestus* mosquito. Tables 1 and 2 summarise the results obtained for all the parameters evaluated.

Type of artificial membrane for optimum feeding

To compare the effect the type of membrane has on the feeding rate of the mosquito, the feeding rate between three different artificial membranes were compared. The one-way ANOVA results revealed that there was a statistically significant difference in the feeding rate between the parafilm (untreated) (M = 57.83 ± 9.725%), parafilm exposed to human odour (M = 69.33 ± 4.590%), and cattle intestine membrane (M = 47.50 ± 10.93%) at the $p < 0.05$ level for the three conditions ($F_{2,15}$ = 9.132, p = 0.0025) (Table 1).

The Tukey's HSD Test for multiple comparisons found that the mean value of the feeding rate was significantly different between the parafilm exposed to human odour and cattle intestine membrane (p = 0.0018, 95% C.I. = 8.557, 35.11). There was no statistically significant difference in the feeding rate between the parafilm membrane (untreated) and the cattle intestine membrane (p = 0.1412). In addition, there was also no significant difference in the feeding rate between parafilm (untreated) and parafilm exposed to human odour (p = 0.0948).

Table 1: Optimisation of the *An. funetus* feeding rate results. [a]ANOVA statistical analysis; [b]unpaired *t*-test

Parameter tested	Total number of blood-fed mosquitoes	Average feeding rate	SDEV	*p*-value
1. Type of artificial membrane:				
A. Parafilm membrane	138	57.80%	± 9.725	0.0025[a]
B. Cattle Intestine membrane	113	47.50%	± 10.93	
C. Parafilm exposed to human odour	166	69.33%	± 4.590	
2. Mosquito density:				
A. 20 mosquitoes/cup (0.06 females/cm^3)	35	29.17%	± 15.94	0.0426[a]
B. 40 mosquitoes/cup (0.11 females/cm^3)	119	49.80%	± 9.020	
C. 80 mosquitoes/cup (0.23 females/cm^3)	211	44.17%	± 13.70	
3. Age of the mosquito:				
A. 3–5 days old	73	30.67%	± 9.688	0.0009[a]
B. 7–10 days old	131	54.83%	± 7.167	
C. 11–13 days old	109	45.67%	± 7.167	
4. Duration and method of starvation:				
A. Dry-starved 18H	71	59.67%	± 2.887	< 0.0001[a]
B. Dry-starved 6H	29	24.33%	± 4.041	
C. Dry-starved 2H	14	12%	± 4.041	
D. Water-starved 18H	17	14.33%	± 1.732	
E. Water-starved 6H	7	6%	± 3.606	
F. Water-starved 2H	7	8.67%	± 3.606	
5. Volume of blood meal:				
A. 100 µl	78	32.83%	± 5.345	0.0758[a]
B. 200 µl	84	35.17%	± 10.71	
D. 400 µl	122	51%	± 14.51	
E. 800 µl	105	44%	± 17.12	
6. Duration of feeding:				
A. 30 minutes	100	41.80%	± 21.83	0.7187[a]
B. 45 minutes	121	50.67%	± 23.49	
C. 60 minutes	119	49.67%	± 14.79	
7. Type of light exposure:				
A. Feeding in the dark	150	62.83%	± 9.411	0.0006[b]
B. Feeding in the light	84	35.17%	± 10.23	
8. Impact of lactic acid on the feeding rate:				
A. 0% Lactic acid (Untreated)	39	49%	± 8.485	0.457[b]
B. 2.5% Lactic acid	41	51.50%	± 16.26	
C. 5% Lactic acid	47	65.50%	± 12.73	
D. 10% Lactic acid	54	68%	± 7.071	

Optimal number of mosquitoes in a feeding cup

To determine the effect the sample size has on the feeding rate of the mosquito, a feeding rate comparison was conducted for the three different sample sizes. The one-way ANOVA results revealed that there was a statistically significant difference in the mosquito feeding rate between the three sample sizes 0.06 female/cm^3 (n = 20) (M = 29.17 ± 15.94%), 0.11 female/cm^3 (n = 40) (M = 49.83 ± 9.020%) and 0.23 female/cm^3 (n = 80) (M = 44.174 ± 13.70%) at the $p < 0.05$ level for the three conditions ($F_{2,15} = 3.923$, $p = 0.0426$) (Table 1).

The Tukey's HSD Test for multiple comparisons found that the mean value of the feeding rate was significantly different between the 0.06 female/cm^3 and 0.11 female/cm^3 sample size ($p = 0.0403$, 95% C.I. = –40.47, –0.8603). There was no statistically significant difference in the feeding rate between the 0.11 female/cm^3 and 0.23 female/cm^3 sample size ($p = 0.7422$) and the 0.06 female/cm^3 and the 0.23 female/cm^3 sample size ($p = 0.1547$).

Mosquito age range with the highest feeding rate

Three different age group ranges were compared to determine the effect age of the mosquito has on the feeding rate. The one-way ANOVA results revealed that there was a statistically significant difference in the feeding rate between 3–5 days old mosquitoes (M = 30.67 ± 9.688%), 7–10 days old mosquitoes (M = 54.83 ± 7.167%), and 11–13 days old mosquitoes (M = 45.67 ± 9.374%) at the $p < 0.05$ level for the three conditions ($F_{2,15} = 11.49$, $p = 0.0009$) (Table 1).

The Tukey's HSD Test for multiple comparisons found that the mean value of the feeding rate was significantly different between the 3–5 days old mosquitoes and the 7–10 days old mosquitoes ($p = 0.0007$, 95% C.I. = –37.39, –10.95). The test also indicated that there was a statistically significant difference in the feeding rate between the 3–5 days and 11–13 days-old mosquitoes ($p = 0.0255$, 95% C.I. = –28.22, –1.781). However, there was no significant difference in the feeding rate between 7–10 days and 11–13 days-old mosquitoes ($p = 0.2028$).

Table 2: Generalised linear mixed effects model fit using Binomial distribution

Parameter tested	Estimate	SE	*t*-test	DF	*p*-value	Lower	Upper	β-value
Cattle intestine	-0.471	0.068668	-6.85909	5710	7.66×10^{-12}	-0.60562	-0.33639	-0.4710
Odour treated Parafilm	0.496837	0.103619	4.794856	5710	1.67×10^{-6}	0.293705	0.699969	0.4968
Density	5.04×10^{-5}	0.002357	0.021391	5710	0.982935	-0.00457	0.004672	0.0001
Age of the mosquito (3–5 days old)	-0.47209	0.103173	-4.57574	5710	4.85×10^{-6}	-0.67435	-0.26984	-0.4721
Age of the mosquito (11–13 days)	0.224081	0.09893	2.265034	5710	0.023548	0.03014	0.418022	0.2241
Starvation method	0.751803	0.101248	7.425372	5710	1.29×10^{-13}	0.553319	0.950288	0.7518
Starvation duration	0.093333	0.011175	8.352	5710	8.34×10^{-17}	0.071426	0.11524	0.0933
Volume of blood meal	0.000721	0.000242	2.981113	5710	0.002884	0.000247	0.001196	0.0007
Duration of feeding	0.00829	0.004961	1.671076	5710	0.094761	-0.00144	0.018015	0.0083
Type of light exposure	0.229562	0.070335	3.263826	5710	0.001106	0.091678	0.367445	0.2296
Untreated (lactic acid) membrane	5.012925	33.11317	0.151388	5710	0.879675	-59.9015	69.92731	5.1228
Lactic acid (2.5%) treated membrane	5.222848	33.11372	0.157725	5710	0.874679	-59.6926	70.13831	5.2228
Lactic acid (5%) treated membrane	5.52648	33.11374	0.166894	5710	0.867459	-59.389	70.44198	5.5265
Lactic acid (10%) treated membrane	5.903724	33.1138	0.178286	5710	0.858505	-59.0119	70.81934	5.9037
Lactic acid (20%) treated membrane	-8.3731	90.86412	-0.09215	5710	0.926582	-186.501	169.7551	-8.3731
Lactic acid (40%) treated membrane	-8.36356	90.48907	-0.09243	5710	0.926363	-185.756	169.0294	-8.3636

Duration and method of starvation

Mosquitoes that were dry-starved for approximately 18 hours had a higher feeding rate in comparison to the mosquitoes that were subjected to different starvation methods and starvation periods. A one-way ANOVA was performed to compare the effect of sugar water starvation and the duration for which mosquitoes starved on the feeding rate of the mosquito. The one-way ANOVA results revealed that there was a statistically significant difference in the feeding rate between the mosquitoes that were dry-starved (had no access to water for two hours, six hours, and approximately 18 hours) and the mosquitoes that were starved while being maintained on water (for two hours, six hours and approximately 18 hours) at the $p < 0.05$ level for the six conditions ($F_{5,12} = 106.9$, $p < 0.0001$) (Table 1).

The Tukey's HSD Test for multiple comparisons found that the mean value of the feeding rate was significantly different between the approximately 18 hours dry-starved mosquitoes ($M = 59.67 \pm 2.887\%$) and the 18 hours water-starved mosquitoes ($M = 14.33 \pm 4.041\%$, $p < 0.0001$, 95% C.I. = 35.97, 54.70). There was also a significant difference in the feeding rate between the mosquitoes that were dry-starved for approximately 18 hours and those mosquitoes that were dry-starved for six hours ($M = 24.33 \pm 4.041\%$, $p < 0.0001$, 95% C.I. = 25.97, 44.70), dry-starved for two hours ($M = 12.00 \pm 1.732\%$, $p < 0.0001$, 95% C.I. = 38.30, 57.03), water-starved for six hours ($M = 6.00 \pm 3.606\%$), $p < 0.0001$, 95% C.I. = 44.30, 63.03) and water-starved for two hours ($M = 6.00 \pm 3.606\%$, $p < 0.0001$, 95% C.I. = 44.30, 63.03).

The results obtained from the Tukey's HSD Test for multiple comparisons also indicated that there was a significant difference in the feeding rate between the mosquitoes that were dry-starved for six hours and those mosquitoes that were water-starved for approximately 18 hours ($p = 0.0341$, 95% C.I. = –19.37, –0.6324), water-starved for six hours ($p = 0.0003$, 95% C.I. = –27.70, –8.966), water-starved for two hours ($p = 0.0003$, 95% C.I. = 8.966, 27.70) and dry-starved for two hours ($p = 0.0083$, 95% C.I. = 2.966, 27.70).

However, there was no significant difference in the feeding rate between the mosquitoes that were water-starved for approximately 18 hours and those mosquitoes that were water-starved for six hours ($p = 0.0923$), water-starved for two hours ($p = 0.0923$) and dry-starved for two hours ($p = 0.0341$). There was also no significant difference in the feeding rate between mosquitoes that were dry-starved for two hours and those mosquitoes that were water-starved for two hours ($p = 0.3254$).

Volume of blood meal

Mosquitoes were provided with a blood meal volume of either 100 µl, 200 µl, 400 µl or 800 µl of blood to determine the effect the volume of blood has on the feeding rate of the mosquitoes. The one-way ANOVA results revealed that there was no statistically significant difference in the feeding rate between mosquitoes that fed on 100 µl ($M = 32.83 \pm 5.345\%$), 200 µl ($M = 34.67 \pm 10.71\%$), 400 µl ($M = 51.00 \pm 14.51\%$) or 800 µl ($M = 44.00 \pm 17.12\%$) of blood at the $p < 0.05$ level for the three conditions ($F_{3,20} = 1.616$, $p = 0.0758$) (Table 1).

Duration of taking a blood meal

Mosquitoes were fed a blood meal for various durations to determine the impact the duration of taking a blood meal has on the feeding rate of the mosquito. The one-way ANOVA results revealed that there was no statistically significant difference in the feeding rate between mosquitoes that fed for 30 minutes ($M = 41.83 \pm 21.83\%$), 45 minutes ($M = 50.67 \pm 23.49\%$) and 60 minutes ($M = 49.67 \pm 14.79\%$) at the $p < 0.05$ level for the three conditions ($F_{2,15} = 0.3377$, $p = 0.7187$) (Table 1).

Feeding in the dark vs feeding in the light

To determine if light exposure has an impact on the feeding rate of the mosquito, an independent sample *t*-test was conducted to compare the feeding rate of mosquitoes that were fed either in the light or dark. The unpaired *t*-test results indicate that there was a significant difference in the feeding rate between the mosquitoes that were fed in the dark ($M = 62.83 \pm 9.411\%$) and the mosquitoes that were fed in the light ($M = 35.17 \pm 10.23\%$) conditions ($t_{(10)} = 4.876$, $p = 0.0006$). These results suggest that *An. funestus* female mosquitoes prefer to feed in the dark as expected.

Effect of lactic acid on the feeding rate of the mosquito

The cattle intestine membrane was treated with lactic acid to determine the impact it has on the feeding rate of the mosquito. Lactic acid at high concentrations (20–80%) has been shown to coagulate the blood and this resulted in a 0% feeding rate of the *An. funestus* mosquito. However, feeding has been observed at lactic acid concentrations of 2.5%, 5% and 10%. A one-way ANOVA was performed to determine if the feeding rate of the mosquito was affected. The one-way ANOVA results revealed that there was no statistically significant difference in the mosquito feeding rate between the three lactic acid-treated membranes 2.5% lactic acid ($M = 51.50 \pm 16.26\%$), 5% lactic acid ($M = 59.00 \pm$

12.73%), 10% lactic acid (M = 68.00 ± 7.071%) and untreated cattle intestine membrane (M = 49.00% ± 8.485%) at the $p < 0.05$ level for the four conditions ($F_{3,4} = 1.066$, $p = 0.4571$) (Table 1).

Combined parameter impact on the feeding rate

To determine the impact all eight parameters have on the feeding rate of the mosquito, a GLMM analysis was conducted. The results obtained indicate that the cattle intestine membrane (estimate ± SE: −0.471 ± 0.0687, β = −0.4710, t = −6.8591, p = 7.6621×10^{-12}, 95% C.I. = −0.60562, −0.33639), parafilm exposed to human odour (estimate ± SE: 0.49684 ± 0.10362, β = 0.4968, t = 4.7949, $p = 1.6692 \times 10^{-6}$, 95% C.I. = 0.2937, 0.69997), 3–5 days old mosquitoes (estimate ± SE: −0.47209 ± 0.10317, β = −0.4721, t = 4.5757, $p = 4.8459 \times 10^{-6}$, 95% C.I. = −0.67435, −0.26984), 11–13 days old mosquitoes (estimate ± SE: 0.22408 ± 0.09893, β = 0.2241, t = 2.265, p = 0.023548, 95% C.I. = 0.03014, 0.41802], starvation method (estimate ± SE: 0.22956 ± 0.070335, β = 0.7518, t = 3.2638, $p = 1.2898 \times 10^{-13}$, 95% C.I. = 0.55332, 0.95029), duration of starvation (Estimate ± SE: 0.093333 ± 0.011175, β = 0.0933, t = 8.352, $p = 8.3412 \times 10^{-17}$, 95% C.I. = 0.071426, 0.11524), volume of blood meal (estimate ± SE: 0.00072135 ± 0.00024197, β = 0.0007, t = 2.9811, p = 0.0028841, 95% C.I. = 0.000247, 0.001196), type of light exposure (estimate ± SE: 0.22956 ± 0.070335, β = 0.2296, t = 3.2638, p = 0.0011057, 95% C.I. = 0.091678, 0.36745), are statistically significant predictors at the 5% significance level (95% C.I. = 0.00024699, 0.0011957) (Table 2).

Additionally, mosquito density (estimate ± SE: 5.0425×10^{-5} ± 0.002356, β = 0.0001, t = 0.021391, p = 0.98293, 95% C.I. = −0.0045709, 0.0046717), duration of feeding (estimate ± SE: 0.0082899 ± 0.0049608, β = 0.0083, t = 1.6711, p = 0.094761, 95% C.I. = −0.0014352, 0.018015), untreated (lactic acid) membrane (estimate ± SE: 5.0129 ± 33.113, β = 5.1228, t = 0.15139, p = 0.87968, 95% C.I. = −59.901, 69.927), lactic acid (2.5%) treated membrane (estimate ± SE: 5.2228 ± 33.114, β = 5.2228, t = 0.15772, p = 0.87468, 95% C.I. = −59.693, 70.138), lactic acid (5%) treated membrane (estimate ± SE: 5.5265 ± 33.114, β = 5.5265, t = 0.16689, p = 0.86746, 95% C.I. = −59.389, 70.442), lactic acid (10%) treated membrane (estimate ± SE: 5.9037 ± 33.114, β = 5.9037, t = 0.17829, p = 0.8585, 95% C.I. = −59.012, 70.819), lactic acid (20%) membrane (estimate ± SE: −8.3731 ± 90.864, β = −8.3731, t = −0.09215, p = 0.92658, 95% C.I. = −186.5, 169.76), lactic acid (40%) membrane (estimate ± SE: −8.3636 ± 90.489, β = −8.3636, t = −0.092426, p = 0.92636, 95% C.I. = −185.76, 169.03) are not statistically significant predictors at the 5% significance level (Table 2).

DISCUSSION

This is the first study where eight different parameters have been evaluated to improve the feeding rate of the main African malaria vector *An. funestus* on an artificial membrane feeding system. Research on this species is under-represented in literature, due to the difficulty in rearing this species and maintaining stable large colonies (Zengenene et al. 2021). This is due, amongst other challenges, to feeding them using an artificial feeding system. As transmission-blocking studies are currently excluding this species, the feeding conditions used for artificial infection of *P. falciparum* in African malaria vectors (Reader et al. 2021; Arendse et al. 2022) were used in this study to allow future infection studies on this species. This study aimed to evaluate how different components of the artificial membrane feeding system can be optimised to improve feeding success.

The first variable evaluated was the type of membrane used. In this study, feeding proportions (feeding rate) did not improve if parafilm or cattle intestine membrane (sausage casing) was used. However, the feeding proportion improved when parafilm that was exposed to human odour (sweat) was used and compared to cattle intestine. A study by Jové et al. (2020) also showed that exposure of human odour to the artificial membrane surface increases the attraction of the membrane to mosquitoes and improves the feeding rate of the mosquito. Researchers have used various membranes in artificial feeding systems to stimulate the skin of humans or animals (Dias et al. 2020). These preferences can be species-specific or be influenced by the colonised strain being evaluated. High feeding rates have been reported for colonised strains of *Aedes aegypti* and *Culex quinquefasciatus* (80.3% and 80.8%, respectively) when using parafilm as a membrane in the artificial feeding system (Dias et al. 2020). Parafilm is often preferred as it is a synthetic membrane and easier to standardisation, however, it can be overstretched impacting the feeding (Coulibaly et al. 2017; Dias et al. 2020). Ox intestine (Goldbeater's skin or Baudruche membranes) is also commonly used for blood-feeding (Coulibaly et al. 2017; Nunn et al. 2022). Coulibaly et al. (2017) compared these two types of membranes using *An. coluzzii*, another main African malaria vector and this colony originated from wild-caught mosquitoes that were reared in the laboratory. The 82nd generation laboratory mosquitoes were used for this study and no significant difference between the parafilm and Baudruche membranes was reported. These results correlate with the results obtained in this study.

Mosquito density under this study conditions indicated that the number of mosquitoes in a 350 ml cup does not significantly increase the feeding rate of the *An. funestus* mosquitoes. However, it was observed that the mosquito density of 0.11 females/cm^3 (n = 40) and 0.23 females/cm^3 (n = 80) improved the average rate of blood meal feeding in comparison to 0.06 females/cm^3 (n = 20). In addition, if the cage size or shape change, differences in the mosquito feeding rate might be observed and needs to be investigated since the surface area and volume of the cage can also influence the feeding rate of the mosquito (Rutledge et al. 1964). Rutledge et al. (1964) used a sample size of 24, 48, 94 and 136 (5–26 days old) of *Aedes aegypti* that were fed on chick blood using Baudruche membrane and the study obtained statistically significant results since the authors observed a decrease in the feeding rate with more mosquitoes per cage. From the results obtained in this study, it can be hypothesised that density does not improve the feeding rate of *An. funestus* mosquitoes but overcrowding a cage can result in a higher number of fed mosquitoes.

In theory, mosquitoes that are injured, defective, too young or were already fed will not feed under natural conditions (Rutledge et al. 1964). However, *An. funestus* female mosquitoes infrequently take a blood meal before mating. Charlwood and authors indicated that 95.77% unfed *An. funestus* female mosquitoes that were dissected were virgins and mated females search for blood since they use their sense of smell for a blood meal (Charlwood et al. 2003). Furthermore, Maharaj et al. (2022) indicated that *An. funestus* female mosquitoes have an optimum mating success 12 days post-emergence. From the results obtained in this study, it can be seen that the mosquitoes aged 3–5 days old had a lower feeding rate in comparison to the *An. funestus* mosquitoes aged 7–10 days old and 11–13 days old. This difference in feeding rate between the age groups could indicate that the younger mosquitoes were unmated in comparison to the older mosquitoes. The increase in feeding between the different age groups may be species-specific since a study conducted by Timinao et al. (2021) indicated no significant difference in the feeding rate of *Anopheles farauti s.s.* between the 3-, 5- and 7-days-old mosquitoes. Thus, mosquitoes that are older than seven days should be used for blood feeding assays to obtain a large sample size of blood fed mosquitoes.

In addition, long starvation periods prior to blood-feeding increase the feeding rate of the mosquito. The overnight duration of starvation was statistically significant when compared to the feeding rate of the other two durations of starvation (two hours and six hours). Furthermore, those mosquitoes who had no

access to water during the starvation period (dry-starved) had a higher feeding rate in comparison to those mosquitoes that were not starved with water. Dry starved mosquitoes (18 hours) had a feeding rate of 51.33% in comparison to the mosquitoes that were water starved for 18 hours (13.67%). This difference in feeding rate was also observed in mosquitoes that were dry-starved and water-starved for two and six hours. These results link with studies that have indicated that water-starved mosquitoes fed more avidly in comparison to water-satiated mosquitoes (Friend and Smith 1977). Several studies utilise overnight starvation while certain studies utilise a starvation period of a minimum of five hours (Timinao et al. 2021). Coulibaly et al. (2017) indicate that the optimum starvation period for *An. coluzzii* species (82nd generation of laboratory-reared mosquitoes) is between 12 and 15 hours for mosquitoes that are three days old as this results in a high feeding, survival, and infection rate. Thus, the starvation period should be long enough for optimal infection but not that it impacts the health of the mosquito. In this study, less than 1% mortality was observed in the mosquitoes that were starved overnight. Since *An. funestus* female mosquitoes have a long-life span, overnight dry starvation can be used in blood-feeding studies as this will improve the feeding rate of the mosquito as well as increase the sample size for blood feeding assays. Furthermore, it was observed that the volume of blood meal during blood-feeding and the presence of lactic acid did not have a significant feeding difference in the feeding rate.

When working with an infectious blood meal it is crucial to know the optimum volume of blood per glass feeder that will result in a high feeding rate since the total volume of parasite blood is limited. The volume of blood varies per study, and it ranges from 250 µl to 1 500 µl (Timinao et al. 2021). In this study, a range of blood volumes (100 µl, 200 µl, 400 µl and 800 µl) were tested and similar feeding rates were obtained for the different blood volumes. Since the average blood-feeding proportions were higher when blood volumes of 400–800 µl were used, it can be suggested that any volume of blood within the range of 400–800 µl of blood can be used for blood-feeding assays. Additionally, feeding the mosquitoes a blood meal for 30 minutes yielded similar feeding rates as feeding the mosquito for 45 minutes and 60 minutes. This correlates with a study conducted by Timinao et al. (2021) as they observed no significant difference in the feeding rate when the mosquitoes were fed for 20–30 minutes. Thus, it can be concluded the duration of feeding is dependent on the feeding schedule of the individual and the duration of feeding does not affect the mosquito's ability to take a blood meal. It was also observed that *An. funestus* mosquitoes that were fed in the dark had a higher feeding proportion in comparison to mosquitoes that were fed in the light. This correlates with other studies that indicate that membrane feeding assays should be conducted in the dark to mimic the natural feeding conditions (Timinao et al. 2021). In nature, these mosquitoes prefer to rest and bite indoors at night (Gillies and De Meillon 1968) and the results obtained from this study confirm this observation.

A study conducted by Steib et al. (2001) indicated that lactic acid combined with calf or goat odour increased the feeding rate to 70%. However, when lactic acid was used in this study, the feeding rate of the *An. funestus* mosquitoes did not improve the average feeding rate for the lactic acid-treated cattle intestine membrane and when a high concentration of lactic acid (20–80%) was used in this study, it was noted that the blood coagulated. This can be expected since the human body odour is made up of volatile chemicals and the skin microbiota plays a huge role in generating these compounds to attract mosquitoes to human sweat. It can be hypothesised that *An. funestus* mosquitoes may be attracted to other chemicals in the human sweat such as carboxylic acids, ketones, sulphides, etc. This can be confirmed by a study conducted by Raji et al. (2019) which indicates that the detection of both carboxylic acid and lactic acid are required for the robust attraction of mosquitoes to humans. It can also be hypothesised that the feeding rate may be hampered by the coagulation of blood. Low concentrations of lactic acid may not have a huge impact on the blood but at high lactic acid concentrations, the effects were noticeable since an increase in the percentage of lactic acid decreases the pH of the blood (Nanang et al. 2018).

This study has limitations that will require additional research in future. Only one colony of *An. funestus* were evaluated, it will be interesting to determine the impact on the blood-feeding rate of wild-collected females or their progeny. The optimisation of the *An. funestus* feeding rate was only conducted using defibrinated cattle blood. Other blood sources such as citrated human blood or ovine blood, rabbit blood etc. was not evaluated in this study. In addition, evaluation of additional membrane types may improve the feeding rate further.

In addition, if starvation is used to improve blood-feeding rates, it will be important to evaluate if this physiological stress to the female does not negatively impact on biological research parameters or phenotypes under investigation.

CONCLUSION

By optimising the artificial membrane feeding parameters, an increase in the feeding rate of the *An. funestus* mosquitoes were observed. From this study, it can be recommended to blood feed the mosquitoes on parafilm that is exposed to human odour (placing the parafilm in a shoe or sock), however, this might add an additional variable (different individual odours) to experimental outcomes since carboxylic acid and fatty acids are the essential compounds of foot volatiles and the level of odour in an individual depends on the microbial action (Dormont et al. 2013). It is recommended to use mosquitoes that are older than seven days, to dry starve the mosquitoes for 18 hours and to allow the mosquitoes to feed in the dark in order to obtain a large number of blood-fed *An. funestus* mosquitoes. Optimising the feeding conditions is crucial for artificial membrane feeding assays since these conditions may be species-specific.

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AUTHOR CONTRIBUTIONS

Conceptualised the study: ASA and LLK; Contributed to study design: ASA, RC and LLK; performed the experiments, analysed the data, and wrote the first draft of the manuscript: ASA; Funding acquisition: LLK; Supervision: RC and LLK; Assisted with data analysis and interpretation: LLK. All the authors contributed to the subsequent versions of the manuscript, read, and approved the final version of the manuscript.

ORCID IDS

Lizette L Koekemoer: https://orcid.org/0000-0003-4236-6345
Ayesha S Aswat: https://orcid.org/0000-0002-5326-6764
Riann Christian: https://orcid.org/0000-0002-6484-1613

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