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ORIGINAL ARTICLE

Laboratory Propagation of *Anopheles funestus* Using Novel Breeding Techniques in Western Kenya

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ABSTRACT

Background: Malaria remains a formidable public health challenge in Sub-Saharan Africa, with *Anopheles funestus* sensu stricto (s.s.) recognised as a key vector driving residual transmission. Despite its reputation, research on this species is hindered by the complexities associated with its laboratory colonisation. This study designed and evaluated a new breeding technique for wild *Anopheles funestus* s.s. and assessed the vectorial competence of laboratory-reared populations.

Methods: Gravid female *Anopheles* mosquitoes were collected from indoor and outdoor sites in Western Kenya. An in-cup forced oviposition method was employed to secure eggs, which were reared in four water types: Lake water, rainwater, purified water, and pre-conditioned *An. gambiae* water. The resultant adults were subjected to membrane feeding assays using *Plasmodium falciparum* infected blood.

Results: The modified in-cup forced oviposition technique yielded an average fecundity of 114.9 eggs per female vector. Raw Lake Victoria water proved to be the most optimal rearing environment, supporting the highest adult emergence rate and the shortest developmental cycle. Conversely, larval development was significantly delayed in water pre-conditioned by *Anopheles gambiae*. Subsequent artificial membrane feeding assays involving 2,076 mosquitoes across eight transmission cycles resulted in zero *Anopheles funestus* s.s. individuals testing positive for *Plasmodium falciparum* sporozoites. However, a small proportion of *Anopheles arabiensis* vectors were infected, demonstrating a mean parasite infection rate of 6.74% with a mean sporozoite load of 1.25 pg/µl via Enzyme-Linked Immunosorbent Assay (ELISA), and a mean infection rate of 22.9% with a mean load of 1.42 copies/cell via real-time quantitative Polymerase Chain Reaction (PCR) diagnosis.

Conclusions: This study demonstrated that in-cup forced oviposition and raw Lake Victoria water can be vital in establishing large *An. funestus* s.s populations for experimental studies. The findings are crucial for understanding the biology of the parasite, testing potential vaccines and drugs and developing effective control measures.

BACKGROUND

The burden of malaria in Sub-Saharan Africa remains an enduring crisis, with millions of clinical cases and hundreds of thousands of deaths recorded annually, despite decades of intensive intervention. About 249 million cases and 608,000 deaths were recorded in 2022 around the globe.[1] Out of these, about 233 million malaria incidences and 580,000 malaria associated deaths were documented in Africa.[1] At the heart of this transmission cycle is the genus *Plasmodium*, a group of parasitic protozoans that exploit *Anopheline* mosquitoes as their definitive hosts.[2] While *Anopheles gambiae* and *Anopheles arabiensis* have traditionally remained the focal point of vector control efforts, *Anopheles funestus s.s.* has emerged as a particularly resilient and dangerous antagonist in the fight for malaria eradication.[3] This species is a member of the *An. funestus* group, a complex consisting of at least thirteen species which exhibit diverse ecological preferences and varying degrees of vectorial capacity across the Afro-tropical region.[4, 5]

Among these, *Anopheles funestus s.s.* is well-known for its high degree of anthropophagy and endophily,[6] traits that ensure frequent and intimate contact with human populations. Its contribution to malaria transmission is often disproportionate to its population density. In parts of Tanzania and Kenya, it has been observed that even when *An. funestus* occurs at much lower densities than *Anopheles gambiae*, it can be responsible for more than 80% of malaria infections.[7] This phenomenon is partly due to its impressive longevity. Recent research suggested that *An. funestus* can live for 42 to 52 days in the wild, significantly longer than its counterparts in the *An. gambiae* complex.[8] This extended lifespan provides the parasite more opportunities to complete sporogony and for the mosquito to deliver multiple infectious bites.

The persistence of *An. funestus* is further exacerbated

by the widespread emergence of insecticide resistance. As long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS) have put intense selective pressure on vector populations, *An. funestus* has developed robust physiological and behavioural resistance mechanisms.[4] In many regions, this vector has resurfaced after years of apparent suppression. These include; East Africa, West, Central and Southern region of Africa.[9,10,11,12] This has sustained malaria transmission even in areas with high intervention coverage.[13]

However, the scientific community's ability to study *An. funestus* has been severely limited by the lack of a standardized and reliable laboratory propagation protocol for this species, particularly from western Kenya.[14] Propagation refers to the rapid expansion of mosquito populations through the four-stage life cycle: Eggs, larvae, pupae and adult stage. Unlike *An. gambiae*, which is relatively easily colonised, *An. funestus* is typically eurygamous, meaning it requires large, open spaces for mating flights, a condition difficult to replicate in standard insectary cages.[13, 15] Furthermore, the aquatic stages of *An. funestus* are notoriously slow-growing and sensitive to the physical and chemical properties of their rearing environment.[15] These challenges have created a significant bottleneck in malaria research, as most laboratory experiments are forced to rely on long-established colonies that may no longer reflect the physiological or genetic realities of wild populations.

To address these historical methodological gaps, this study introduces a robust breeding protocol for wild *Anopheles funestus s.s.* from Western Kenya by combining a novel "in-cup" forced oviposition technique with optimized water sources. This standardized approach generates high-quality F1 generations from field-caught specimens. This allows for the precise evaluation of their vectorial competence through artificial membrane feeding assays with naturally acquired *Plasmodium falciparum* gametocytes. Ultimately, this specialized protocol provides the reliable biological tool required to directly test transmission-blocking interventions and accurately quantify malaria transmission dynamics.

MATERIALS AND METHODS

Study Site

This study was conducted at the International Centre of Insect Physiology and Ecology (ICIPE) located in Mbita, Western Kenya. This location was preferred due to its proximity to diverse *Anopheline* breeding sites and its state-of-the-art insectary facilities. The region around Ahero rice irrigation scheme in Kisumu County served as the primary field site for mosquito collection. This area is characterised by intensive irrigation, which provides stable, year-round breeding habitats for both *An. funestus* and *An. gambiae*, leading to high malaria endemicity.

Mosquito Sampling

Field collections targeted blood-fed and gravid *Anopheline* female mosquitoes to ensure a high probability of successful egg production in the laboratory. Sampling was performed early in the morning, between 06:30 and 10:00, to capture mosquitoes while they were resting after a night of feeding. Indoor collections utilized mouth aspiration in residential houses, focusing on walls, ceilings, and dark corners where *An. funestus* is known to rest. Outdoor collections were facilitated by cattle-baited traps, which exploit the occasional zoophilic tendencies of the species complex and provide a broader sample of the local vector population.

Collected specimens were transported in insulated containers to the Mbita insectary. During the initial 3 to5 day holding period, the mosquitoes were maintained on a 6% glucose solution provided via moist cotton wool.

Morphological and Molecular Species Identification

Initial taxonomic screening was performed to separate members of the *Anopheles funestus* group from other *Anopheline* species. Field-collected adult female mosquitoes were examined microscopically and identified morphologically based on diagnostic external features, which included; wing maculation, palpal banding patterns and leg ornamentation. This was done using the standard dichotomous and pictorial keys for *Anopheline* mosquitoes.[16, 17, 18] Upon completion of morphological identification, the identities of the parent females were molecularly confirmed using a multiplex PCR assay. This secondary verification was essential to isolate *Anopheles funestus* s.s. from non-vector sibling species.

Characterization of Experimental Water Sources

The choice of water for larval rearing is a critical determinant of colonisation success. In this study, four distinct water types were evaluated for their impact on the life-history traits of *An. funestus s.s.*:

Raw Lake Victoria Water: This source was collected directly from the lake, providing a natural microbial and mineral profile that reflects a potential wild habitat.

Rainwater: Harvested from roof runoff within the ICIPE campus during the rainy season, representing a common seasonal source of breeding water.

Purified Water: Sourced from a PURELAB Elga DV 25 purification system, serving as a controlled, contaminant-free baseline.

***Anopheles gambiae* Rearing Water:** This was obtained from active rearing pans where *An. gambiae s.s.* larvae had been maintained for 5 to7 days. The water was filtered through a 150µm mesh and cotton cloth to remove large debris and larvae, while retaining the chemical signatures and metabolic by products of a competing vector species.

Prior to use, the physico-chemical parameters of the water were measured using Macherey-Nagel Quantofix colorimetric test kits. Parameters included; nitrate (NO_3), nitrite (NO_2), ammonia (NH_4), and carbonate hardness. Potential of hydrogen (pH) levels were determined using a Multi 340 probe. To maintain experimental consistency, water temperatures in the rearing trays were monitored three times daily and maintained between 24.5 °C and 25.7 °C.

In-cup Forced Oviposition

To overcome the eurygamous mating barrier, the study focused on producing F1 progeny from wild-caught gravid females using a modified forced oviposition technique (Figure 1). Eighty gravid females were randomly assigned to the four test waters, with twenty replicates per water type. Each female was placed individually in a paper cup

containing 30 ml of the assigned water. This "in-cup" method relies on the physiological stress of confinement and the proximity of the water substrate to stimulate oviposition in individuals that might otherwise refuse to lay eggs in a larger cage environment.

Oviposition cups were checked at 08:00 and 20:00 daily. Upon the discovery of an egg batch, the parent female was removed and preserved for molecular confirmation. The eggs were counted using a magnifying glass and left in the cups to hatch. The duration from oviposition to the first appearance of first-instar larvae (L1) was recorded as the egg incubation period.

Maintenance of Larvae

Upon hatching, all larvae from a single mother (an isofemale line) were transferred to a 2-litre plastic container filled with 1 litre of the same water type used for oviposition. This approach allowed for the tracking of developmental success on a per-female basis. Larvae were fed daily with 0.10 to0.15 grams of finely ground TetraMin baby fish food, a high-protein diet designed to support rapid growth. The containers were inspected daily, and the number of pupae formed was recorded. Pupae were then transferred to emergence cups and monitored until the adults successfully emerged (Figure 1).

Artificial Infection through Membrane Feeding Assays

The vectorial competence of the laboratory-reared F1 generation was assessed by their ability to acquire and support the development of *P. falciparum*. Healthy F1 adults (3 to5 days old) were selected and starved for 12 hours prior to the infection assay. Naturally infected blood was obtained from volunteer children (aged 5 to15 years) in the local community who were found to be gametocyte-positive during routine screening. The feeding assays utilized an artificial membrane glass feeder system maintained at 38 °C. Heparinized blood containing *P. falciparum* gametocytes was offered to the mosquitoes for a period of 30 minutes. Following the meal, fully engorged females were separated and maintained in a temperature-controlled holding room at 23 to27°C and 70 to80% relative humidity. They were provided with a 6% glucose solution for 10 days post-infection to allow for the completion of the sporogonic cycle.

Transmission Assays

To evaluate and compare parasite development across different vector species, mosquito infections were carried out using the membrane feeding method. A total of eight transmission assays were completed utilizing blood donated by eight distinct *Plasmodium falciparum* gametocyte carriers. These carriers were recruited from several local sites; which included; Genge Primary School (2), Rambim Primary School (1), Alero Primary School (1), Kelly Primary School (1), Saroshine Primary School (1), and Olambwe beach (2).

A total of 2,076 field-colony mosquitoes were used across these assays, consisting of 1,170 *Anopheles funestus* s.s. and 906 *Anopheles gambiae* s.l. On the tenth day post-feeding, surviving mosquitoes were euthanized and dissected to assess infection status. A subset pool of 283 mosquitoes survived to this 10-day post-infection endpoint and was stored for downstream ELISA and PCR processing. This processed pool comprised of; 95 *An. funestus* s.s. (F1 generation from wild-caught mothers), 155 *An. arabiensis* (F1generation from wild-caught mothers), and 33 *An. arabiensis* sourced from the main insectary in Mbita.

Sporozoite Detection and Molecular Screening

During dissection, the head and thorax of each mosquito were separated from the abdomen to effectively distinguish between a localized midgut infection and a salivary gland invasion. Molecular screening and sporozoite detection of *P. falciparum* was then performed using a robust, two-tiered diagnostic approach to ensure high sensitivity and specificity in detecting even low-level infections.

First, an ELISA was employed for the initial screening of Circumsporozoite protein (CSP). For this assay, Optical Density (OD) was measured spectrophotometrically at a wavelength of 405 nm. A sample was classified as positive if its absorbance value was equal to or greater than twice the average OD of the negative controls. To eliminate false positives, all initially positive samples underwent re-testing in a secondary assay.

Secondly, a real-time qPCR was conducted on DNA extracted from the separate mosquito sections, targeting the cytochrome b (cyt b) mitochondrial gene of human *Plasmodium* species. The qPCR amplification conditions consisted of an initial denaturation phase at 95°C for 15 minutes, followed by 35 cycles of denaturation, annealing at 60°C, and extension at 70°C. Samples demonstrating an exponential amplification curve with a quantification cycle (Cq) value of less than 35 were classified as positive, whereas samples yielding no amplification or Cq ³35 were deemed negative. To safeguard data accuracy and verify product purity, a final melt curve acquisition was performed; a true positive required a single, distinct melting temperature peak (Tm) that aligned precisely with the *P. falciparum* positive control.

Data Analysis

Data management was conducted in Microsoft Excel, while all formal statistical procedures were executed in SPSS version 26. The number of eggs laid per female and the count of pupae collected were modelled using a generalized linear model (GLM) assuming a negative binomial distribution. This distribution was selected to account for the high degree of over-dispersion typically observed in biological count data, utilizing a log link function to relate the linear predictors to the mean response. Prior to final model selection, assumptions of over-dispersion and goodness-of-fit were rigorously assessed using the Pearson chi-square ratio ($\chi 2/df$).

For proportional data, such as the emergence rates from pupae to adults, a quasi-binomial GLM was employed with a logit link function to model the probability of emergence. Over-dispersion for this model was evaluated and adjusted for by estimating the scale parameter from the Pearson chi-square statistic.

Continuous variables; which included egg hatching duration, mean larval duration, and pupae stage duration, were analyzed using Analysis of Variance (ANOVA). The underlying assumptions of ANOVA, specifically the normality of residuals and homogeneity of variances, were validated using Shapiro-Wilk and Lavene's tests, respectively, prior to hypothesis testing. Where significant

FIGURE 1: Wild *An. funestus s.s* Population Rearing in the Laboratory



Forced Oviposition of eggs (a), Larvae rearing (b), Pupae collection (c) and Emergence and Maintenance of F1 adults (d).

differences were detected, Tukey's student range test was applied for post-hoc mean separation at a 5% level of significance.

Ethical Considerations

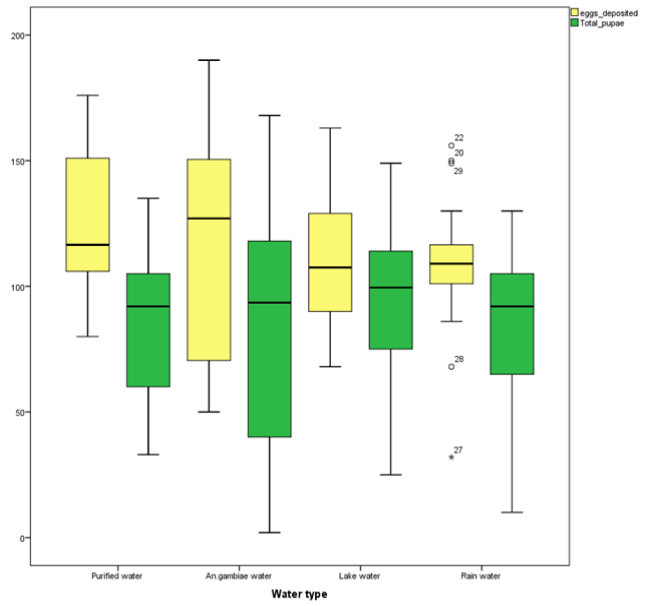
Approval for this study was obtained from Kenya Medical Research Institute (KEMRI) Scientific and Ethics Review Unit (SERU) under the approval number 545. Informed verbal consent was sought from household owners/heads to carry out mosquito collections in their homes. Informed formal consent was sought before screening children for malaria and additional consenting done before blood collection from children positive with *P. falciparum* gametocytes. A community liaison person was always present to assist in obtaining consent. Consenting was done after explaining study objectives to participants.

RESULTS

Oviposition and Immature Development Outcomes

Oviposition Success: Substrate specific oviposition success rates were 20/20 (100%) in *An. gambiae s.s.* rearing water, 19/20 (95%) in rainwater, and 18/20 (90%) in both purified and lake water types (Figure 2). However, the overall proportion of gravid females successfully laying eggs did not differ significantly across the four experimental water types (Pearson X^2 = 2.35, d.f = 3, *P*=504).

FIGURE 2: Boxplots Showing Egg Production and Pupae Collected in the Four Water Types



Fecundity: A total of 8,620 eggs were deposited by the 75 ovipositing females. The egg counts per individual female exhibited was skewed, fecundity is described using medians and interquartile ranges (IQR): *An. gambiae* s.s. rearing water, Median = 127 eggs (IQR = 74); Purified water: Median = 117 eggs (IQR = 44); Rainwater: Median = 109 eggs (IQR = 16) and Lake water: Median = 108 eggs (IQR = 37).

The generalized linear model (GLM) assuming a negative binomial distribution demonstrated that the number of eggs laid per individual female was not significantly influenced by the type of oviposition substrate (Wald X^2 = 3.25; d.f = 3, *P*=.52). Therefore, fecundity levels remained statistically uniform across all four evaluated water environments.

Pupae Production: Following the rearing of individual isofemale lines, pupae yields were tracked across the different test water treatments (Figure 2). Just like the fecundity patterns, the overall number of pupae collected from the lines did not vary significantly among the water types (Wald X^2 = 2.83, d.f = 3, *P*=.32).

Larval Development to Adult Emergence

Egg Hatching Dynamics: There was no egg batch that hatched across any of the water treatments before 24 hours had elapsed. Because mean or median hatching times were not calculated for individual treatments, the distribution of hatching onset across isofemale lines and egg batches was summarized in Table 1.

Larval Development Duration: One-way ANOVA demonstrated that the mean larval development duration differed significantly across the four water types (F=36.124, d.f.=3,*P*=.00). Pairwise post-hoc comparisons using purified water as the reference group revealed significant differences in development time for *An. gambiae* water (*P*=.000) and rainwater (*P*=.000). However, the mean larval duration in purified water was not statistically distinct from that in lake water (*P*=.805).

The developmental timelines associated with each water type were recorded in Table 2 and presented in as a box plot in Figure 3. The longest larval duration was statistically tied to the rainwater treatment.

FIGURE 3: Boxplots Showing Mean Larval and Pupation Duration of Eggs Reared in Different Water Types

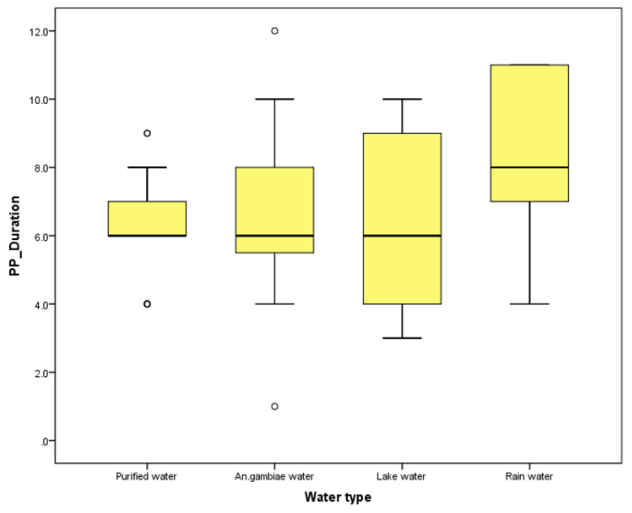


TABLE 1: Egg Hatching Dynamics in Different Treatment Waters

Water Type	Range of Hatching Onset (Hours)	Distribution of Hatching Onset Across Batches/Lines
Purified Water	48 – 72 hours	1 batch at >48 hrs; 15 batches at >60 hrs; 2 batches at >96 hrs
An. gambiae	48 – 144 hours	7 batches at >48 hrs; 12 batches at >60 hrs; 1 batch at >144 hrs
Lake Water	60 – 96 hours	17 batches at >60 hrs; 1 batch at >96 hrs
Rainwater	48 – 72 hours	2 batches at >48 hrs; 13 batches at >60 hrs; 3 batches at >72 hrs; 1 batch at >96 hrs

TABLE 2: Larvae Development Durations in Different Treatment Waters

Water type	Range (Days)	Median (Days)	Mean (Days)
Purified water	10-14	12	12
An. gambiae water	12-17	14	14
Lake water	10-13	11	12
Rainwater	11-18	16	15

Pupation Metrics and Rearing Duration: The total numbers of pupae collected did not differ significantly among the different isofemale lines across the four water types (F=.606, d.f.=56, *P*=.92). The total pupae yields and line-specific summary statistics included:

- Purified Water: 1,538 total pupae (median: 92; IQR: 41).
- *An. gambiae* s.s. Water: 1,615 total pupae (median: 94; IQR: 74).
- Lake Water: 1,685 total pupae (median: 100; IQR: 37.8).
- Rainwater: 1,582 total pupae (median: 92; QR: 40).

Nonetheless, the actual duration of the pupation stage varied significantly by water type (*P*=.004) (Figure 3). The average time required for pupation was shortest in lake and purified water (6 days each) and longest in rainwater (8 days). The exact ranges and medians across treatments were:

- Purified Water: Range: 4 to9 days (median: 6 days).
- *An. gambiae* Water: Range: 1 to12 days (median: 6 days).
- Lake Water: Range: 3 to 10 days (median: 6 days).
- Rainwater: Range: 4 to 11 days (median: 9 days).

Adult Emergence and Overall Life-Cycle Performance: Statistical analysis indicated significant variations in the total number of adults emerging across the four treatments *P*=.000). Total emergence counts alongside line-specific medians were:

- Lake Water: 1,609 adults (median: 91; IQR: 38.3).
- *An. gambiae* s.s. Water: 1,570 adults (median: 89; IQR: 71.8).
- Purified Water: 1,441 adults (median: 88; IQR: 42.5).
- Rainwater: 1,420 adults (median: 80; IQR}: 33).

Cumulatively, the average rearing time for *An. funestus* s.s. from the egg stage to the emergence of the first pupae was 14.5 days in purified water, 14.6 days in lake water, 16.5 days in *An. gambiae* water, and 17.6 days in rainwater.

Stage Specific Survival and Suitability Analysis

To evaluate the developmental suitability of each water medium, survival rates across distinct life cycle transitions were compared statistically (Figure 4 and 5):

- Egg-to-Pupae and Egg-to-Adult Stages: Lake water treatments yielded a significantly higher proportion of individuals successfully surviving from egg to pupae (P=.001) and from egg to adult P=.001). Conversely, *An. gambiae* water supported the lowest proportion of surviving individuals across these cumulative early life stages.
- Pupae-to-Adult Stage: When isolating the final developmental transition, *An. gambiae* water supported the highest rate of development from pupae to adult (P=.0001), which was not statistically different from the rates observed in lake water. Rainwater was demonstrated to be the least statistically suitable medium for supporting survival from the pupae to the adult stage.

Physical Chemical Properties of Rearing Water Samples

The physical-chemical parameters of the four water types used for rearing *Anopheles funestus* s.s. are summarized in Table 3. Test results were determined and interpreted using colorimetric charts from stage-specific test kits.

- Nitrates (NO_3-): Present in all natural water types but absent in purified water.
- Nitrites (NO_2-): Detected exclusively in lake water, ranging from 0.000 to 0.025 mg/L.

- Ammonia (NH_3): Absent in purified water. The highest concentrations were recorded in *Anopheles gambiae* rearing water, ranging from 0.2 to 0.4mg/L.
- Water Temperature: Remained stable across all rearing trays throughout the day, ranging from 24.5 to 25.7 °C ± 1.0 °C.

FIGURE 4: Proportion of Pupae and Emerged Adults Compared to the Number of Eggs in Different Water Types

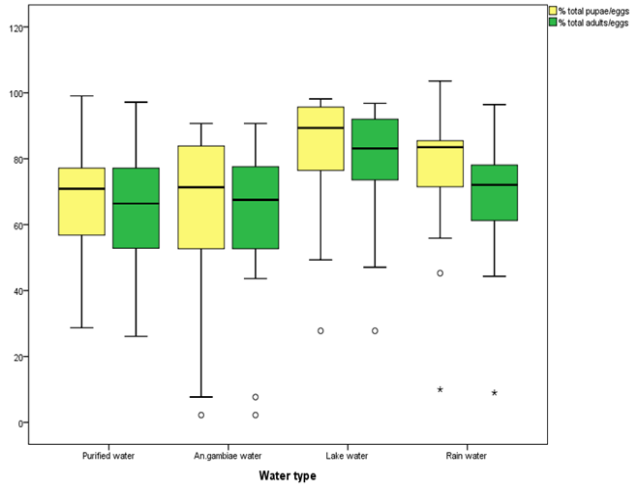
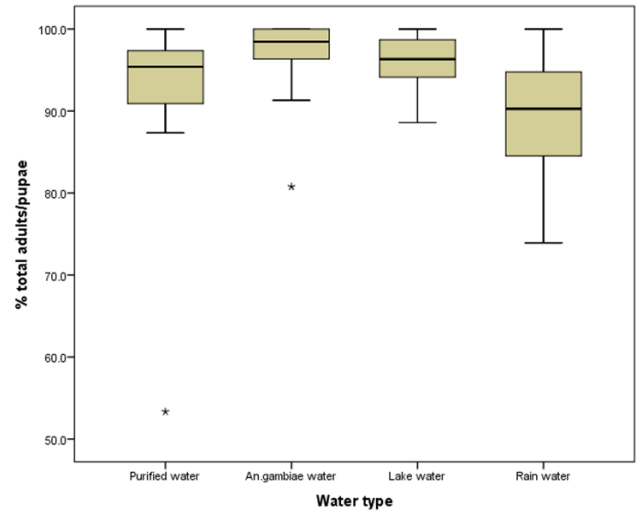


FIGURE 5: Proportion of Adults Emerged Compared to the Number of Pupae Obtained From Different Water Samples



Vectorial Competence of *Plasmodium* spp. in *An. funestus* s.s and *An. gambiae*

Out of the stored mosquitoes that survived 10 days post-infection, a total of 283 individuals were analyzed using ELISA and PCR diagnostics. Among these, only 5 *Anopheles arabiensis* individuals tested positive for the parasite by both detection methods. When broken down by their colony origin, 4 of the positive individuals were from the F1 generation reared from wild-caught mothers, while the remaining positive mosquito was obtained from the main insectary in Mbita. The overall distribution and percentage of the different mosquito species found to be sporozoite-positive by ELISA and PCR are structurally detailed in Figure 6 and Figure 7, respectively, with comprehensive comparative data illustrated in Table 4.

FIGURE 6: Percentages of Different Species from Membrane Feeding Assays Positive by ELISA

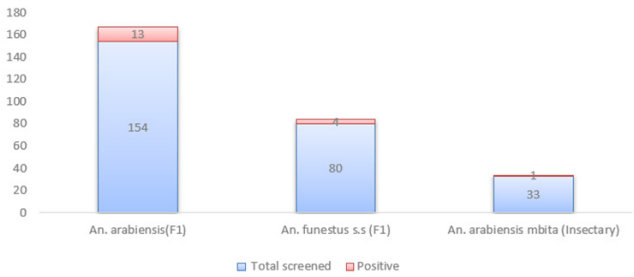
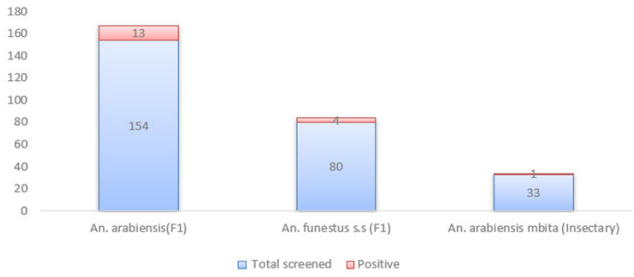


FIGURE 7: Percentages of Different Species from Membrane Feeding Assays Positive by qPCR



Diagnostic performance and infection metrics varied notably between the two assays across the eight transmission cycles.

When evaluated using ELISA, the mean percentage of infected mosquitoes across the eight assays was 6.74%, determined by the total Circumsporozoite protein (CSP) positive individuals out of the total screened population. This assay yielded a mean sporozoite infection dose of 1.25 pg/µl, with an observed range between 1.076 pg/µl and 3.589 pg/µl. Conversely, diagnostic screening using qPCR revealed a higher mean infection rate of 22.9%, calculated as the total sporozoite-positive individuals out of the total screened population. The mean sporozoite infection dose measured by qPCR was 1.42 copies/cell, covering a quantitative range from 0.0096 copies/cell to 56.0927 copies/cell.

TABLE 3: Physicochemical Parameters of Water Samples Used in the Study

Chemical properties	Water type			
	An. gambiae rearing water	Lake water	Purified water	Rainwater
pH	6.8	7.5	7.9	7.6
Nitrate (NO3-) (mg/l)	0 – 10	0 – 10	0	0-10
Nitrite (NO2-) (mg/l)	0	0 – 0.025	0	0
Ammonia (NH4) (mg/l)	0.2 – 0.4	0 – 0.2	0	0
Carbonate hardness mm/l	0.4	1.2	3.4	0.4

TABLE 4: Proportions of Sporozoites by ELISA and qPCR

Diagnostic Assay	Mean Infection Rate (Positive / Total Screened)	Mean Sporozoite Load (Infection Dose)	Range
ELISA (CSP)	6.74%	1.25 pg/µl	1.076 – 3.589 pg/µl
qPCR (DNA)	22.9%	1.42 copies/cell	0.0096 – 56.0927 copies/cell

DISCUSSION

This study aimed to investigate the life parameters, colonization potential and vector competence of *Anopheles funestus* s.s. strains in Western Kenya by evaluating local water substrates, optimizing oviposition techniques and assessing susceptibility to *Plasmodium* infection. Because the immature stages of mosquitoes complete their life cycle entirely in water, the specific quality of water required for development varies significantly from one species to another. The findings of this study demonstrate that local water sources; such as lake, rain and purified water, supported the development of immature *An. funestus* from eggs to pupae, and ultimately to adults. Conversely, water previously used for *Anopheles gambiae* rearing only performed well during the transition from pupae to adult stages. *Anopheles gambiae* larval conditioning introduces specific chemical signals, metabolic waste build-up, or semiochemical competitors which act as an allomonal deterrent, actively retarding the early larval growth stages of *An. Funestus*.[19]

Among the substrates tested, raw Lake Victoria water presented an extra advantage by yielding the shortest developmental time coupled with the highest adult emergence rate from the eggs produced. This performance makes lake water the most appropriate and optimal substrate for *An. funestus* s.s. breeding. Unlike mineral bottled water, clean de-chlorinated tap water, or spring water, which are routinely used for rearing *Anopheles* mosquitoes but are highly uneconomical for maintaining large-scale colonies, raw lake water serves as a cost-effective and ecologically realistic alternative. From a biological point of view, natural permanent and semi-permanent water bodies inherently provide a rich, micro-ecological matrix containing essential organic matter, microbial communities, and dissolved minerals that mirror natural larval habitats. This naturally occurring nutrient profile likely enhances larval nutrition and physiological development far better than artificial or purified substrates, driving the observed accelerated generation times and superior emergence success without altering the baseline egg hatching rates. These results closely concur with previous literature suggesting that *An. funestus* s.s. naturally breeds in permanent or semi-permanent water bodies characterized by floating or emergent vegetation.[19, 20]

While physical-chemical parameters of rearing water have been documented not to affect mosquito egg hatching rates,[21]an observation mirrored in this study by the lack of significant differences in hatching times across water types, water source selection remains highly consequential. In *An. gambiae* s.l., for instance, the aquatic environment significantly influences adult size, quality, wing length, and overall developmental duration, with populations usually peaking during the rainy season.[22]

Apart from the aquatic substrate, ambient temperature plays a fundamental role in regulating vector populations, with *An. gambiae* s.l. and *An. funestus* s.l. responding uniquely to distinct thermal ranges.[23] The larval water temperature of 24.5°C to 25.7°C maintained during this study proved highly suitable for *An. funestus* s.s. The development of this species is known to occur at an optimum temperature of about 25°C.[22] This is distinct from *Anopheles arabiensis*, which exhibits a broader temperature tolerance compared to *An. gambiae* s.s.[24]

Fecundity is determined by the number of eggs or the asexual propagates. A female *An. funestus* s.s. lays an average of 123 eggs per gonotrophic cycle.[25] However, a lower average of 81.7 eggs per female was harvested from field-collected mosquitoes in Tanzania.[15] In the current

study, the observed output reached an average of 114.9 eggs per female, aligning closely with these baseline averages reported in previous literature.[15,25] To bypass the challenges associated with colonizing this difficult vector, this study successfully designed and deployed a modified "in-cup" forced oviposition technique. While standard forced-egg laying techniques are known efficient tools for producing eggs from wild adult mosquitoes,[21] our modified in-cup method utilizes restricted spatial confinement to induce strategic stress in gravid females, compelling them to lay their non-drought-resistant eggs singly and directly onto the water surface.[26]

This approach addresses a critical bottleneck: Unlike the larvae of *An. gambiae*, *An. funestus* s.s. larvae are notoriously difficult to breed and rarely collected in nature. Consequently, colony establishment relies heavily on wild-caught gravid or blood-fed adults. Because traditional collection and breeding methods frequently suffer from low egg production and poor larval survival,[26] this high-yielding and easy-to-implement in-cup technique represents substantial progress for malaria vector research. Using this method alongside standard *Anopheline* rearing protocols, each batch of eggs yielded a large adult population across the different water types. Morphological identification of larval stages was confirmed using keys developed by Gillies and Coetzee.[17] The developmental and survival rates of immature stages are crucial, as they affect population growth, adult traits, and general colonization capabilities.[27] Due to strain variations, different estimated generation times have been documented across experiments. In the current study, the generation time from egg to adult was approximately 15 to 17 days, which strongly concurs with another survey that reported an average 16-day rearing duration.[28]

The artificial vector infection assays provided key insights into the vector-parasite relationship, which is critical for evaluating transmission-blocking interventions like vaccines or insecticide-treated nets. Transmission of *Plasmodium* parasites from human to vector hosts is heavily influenced by the presence of mature gametocytes in peripheral human blood.[29] Even so, ingestion of an infectious blood meal did not uniformly translate into active sporozoite infections across all screened vectors in this study. While a few *An. arabiensis* mosquitoes were successfully infected, none of the *An. funestus* s.s. individuals tested positive. The failure to infect *An. funestus* could probably have been driven by a clear feeding preference for live hosts over artificial membranes, reducing overall blood meal uptake in the F1 cohorts, hence limited sporogony. This observation is consistent with previous surveys which revealed that sporogony can be suppressed at any developmental stage by a network of interacting factors.[30] These limiting factors include vector sex-ratio, density, packed cell volume, gametocyte maturity, parasite intrinsic traits, human and mosquito immune factors, and the presence of transmission-modulating elements such as antimalarial drug levels. Therefore, ingested gametocytes may not necessarily result in a sporozoite infection.[31, 32, 33, 34, 35]

This study has advanced a novel forced oviposition technique and delivered a report on life parameters of *An. funestus* s.s strain in Western Kenya. The study provides base-line information, essential for further studies towards better understanding of population dynamics of *An. funestus* under local and regional conditions. In this study, the possibility of using local sources of water in breeding *Anopheles* vectors has been investigated. Artificial infection of mosquitoes with *Plasmodium* parasites enables studies of various aspects on malaria transmission, including development and behavior of parasites within the mosquito vector, factors influencing mosquito susceptibility to infection, and effectiveness of interventions aimed at blocking transmission, such as insecticide-treated bed nets or vaccines targeting the parasite. It is crucial for understanding interactions between the vector and parasite.

Study Limitations

A number of limitations must be acknowledged when interpreting the findings of this study. First, the wild-caught female *An. funestus* vectors utilized for oviposition were of unknown age, historical oviposition cycle and prior blood-feeding frequency. Because factors such as female body size, adult diet and environmental dynamics significantly influence mosquito reproduction, these uncontrolled variables likely suppressed the full physiological expression of their baseline fecundity. Furthermore, establishing subsequent, long-term transmission assays was severely constrained by difficulties in securing consistent gametocyte carriers, even when colonies were ready for testing. This limitation was directly tied to the localized impact of indoor residual spraying implemented by the County Government of Homa Bay, which restricted field sampling and carrier recruitment.

CONCLUSIONS

This study has established a reliable and efficient technique for laboratory colonisation of *Anopheles funestus s.s*. from wild populations in Western Kenya. The integration of in-cup forced oviposition and the use of raw Lake Victoria water as a rearing substrate optimize both fecundity and developmental success. These findings provide a vital tool for the malaria research community, enabling the production of high-quality vector populations for the study of biology, resistance, and competence.

While challenges in artificial membrane feeding persist, the work highlights the importance of ecological realism in laboratory protocols. The evidence of chemical interference from *An. gambiae* rearing water suggests a complex inter-species dynamic that warrants further investigation, as it may influence vector succession following public health interventions. Ultimately, the standardisation of these breeding techniques is a critical step toward a deeper understanding of one of Africa's most significant malaria vectors and the development of more effective measures for its control.

RECOMMENDATIONS

1. Raw Lake Victoria water should be standardized as the primary larval substrate, as it supports faster development and higher adult emergence, making it the most effective and economical option for *An. funestus* colonisation; 2. The in-cup forced oviposition technique should be adopted to reliably obtain eggs from wild gravid females and overcome laboratory colonisation challenges

associated with this species; 3. There is need for optimized membrane-adaptation protocols in future trials to better assess *Plasmodium falciparum* susceptibility and strengthen laboratory-based vector competence studies.

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