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Elison Elieza Kemibala

elisonmwebembezi@gmail.com

Muheza College of Health and Allied Sciences

Magreth Shayo

Kilimanjaro Christian Medical University

Innocent Leonard Swai

Muheza College of Health and Allied Sciences

Bahati Hakili Kilagwile

Muheza College of Health and Allied Sciences

David Hamis Abdiel

Muheza College of Health and Allied Sciences

Angela Munishi

Muheza College of Health and Allied Sciences

Neema Adam

Muheza College of Health and Allied Sciences

Josephina Rutayagi

Muheza College of Health and Allied Sciences

Ramadhani Bofu

Ministry of Health

Jennifer Thomas Mbise

Muheza College of Health and Allied Sciences

Fadhili Lyimo

Ministry of Health

Saitore Ole Laizer

Ministry of Health

Teun Dekker

Swedish University of Agricultural Sciences

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Effects of natal habitat odour on oviposition site selection by *Anopheles gambiae* sensu lato

Authors

Elison Elieza Kemibala[1]†, Magreth Shayo[3], Innocent Leonard Swai[1], Bahati Hakili Kilagwile[1], David Hamis Abdiel [1], Angela Munishi[1], Neema Adam[1], Josephina Rutayagi[1], Ramadhani Bofu[4], Jennifer Thomas Mbise[1], Fadhili Lyimo[5], Saitore Ole Laizer[5], Teun Dekker[6]

†Corresponding author
email: elisonmwebembezi@gmail.com

Affiliations

[1] Department of Vector Control, Muheza College of Health and Allied Sciences, Muheza, Tanzania
[3]Pan-African Malaria Vector Research Consortium, Kilimanjaro Christian Medical University,Moshi, Tanzania.
[4] Dodoma Institute of Health and Allied Sciences, Dodoma, Tanzania
[5] Directorate of Human Resource Development, Ministry of Health, Government City, Mtumba, Dodoma, Tanzania
[6] Division of Chemical Ecology, Department of Plant Protection Biology, Swedish University of Agricultural Sciences, Alnarp, SE-230 53, Sweden

Abstract

Background

The mechanisms underlying oviposition site selection by malaria vectors remain poorly understood. Understanding the behavioural cues that guide gravid mosquitoes in selecting breeding habitats is essential for developing improved surveillance and vector control tools, particularly those targeting gravid females and aquatic stages.

Methods
Laboratory colonies of *Anopheles gambiae* s.s. and *Anopheles arabiensis* were reared separately in water collected from two natural breeding habitats in Muheza District, Tanzania, and in dechlorinated laboratory tap water. Upon becoming gravid, females from each rearing group were simultaneously offered three oviposition substrate choices corresponding to the different rearing waters under both laboratory cage bioassays and semi-field mosquito sphere experiments. Oviposition preference was determined by comparing the proportion of eggs deposited in each substrate.

Results

A total of 1,080 gravid mosquitoes were evaluated under laboratory conditions and 3,600 under semi-field conditions. Both *An. gambiae* s.s. and *An. arabiensis* demonstrated a tendency to oviposit preferentially in water resembling their natal habitat. In laboratory assays, *An. arabiensis* showed significant preference for its natal habitat water when reared in natural breeding-site substrates, while *An. gambiae* s.s. exhibited significant preference for both natal natural habitat and laboratory-rearing water. Semi-field experiments showed similar behavioural trends, although the responses were less consistent because of increased environmental variability. Overall, the results indicate that larval experience significantly influences oviposition site selection in adult mosquitoes.

Conclusions
These findings suggest that exposure to natal habitat odours during larval development influences subsequent oviposition site selection by adult mosquitoes. Natal habitat odours therefore represent promising semiochemicals candidates for improving gravid mosquito surveillance and control.

Keywords: *Anopheles gambiae* s.s.; *Anopheles arabiensis*; oviposition behaviour; natal habitat; olfactory learning; memory; mosquito ecology; gravid mosquitoes; ovitraps; malaria vector surveillance.

Background

Malaria remains one of the world's most important vector-borne diseases and continues to impose a substantial burden on public health, particularly in sub-Saharan Africa. According to the World Health Organization (WHO), an estimated 263 million malaria cases and 597,000 malaria-related deaths occurred globally in 2023, representing a slight increase compared with previous years. The WHO African Region accounted for approximately 94% of global malaria cases and 95% of malaria deaths, with children under five years of age and pregnant women being the most vulnerable populations (WHO, 2024). Beyond its health consequences, malaria imposes considerable socioeconomic costs through increased healthcare expenditure, reduced productivity, school absenteeism, and impeded economic development, particularly in low-income countries (Gallup & Sachs, 2001; Sachs & Malaney, 2002; WHO, 2024).

Vector control remains the cornerstone of malaria prevention and elimination programmes. Current interventions primarily rely on insecticide-treated nets (ITNs), indoor residual spraying (IRS), larval source management in appropriate settings, and integrated vector management approaches. These interventions have contributed substantially to reductions in malaria transmission over the past two decades, although their effectiveness is increasingly threatened by insecticide resistance, behavioural adaptations of vectors, urbanization, climate variability, and ecological changes (Bhatt et al., 2015; WHO, 2024). Consequently, effective vector

surveillance systems are increasingly recognized as essential components of evidence-based malaria control programmes.

The principal malaria vectors in sub-Saharan Africa belong to the Anopheles gambiae complex and the Anopheles funestus group. Within the An. gambiae complex, Anopheles gambiae sensu stricto and Anopheles arabiensis are among the most efficient malaria vectors because of their high susceptibility to Plasmodium parasites, preference for human hosts, and close association with human settlements (Coetzee et al., 2013; Sinka et al., 2020). A comprehensive understanding of their biology, ecology, behaviour, and population dynamics has greatly informed the development and implementation of current malaria vector control strategies and continues to support innovation in surveillance and intervention technologies (Takken & Lindsay, 2019; WHO, 2024).

The World Health Organization emphasizes that effective malaria vector control should be supported by robust entomological surveillance capable of monitoring vector species composition, abundance, behaviour, insecticide resistance, and ecological changes over time (WHO, 2022; WHO, 2024). Developing sensitive and reliable surveillance tools requires a detailed understanding of mosquito behaviour throughout all stages of their life cycle, including host seeking, resting, mating, and oviposition behaviour (Ferguson et al., 2010; Govella & Ferguson, 2012).

Oviposition site selection is a critical behavioural process in mosquitoes because immature stages are entirely dependent on aquatic habitats for development. Female mosquitoes are therefore expected to maximize offspring survival by selecting breeding habitats that provide favourable environmental conditions for larval growth and development (Bentley & Day, 1989; Blaustein et al., 2005). Consequently, understanding the mechanisms underlying oviposition site selection offers opportunities to develop innovative surveillance tools and environmentally sustainable vector control strategies that exploit mosquito reproductive behaviour (Dugassa et al., 2013; Lindh et al., 2015).

Selection of oviposition sites by Anopheles mosquitoes is influenced by a complex interaction of physical, chemical and biological cues. Previous studies have demonstrated that gravid females may discriminate among potential breeding habitats based on characteristics such as water colour, turbidity, sunlight exposure, presence of vegetation, microbial communities, organic matter content, and volatile organic compounds produced by aquatic microorganisms (Minakawa et al., 1999; Huang et al., 2007; Mwangangi et al., 2007; Ponnusamy et al., 2015). In addition, several studies have demonstrated that semiochemicals produced by microbial activity can either attract or repel gravid mosquitoes depending on their chemical composition

and concentration, highlighting the critical role of olfactory cues in oviposition site selection (Lindh et al., 2015; Ignell & Hill, 2020; Raji & DeGennaro, 2022).

Chemical ecology has become increasingly important in mosquito surveillance and vector control because gravid mosquitoes rely on habitat-associated semiochemicals to locate suitable oviposition sites. Volatile compounds originating from aquatic microorganisms, organic matter and vegetation, together with the physicochemical characteristics of breeding habitats, influence oviposition behaviour and larval performance. These chemical cues have considerable potential for the development of odour-based surveillance tools and environmentally sustainable vector control strategies, particularly for malaria vectors (Mwingira et al., 2020; Hill & Ignell, 2021). Knowledge of oviposition behaviour has successfully facilitated the development of gravid traps and ovitrap-based surveillance systems for important vectors of dengue, yellow fever, Zika, chikungunya, and West Nile virus, particularly Aedes and Culex mosquitoes (Fay & Eliason, 1966; Barbosa et al., 2010; Eiras & Resende, 2009). In contrast, relatively limited progress has been made toward developing equally effective oviposition-based surveillance tools for African malaria vectors.

Gravid traps and ovitraps are designed to attract and capture gravid mosquitoes actively searching for suitable aquatic habitats in which to lay eggs. These tools have become standard surveillance methods for Aedes and Culex mosquitoes because they provide sensitive measures of adult female populations and arbovirus transmission risk (WHO, 2023). However, comparable surveillance systems for Anopheles gambiae complex mosquitoes remain underdeveloped. One of the principal challenges has been the incomplete understanding of the semiochemicals and behavioural mechanisms that mediate oviposition site selection in these vectors (Dugassa et al., 2013; Lindh et al., 2015).

Recent evidence suggests that mosquitoes possess greater behavioural plasticity and learning capacity than previously recognized. Experimental studies indicate that mosquitoes can acquire and retain olfactory information associated with feeding and breeding habitats, enabling them to modify subsequent behavioural responses based on previous experiences (Vinauger et al., 2018; Raji & DeGennaro, 2022). Such learning mechanisms may influence habitat fidelity, dispersal, and oviposition site selection, although these processes remain poorly understood in members of the Anopheles gambiae complex.

Therefore, this study aims to investigate whether natal habitat odours influence oviposition site selection through learning and memory in Anopheles gambiae sensu stricto and Anopheles arabiensis. Specifically, the study seeks to determine whether gravid females exhibit a preference for oviposition sites containing odour cues associated with their larval habitat. Improved

understanding of these behavioural mechanisms could facilitate the development of novel, cost-effective gravid traps and odour-based surveillance tools for malaria vectors, thereby strengthening entomological surveillance and supporting integrated malaria vector management.

Material and Methods

Study area

The study was conducted at the Amani Medical Research Centre of the National Institute for Medical Research (NIMR), located in Muheza District, north-eastern Tanzania (5°13′S, 38°39′E; altitude 193 m above sea level). Muheza receives an average annual rainfall of approximately 1,000 mm, characterized by a bimodal rainfall pattern with the main rainy season occurring between March and May and a shorter rainy season between November and December. The mean annual temperature is approximately 26°C, with relatively cooler months extending from June to September and warmer conditions prevailing from October to May.

Study design

This experimental study was conducted under laboratory and semi-field conditions using mosquito spheres. Three subpopulations each of *Anopheles gambiae* sensu stricto and *Anopheles arabiensis* were reared separately and simultaneously using different larval rearing substrates. After the adult females became gravid, they were offered multiple oviposition choices, including their respective natal substrate, to assess their oviposition preferences and determine whether natal habitat influenced oviposition site selection.

Sources of mosquitoes rearing material

Genge natural breeding site

One of the larval rearing substrates was collected from a natural mosquito breeding site located at S 05.171540, E 038.793700, at an elevation of 180 m above sea level. The breeding habitat

was situated approximately 15 m from the nearest human settlement and was surrounded by natural vegetation. The substrate consisted predominantly of greyish clay soil. The water was milky in appearance and had a characteristic odour of decomposing grass. Physicochemical characteristics of the breeding water, including temperature, pH, electrical conductivity, and turbidity, were measured in situ using a portable multiparameter meter (HI98129 Combo®, Hanna Instruments, Woonsocket, RI, USA) and a portable turbidity meter (2100Q Portable Turbidimeter, Hach Company, Loveland, CO, USA). At the time of collection, the water temperature was 26.7°C, pH 7.3, turbidity 0.49 NTU, and electrical conductivity 0.94 mS/cm. Water samples were collected from a section of the habitat measuring approximately 3 m in diameter with an average depth of 1 m.

Kilima ntengwe natural breeding site

The second natural breeding site was located at S 05.163140, E 038.766210, at an elevation of 201 m above sea level. This habitat was an abandoned quarry characterized by sparse vegetation cover, with the nearest human settlement located approximately 100 m away. The water was reddish-brown in colour. Physicochemical parameters were measured using the same instruments described above, at the time of sampling showed a temperature of 27.2°C, pH 7.9, turbidity 0.06 NTU, and electrical conductivity 0.12 mS/cm. The breeding habitat had an average water depth of approximately 10 cm.

Tap water from NIMR insectary

The third larval rearing medium consisted of tap water obtained from the NIMR insectary. Prior to use, the tap water was dechlorinated by boiling and subsequently allowed to cool to room temperature. Water quality parameters were measured using the same portable multiparameter meter (HI98129 Combo®, Hanna Instruments, Woonsocket, RI, USA) and portable turbidity meter (2100Q Portable Turbidimeter, Hach Company, Loveland, CO, USA). The water had a temperature of 26.6°C, pH 7.3, turbidity 0.49 NTU, and electrical conductivity of 0.94 mS/cm.





Figure 1: Kilimantengwe breeding site **Figure 2**: Genge breeding site

Mosquito colonies and rearing

Laboratory colonies of *Anopheles gambiae sensu stricto* (Kisumu strain) and *Anopheles arabiensis* (TPHPA strain) maintained at the National Institute for Medical Research (NIMR) - Amani Centre, Tanzania, were used throughout the study. The *An. gambiae* Kisumu strain is a well-established insecticide-susceptible reference colony originally obtained from Kisumu, Kenya, whereas the *An. arabiensis* TPHPA strain originated from the Tanzania Plant Health and Pesticides Authority (TPHPA), Tanzania. Both colonies were maintained under standard insectary conditions following established procedures for rearing Anopheles mosquitoes (MR4, 2014; Benedict, 2007; WHO, 2016).

For each mosquito species, larvae were divided into three independent rearing groups according to the source of larval habitat water: (i) Kilima Ntengwe natural breeding site water, (ii) Genge natural breeding site water, and (iii) dechlorinated laboratory tap water obtained from the NIMR insectary. All other rearing conditions, including larval density, feeding regime, temperature, relative humidity, and photoperiod, were maintained uniformly across the three groups to minimize experimental variation.

Mosquitoes were maintained at 27 ± 2°C, 75 ± 10% relative humidity, and a 12:12 h light: dark photoperiod. Larvae were fed daily with Tetramin® fish food (Tetra GmbH, Germany), while adults were provided continuous access to 10% sucrose solution. Water from each respective

larval rearing habitat was collected separately in clean, labelled containers and preserved for subsequent use as the oviposition medium during the behavioral experiments. Females aged 3–5 days were blood-fed on restrained rabbits in accordance with institutional animal welfare guidelines and used for oviposition assays 72 h post blood meal.

Oviposition choice bioassay in the laboratory

Water collected from each larval rearing habitat water was diluted with distilled water at a ratio of 1:3 (v/v; habitat water: distilled water) ratio to minimize rapid decomposition of organic matter while retaining the physicochemical characteristics and odour cues of the original habitat. The diluted water samples were stored in clean, labelled containers at room temperature in the insectary until use in the oviposition bioassays. The use of habitat water as an oviposition stimulus is based on the premise that gravid mosquitoes rely on chemical cues originating from aquatic habitats to identify suitable oviposition sites (Bentley & Day, 1989; Lindh et al., 2015). For each mosquito species, three subpopulations reared in Genge, Kilimantengwe, and laboratory tap water were evaluated simultaneously in oviposition choice experiments. The assays were conducted in 30 × 30 × 30 cm screened mosquito cages under standard insectary conditions (26 ± 1°C, 78 ± 4% relative humidity, and a 12:12 h light: dark photoperiod) following standard procedures for laboratory maintenance and behavioral assays of Anopheles mosquitoes (Benedict, 2007).

A modified double-cup oviposition technique was used to evaluate the influence of habitat-associated odour cues on oviposition site selection while preventing gravid mosquitoes from making direct contact with the test substrates. This design ensured that oviposition responses were mediated primarily by volatile chemical cues emitted from the tested materials rather than by visual or contact stimuli.

For each oviposition choice, 20 mL of the test substrate was placed into a transparent plastic container (6.8 cm diameter × 7.5 cm depth) (**Figure 3**). Two perforated paper cups (5 cm

diameter × 5 cm depth) were used to construct the odour delivery system. The lower paper cup contained four perforations (0.3 cm diameter) at its base, whereas the upper cup contained four perforations of the same diameter around its upper edge (**Figures 4 and 5).** The cups were assembled by inserting the upper cup into the lower cup, thereby creating a double-cup chamber that allowed volatile compounds from the test substrate to diffuse through the perforations while preventing mosquitoes from directly contacting the substrate **(Figure 6),** Similar odour-based oviposition bioassay designs have previously been used to evaluate attractants for gravid Anopheles mosquitoes (Dugassa et al., 2013; Lindh et al., 2015).

The assembled paper cups were carefully positioned inside the plastic container without touching the test substrate. A smaller plastic oviposition cup (4.1 cm diameter × 3.0 cm depth) containing 2 mL of distilled water was then placed into the opening of the double-cup assembly **(Figure 7).** Consequently, gravid mosquitoes deposited eggs into the distilled water contained in the small oviposition cup after being attracted by volatile odors released from the test substrate in the larger container **(Figure 8).**

Each experimental cage (30 × 30 × 30 cm) contained four double-cup oviposition devices (**Figure 9)** corresponding to the four oviposition choices: Genge habitat water, Kilimantengwe habitat water, laboratory rearing water, and fresh dechlorinated tap water (control). The positions of the four oviposition devices within each cage were randomized for every experimental replicate to minimize positional bias **(Figure 10).** Overlapped paper cups were placed inside the plastic cup taking precaution not to contact materials inside the plastic cup. Then the small plastic cup (4.1cm diameter, 3cm depth) (**Figure 10**) filled with 2mls of distilled were inserted in the opening of overlapped cups, hence mosquitoes were ovipositing in distilled contained in a small plastic cup after being attracted by the odour emitted from the material contained in the large plastic cup and odour passed through the holes of the two overlapped paper cups (Bentley & Day, 1989; Lindh et al., 2015).

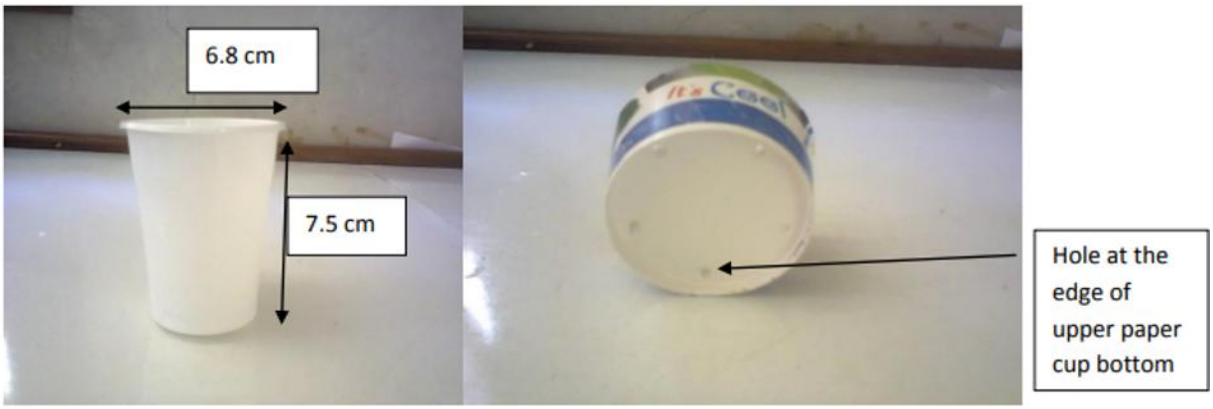

Figure 3 & 4

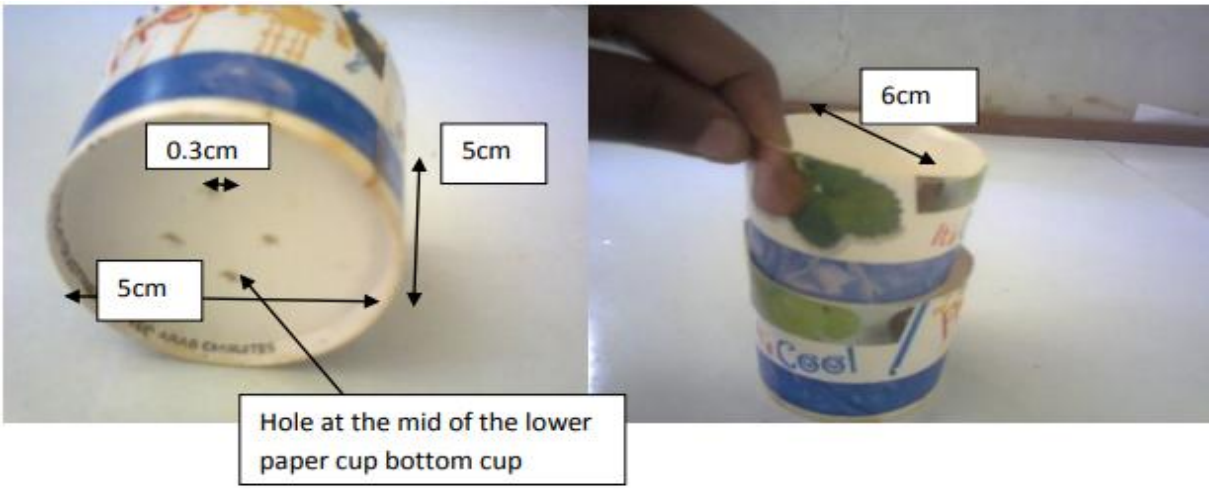

Figure 5 &6

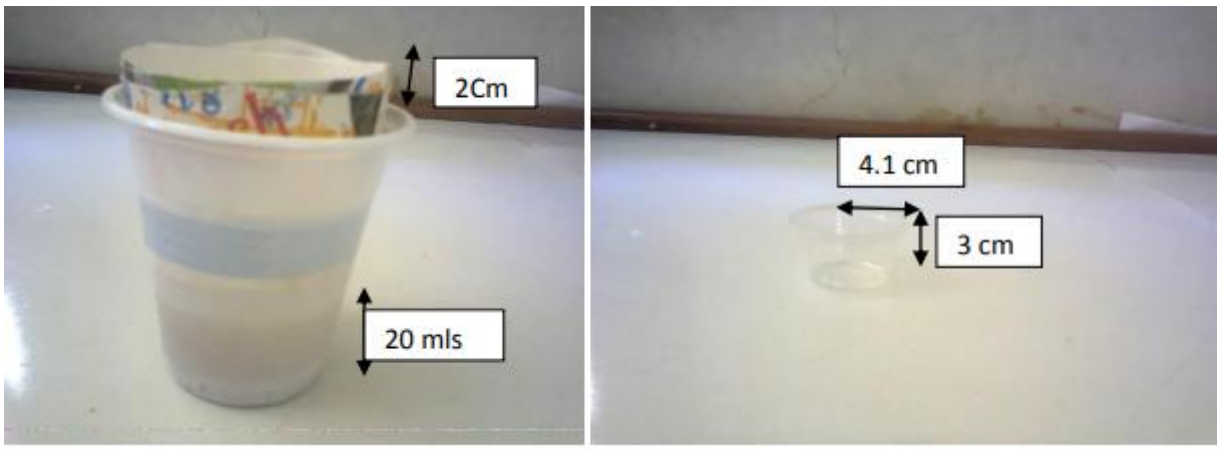

Figure 7 & 8

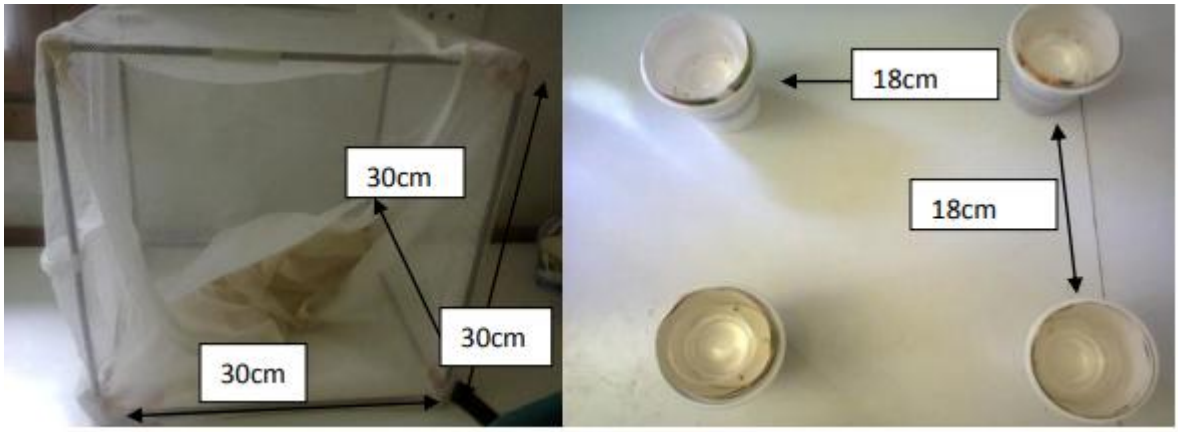

Figure 9 & 10

For each oviposition assay, 10 gravid female mosquitoes (3–5 days post-blood meal) from a single larval rearing subpopulation were released into an experimental cage and allowed to oviposit for 24 h under standard insectary conditions (26 ± 1°C, 78 ± 4% relative humidity, and a 12:12 h light: dark photoperiod). Each assay was performed in six independent biological replicates simultaneously for the three larval rearing subpopulations of each mosquito species. The complete experiment was repeated three times for each species, resulting in a total of 18 replicates per subpopulation and 180 gravid females tested per subpopulation (10 females × 18 replicates). Overall, 540 gravid females were evaluated for each mosquito species.

Following the oviposition period, eggs deposited in each oviposition cup were counted under a stereomicroscope. Oviposition preference was quantified by calculating the proportion of the total eggs laid in each of the four oviposition choices relative to the total number of eggs deposited within each experimental cage. This approach enabled comparison of oviposition preferences among gravid females originating from different larval habitats and assessment of the influence of natal habitat experience on oviposition site selection.

Semi-field experiments setup (In the mosquito's sphere)

Semi-field oviposition experiments were conducted in mosquito spheres, which are double-layered enclosures constructed from dark-green shade netting that permits natural airflow and rainfall while preventing the escape of released mosquitoes and the entry of wild mosquito populations (Knols *et al*., 2002; Ferguson *et al*., 2008). This semi-field system provides

environmental conditions that closely resemble natural settings while maintaining experimental control.

Locally manufactured reddish-brown clay pots (22 cm diameter × 9 cm depth) were used as artificial oviposition habitats **(Figure 11).** Each clay pot was embedded into the earthen floor of the mosquito sphere, leaving approximately 1 cm of the rim above the ground surface to simulate natural ground-level breeding habitats. Each pot was filled with 500 mL of the designated oviposition substrate.

A total of 30 clay pots were used in each experiment and arranged at 1 m intervals throughout the mosquito sphere. The pots were assigned identification numbers from 1 to 30 using a random allocation procedure. The three oviposition substrates corresponding to the larval rearing habitats (Genge habitat water, Kilimantengwe habitat water, and laboratory rearing water) were evaluated simultaneously, with 10 clay pots assigned to each substrate. The spatial positions of the clay pots were randomized between experimental replicates to minimize positional bias **(Figure 12**).

Each larval rearing subpopulation of Anopheles gambiae sensu stricto and Anopheles arabiensis was evaluated separately. Experiments were conducted in three mosquito spheres, with one subpopulation tested in each sphere at a time. To minimize any potential carry-over effects resulting from residual odors or environmental contamination, subpopulations were rotated among the three spheres after a 2-day washout period.

For each mosquito species, three independent biological replicates were conducted for each larval rearing subpopulation. In each replicate, 200 fully gravid females were released into the mosquito sphere at 18:00 h and allowed to move freely overnight in search of suitable oviposition sites under ambient semi-field conditions. The following morning, eggs deposited in each clay pot were collected and counted under a stereomicroscope.

Oviposition preference was quantified by comparing the total number and proportion of eggs deposited in clay pots containing each oviposition substrate. This experimental design enabled assessment of whether gravid females preferentially selected oviposition sites associated with their natal larval habitat under semi-field conditions that more closely approximated the natural environment.

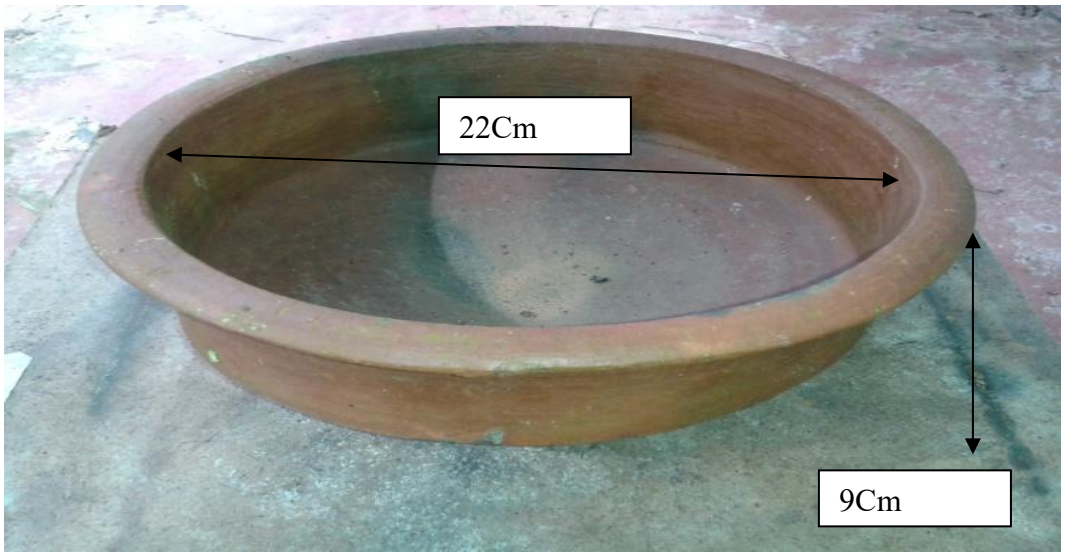

Figure 11

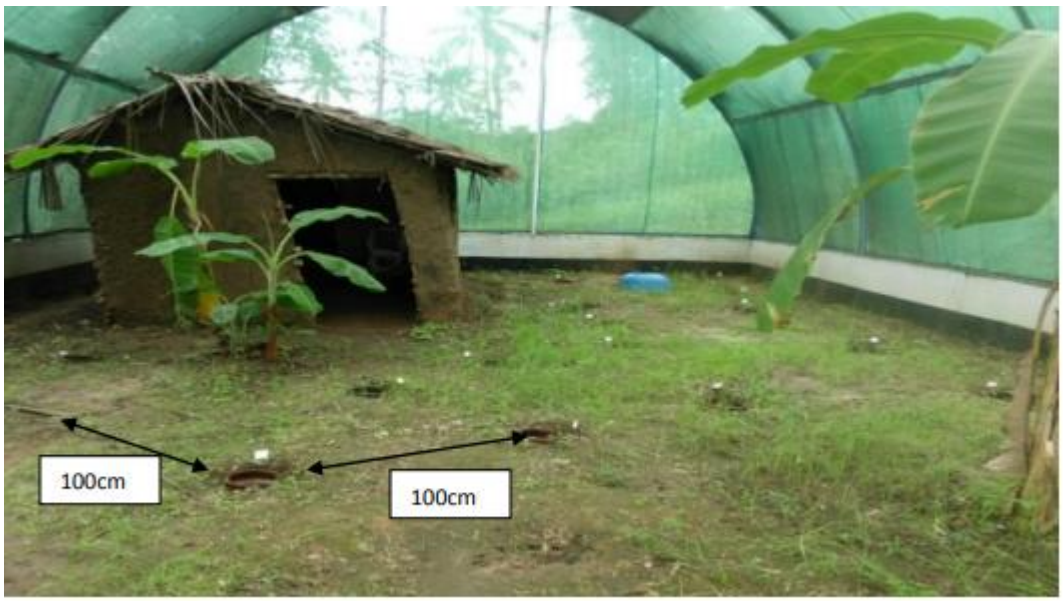

Figure 12

Data collection procedures

Oviposited eggs in each substrate were assessed the following day in both laboratory and mosquitoes same field settings. Eggs counting was aided by magnifying lance and tally counter then scores recorded in special designed form with the details on temperature, humidity, responsible technician and counting time and date. Proportional of oviposited mosquitoes also were recorded.

Data analysis

Data were entered into Microsoft Excel 2021, checked for completeness and consistency, and analysed using Stata Statistical Software version 18.0 (Stata Corp LLC, College Station, TX, USA). For the laboratory oviposition choice bioassays, the total number of eggs laid in each oviposition substrate was summarized as frequencies and proportions. Oviposition preference was expressed as the proportion of eggs deposited in each substrate relative to the total number of eggs laid within each experimental cage. Pairwise differences in oviposition preferences among substrates for each mosquito species and larval rearing habitat were assessed using two-sample tests of proportions with 95% confidence intervals. For the semi-field (mosquito sphere) experiments, the distribution of egg count data was first assessed using the Shapiro–Wilk test for normality. Because the data were not normally distributed, non-parametric statistical methods were employed. The Scheirer–Ray–Hare test, a non-parametric alternative to two-way analysis of variance (ANOVA), was used to evaluate the interaction between larval rearing habitat and oviposition substrate on oviposition preference. Where significant differences were detected, the Kruskal–Wallis test was used to compare egg deposition among substrates, followed by Dunn's multiple comparison test with Bonferroni adjustment for pairwise comparisons. All statistical tests were two-tailed, and statistical significance was determined at a 5% significance level ($p < 0.05$).

RESULTS

Laboratory results

A total of 303 eggs of *Anopheles gambiae* s.s. were collected from mosquitoes reared in Genge habitat water. Of these eggs 86 (28.4%) were collected in the bowl with Kilimantengwe water, 41 (13.5%) in Genge water, 98 (32.3%) in laboratory rearing water and 78 (25.7%) in clean laboratory water. For *Anopheles arabiensis*, a total of 483 eggs were collected from mosquitoes reared in Genge habitat water. Of these eggs, the highest proportion 37.5% (n = 181) were oviposit in Genge water, 113 (23.4%) in Kilimantengwe water, 102 (21.1%) in laboratory rearing water, and 87 (18.0%) in laboratory clean water. Both species reared in Kilimantengwe habitat water, they all preferred to oviposit in Kilimantengwe substrate water (*Anopheles gambiae* s.s. = 31.6% and *Anopheles arabiensis* = 34.5%) as compared to other substrates. The same behavior was shown for mosquitoes reared in laboratory water, more than 29% of eggs were oviposit in either in laboratory rearing water or clean laboratory water (**Table 1**)

Table 1. Number and percentage of eggs laid by *Anopheles gambiae s.s*. and *Anopheles arabiensis* in different oviposition substrates according to larval rearing habitat in laboratory choice bioassays.

Mosquitoes	Substrate	*Anopheles gambiae s.s.*		*Anopheles arabiensis*	
		Eggs oviposit	%	Eggs oviposit	%
Reared in Genge habitat water	Kilimantengwe water	86	28.4	113	23.4
	Genge water	41	13.5	181	37.5
	Lab rearing water	98	32.3	102	21.1
	Clean Lab water	78	25.7	87	18.0
Reared in Kilimantengwe habitat water	Kilimantengwe water	141	31.6	173	34.5
	Genge water	74	16.6	107	21.4
	Lab rearing water	120	26.9	108	21.6
	Clean Lab water	111	24.9	113	22.6
Reared in Lab water	Kilimantengwe water	112	23.0	114	21.8
	Genge water	85	17.5	102	19.5
	Lab rearing water	146	30.0	157	30.0
	Clean Lab water	144	29.6	150	28.7

Proportion difference of oviposition preference

The immediate form of two-sample test of proportions was used to test the equality of proportions of mosquitoes laying eggs in difference substrates with 95% confidence intervals.

Anopheles arabiensis

Two-sample test of proportions was used to determine any significance difference of oviposition preferences of mosquitoes reared in Genge, Kilimantengwe and Laboratory water. Anopheles arabiensis reared in Genge habitat water significantly preferred to oviposit in Genge substrate water as compared to Kilimantengwe water (p-value = 0.012), Lab rearing water (p = 0.004) and Clean laboratory water (p = 0.001). The same trend was observed for Anopheles arabiensis reared in Kilimantengwe habitat water. The proportion of mosquitoes ovipositing in Kilimantengwe substrate water was significantly higher as compared to Genge substrate water (p = 0.020), Laboratory rearing water (p = 0.025) and clean laboratory water (0.032). On the other hand, Anopheles arabiensis reared in Laboratory water did not show any statistically significant preference of substrate to lay their eggs. (**Figure 13**)

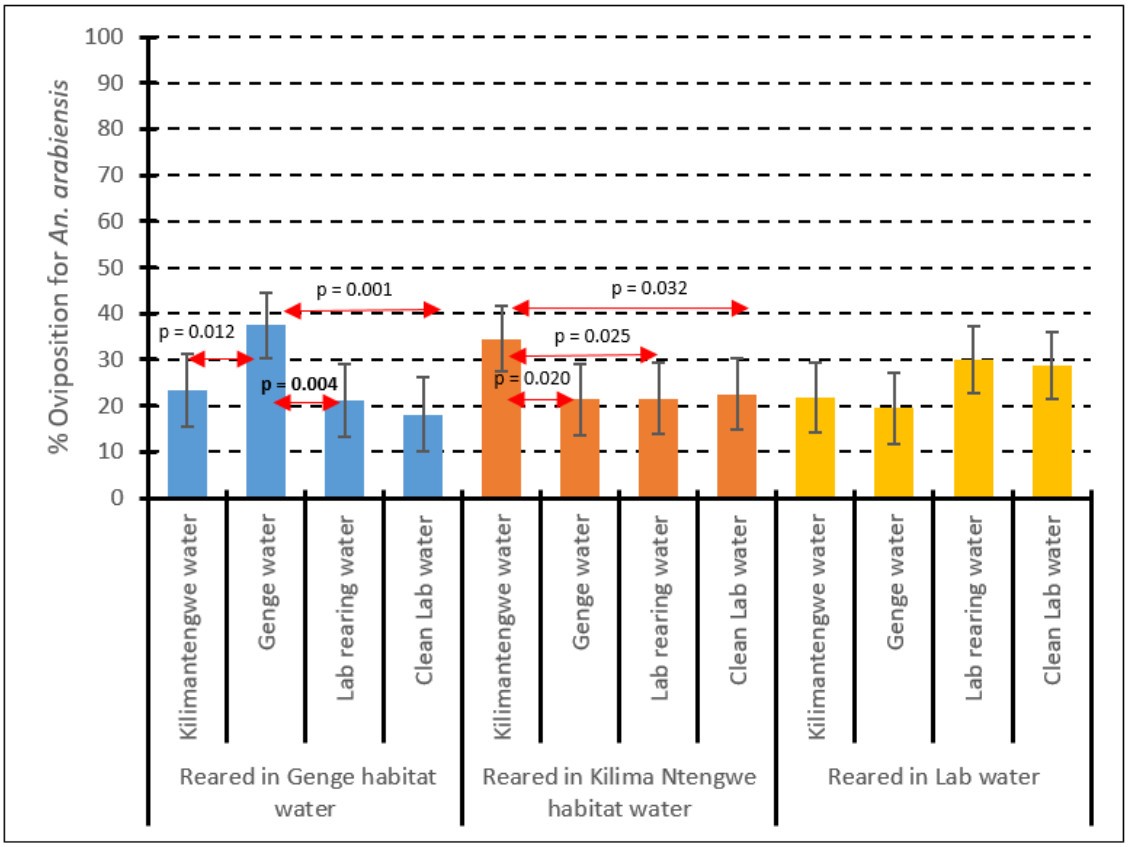

Figure 13. Oviposition preferences of gravid *Anopheles arabiensis* reared in Genge, Kilimantengwe, or laboratory water. Bars represent the proportion of eggs deposited in each oviposition substrate. Significant pairwise differences were determined using two-sample tests of proportions ($p < 0.05$)

Anopheles gambiae

The proportion of *Anopheles gambiae s.s.* reared in Kilimantengwe habitat water and going to oviposit in Kilimantengwe substrate water was statistically higher than those ovipositing in Genge substrate water (p-value = 0.018). Mosquitoes reared in Laboratory water, statistically preferred to oviposit in Laboratory rearing water or Clean laboratory water rather than Genge of Kilimantengwe substrate water ($p>0.02$) as shown in **Figure 14.**

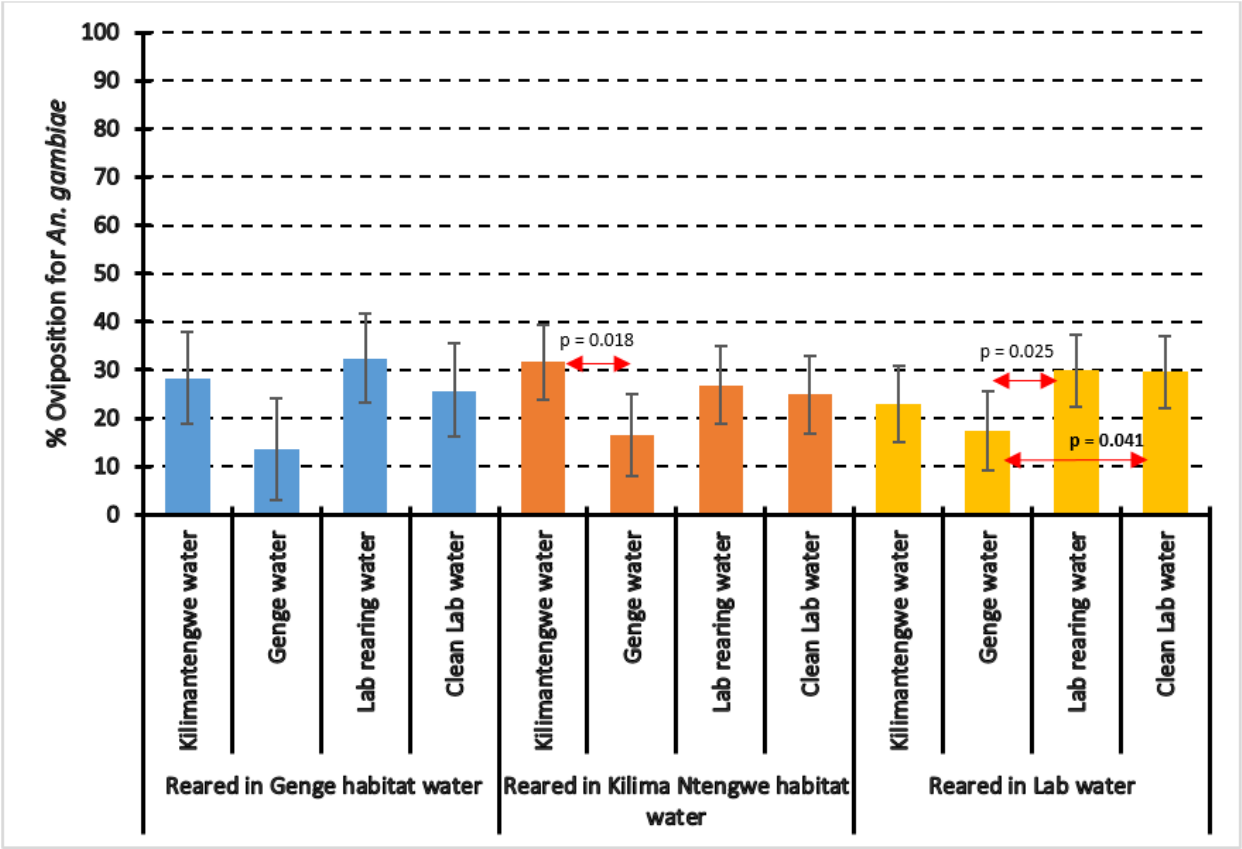

Figure 14. Oviposition preferences of gravid *Anopheles gambiae* s.s. reared in Genge, Kilimantengwe, or laboratory water under laboratory conditions. The figure shows the proportion of eggs deposited in each oviposition substrate. Significant differences among substrates were assessed using two-sample tests of proportions ($p < 0.05$)

Semi-field study (Mosquito spheres)

Normality testing

We tested if total eggs laid by *An. arabiensis* and *An. gambiae s.s.* were normally distributed across different substrates in the sphere (water sourced from Genge, Kilimantengwe and tap water). Shapiro-Wilk W test for normal data was used and indicated a significant departure from normality for both species; *An. arabiensis* (W = 0.955, p-value = 0.006) and *An. gambiae* (W = 0.959, p-value = 0.009). For this reason, this analysis employed non-parametric tests.

Testing for interaction effect

We used Scheirer-Ray-Hare Test (non-parametric alternative to two-way ANOVA) to test if the mosquitoes reared in Genge, Kilimantengwe and laboratory water prefer to oviposit in water substrates obtained from respective places. There was statistically significant interaction effect between the place where mosquitoes were reared and preferred place for egg laying in both species. (Table 1)

Table 2: Results of the Scheirer–Ray–Hare test evaluating the effects of larval rearing habitat, oviposition substrate, and their interaction on oviposition preferences of *Anopheles gambiae* s.s. and *Anopheles arabiensis* under semi-field conditions.

Source	df	H	Prob>chi2
Anopheles arabiensis (n = 242)			
Substrate used to rear larvae	2	7.589	0.023
Prefered substrate for oviposition	2	1.228	0.541
Substrate of rearing larvae # Prefered substrate for oviposition	4	9.207	0.056
Anopheles gambiae s.s. (n = 250)			
Substrate used to rear larvae	2	1.812	0.404
Prefered substrate for oviposition	2	0.130	0.937
Substrate used to rear larvae # Prefered substrate for oviposition	4	20.355	0.000

Oviposition preferences using Kruskal-Wallis test

Anopheles arabiensis

The Kruskal-Wallis test indicated a significant difference among substrates (chi-squired test = 10.748, p-value = 0.0046). Post hoc pairwise comparisons using Dunn's test with a Bonferroni correction showed that *Anopheles arabiensis* reared in Kilimantengwe water significantly preferred to oviposit their eggs in pots with Kilimantengwe water as compared to Genge and laboratory water. (Adjusted p-value = 0.0016). Other pairwise comparisons showed the same trend but was not statistically significance (**Figure 15**)

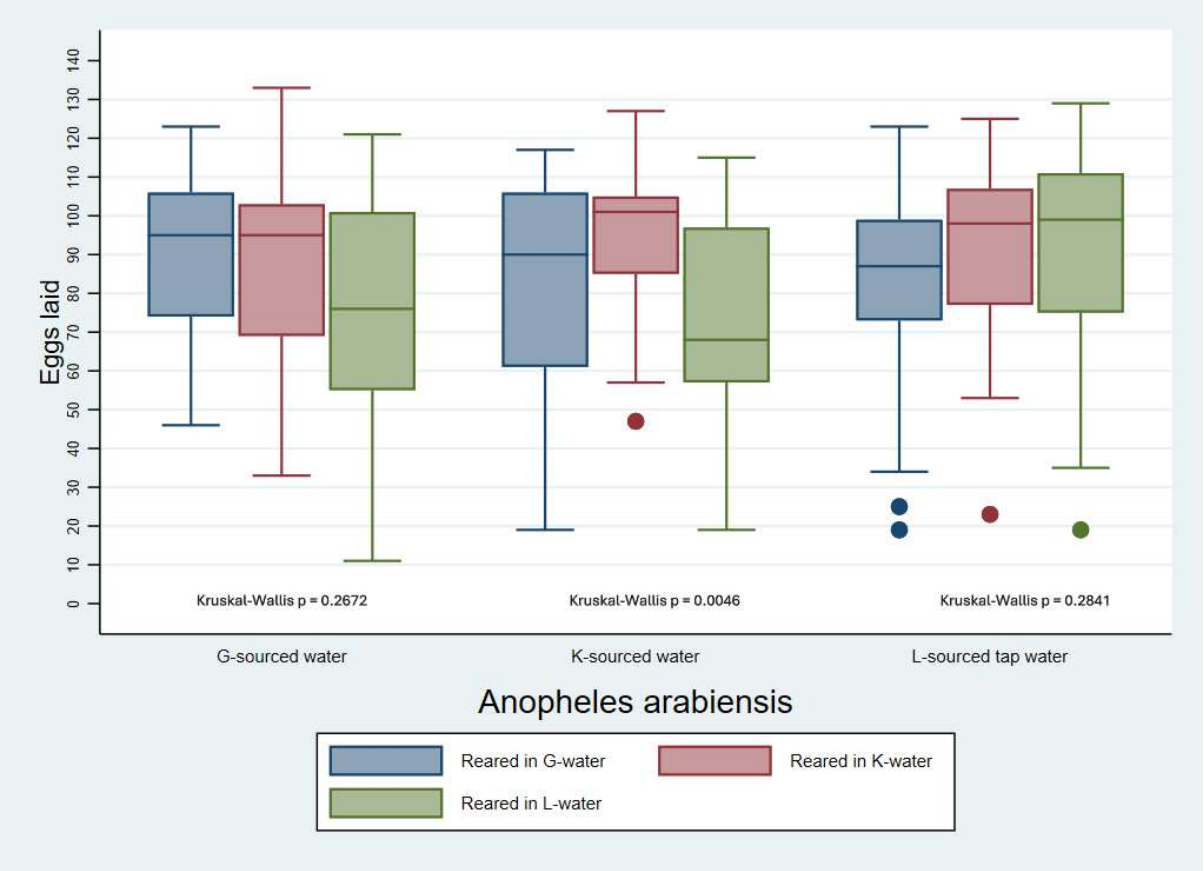

Figure 15. Oviposition preferences of gravid *Anopheles arabiensis* reared in Genge, Kilimantengwe, or laboratory water under semi-field (mosquito sphere) conditions. The figure shows the proportion of eggs deposited in Genge-, Kilimantengwe-, and laboratory-sourced water substrates. Differences among oviposition substrates were assessed using the Kruskal–Wallis test, followed by Dunn's post hoc test with Bonferroni correction for pairwise comparisons ($p < 0.05$).

Anopheles gambiae s.s.

For *Anopheles gambiae s.s.*, mosquitoes reared in laboratory water significantly preferred to oviposit their eggs in pots with laboratory water as compared to Genge and Kilimantengwe waters (Kruskal-Wallis test: $\chi^2 = 13.127$, p-value = 0.0014). The Dunn's test and Bonferroni post estimation tests also indicated a significant pairwise comparisons (p-value = 0.0017) as shown in **Figure 16**.

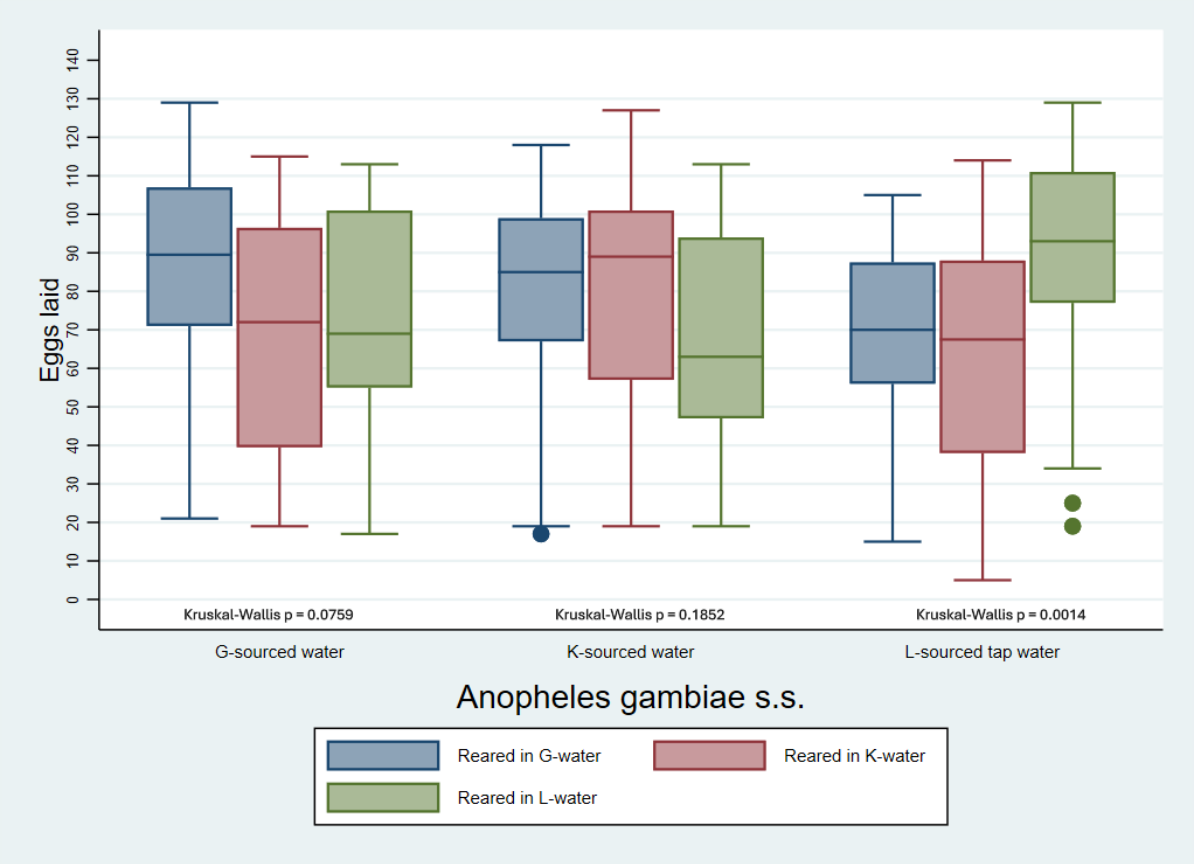

Figure 16. Oviposition preferences of gravid *Anopheles gambiae* s.s. reared in Genge, Kilimantengwe, or laboratory water under semi-field (mosquito sphere) conditions. The figure shows the proportion of eggs deposited in Genge-, Kilimantengwe-, and laboratory-sourced water substrates. Differences among oviposition substrates were assessed using the Kruskal–Wallis test, followed by Dunn's post hoc test with Bonferroni correction for pairwise comparisons ($p < 0.05$).

DISCUSION

The present study demonstrates that the larval aquatic environment can influence subsequent oviposition site selection by adult *Anopheles gambiae* s.s. and *Anopheles arabiensis*. Females reared in habitat water collected from Genge, Kilimantengwe, or the laboratory generally showed a greater tendency to oviposit in substrates associated with their natal larval habitat than in alternative substrates. These findings suggest that exposure to habitat-specific physicochemical and microbial cues during immature development influences adult oviposition behaviour

through pre-imaginal conditioning or associative learning. In insects, learning is defined as a relatively persistent change in behaviour resulting from experience, whereas memory refers to the acquisition and retention of information that modifies subsequent behavioral responses (Thorpe, 1963; Immelmann & Beer, 1989). Although the neural mechanisms responsible for this phenomenon in malaria vectors remain poorly understood, our findings indicate that experiences acquired during larval development may contribute to oviposition decisions in adulthood.

In the laboratory bioassays, both *An. gambiae s.s*. and *An. arabiensis* consistently preferred oviposition substrates corresponding to their larval rearing environment. This response was more pronounced in *An. gambiae s.s*, suggesting possible interspecific differences in sensitivity to habitat-associated olfactory cues. These differences may reflect species-specific ecological adaptations because *An. gambiae s.s*. is generally associated with smaller, more stable breeding sites than *An. arabiensis*, which occupies a broader range of aquatic habitats. Gravid Anopheles mosquitoes are known to rely primarily on volatile semiochemicals emitted from aquatic habitats when selecting oviposition sites, and several microbial-derived volatile organic compounds have been identified as important attractants or deterrents (Bentley & Day, 1989; Lindh et al., 2015; Wondwosen et al., 2018). The stronger attraction observed for Kilimantengwe water may therefore reflect differences in its physicochemical properties, microbial communities, and the composition of volatile compounds released from this habitat. Further chemical and microbiological characterization of the breeding sites would help identify the specific cues responsible for the observed behavioural responses.

The behavioural responses observed under laboratory conditions were less consistent in the mosquito sphere experiments. Although similar trends were evident, particularly for *An. gambiae s.s*., some comparisons were not statistically significant, especially for *An. arabiensis*. This reduced consistency is likely attributable to the greater environmental complexity of the semi-field system, where fluctuating temperature, humidity, airflow, and competing environmental odours may reduce the relative strength of habitat-derived olfactory cues. Semi-field systems nevertheless provide more realistic ecological conditions than laboratory assays and therefore remain valuable for assessing mosquito behaviour under conditions that better approximate natural environments.

Our findings are consistent with recent studies demonstrating that environmental cues encountered during the aquatic stage can influence subsequent oviposition behaviour in malaria mosquitoes. Gravid Anopheles females integrate chemical signals associated with larval habitats, including odours produced by conspecific larvae and aquatic microorganisms, when selecting oviposition sites, indicating that habitat-associated olfactory cues contribute to

reproductive decision-making (Schoelitsz et al., 2020; Takken et al., 2024). Similarly, McCall and Eaton (2001) demonstrated that *Culex quinquefasciatus* reared in water containing skatole subsequently preferred this normally repellent compound during oviposition, indicating that larval exposure altered adult odour preference. Behavioural plasticity and learning have also been demonstrated in mosquitoes, including *Aedes aegypti*, where previous environmental experience and sublethal exposure to chemical stimuli can modify subsequent behavioural responses, including oviposition and avoidance behaviour (Sougoufara et al., 2022; Mulatier et al., 2022). Collectively, these studies and the present findings support the hypothesis that early-life exposure to environmental cues can modify adult behavioural responses across several mosquito genera.

The present study was specifically designed to evaluate the role of olfactory cues in oviposition site selection. The modified double-paper-cup technique prevented gravid females from contacting or visually assessing the test substrates, thereby restricting behavioural responses primarily to volatile compounds diffusing from the habitat water. This design minimized potential confounding effects of tactile and visual stimuli and therefore provided a robust approach for investigating odour-mediated oviposition behaviour. Similar approaches have been successfully employed in previous studies investigating mosquito oviposition responses to habitat-associated odours (Lindh et al., 2015; Dugassa et al., 2013).

The mechanisms underlying retention of larval habitat information remain uncertain. Several hypotheses have been proposed, including persistent chemical exposure during development (the "chemical legacy" hypothesis), changes in sensory sensitivity, habituation, or neural plasticity retained through metamorphosis (Corbet, 1985; Anderson, 2016). Evidence from Drosophila demonstrates that odour-associated memories acquired during larval stages can persist into adulthood, suggesting that complete metamorphosis does not necessarily erase all forms of learned information. Whether similar neural mechanisms operate in malaria vectors remains an important question for future neurophysiological and molecular investigations.

Understanding how larval experience influences oviposition behaviour has important implications for malaria vector surveillance and control. If gravid females preferentially respond to odours associated with their natal habitats, these habitat-specific volatile blends could be exploited to improve gravid traps, oviposition surveillance tools, and attract-and-kill strategies. Such approaches have already shown promise through the identification of oviposition attractants for malaria vectors and may contribute to more targeted vector monitoring and integrated vector management programmes (Lindh et al., 2015; Wondwosen et al., 2018). Further studies combining behavioural assays with chemical ecology and microbial profiling of

breeding habitats are needed to identify the specific semiochemicals responsible for the observed oviposition preferences and to determine their potential application in malaria vector control.

Conclusion

Laboratory populations of *Anopheles gambiae s.s*. and *Anopheles arabiensis* preferentially oviposited in substrates with odour profiles similar to those experienced during their larval development, indicating that natal habitat odours can influence adult oviposition site selection. Although the preference was moderate rather than absolute, its consistency across both species suggests that larval exposure may induce persistent physiological or behavioural changes, possibly through altered olfactory sensitivity or learning processes retained through metamorphosis. To our knowledge, this study provides one of the first experimental demonstrations that natural breeding-site odours can influence oviposition site selection through natal habitat experience in Anopheles mosquitoes while minimizing the influence of contact, visual, and other non-olfactory cues. These findings further suggest that differences in larval rearing environments may contribute to variation in behavioural responses observed among laboratory mosquito colonies. From an applied perspective, identification and characterization of habitat-specific volatile semiochemicals may facilitate the development of more effective gravid traps and attract-and-kill strategies for malaria vector surveillance and integrated vector management.

Suggestion for further work

Further studies are needed to determine whether the observed differences in oviposition preferences between *Anopheles gambiae* s.s. and *Anopheles arabiensis* are attributable to differences in olfactory sensitivity, associative learning, or other forms of behavioural plasticity. Future research should also identify the developmental stage at which habitat-associated information is acquired, whether during specific larval instars, the pupal stage, or shortly after adult emergence. In addition, future studies should combine behavioural assays with chemical and microbial analyses to identify the compounds responsible for the observed oviposition preferences and evaluate their performance under natural field conditions for potential application in odour-based malaria vector surveillance and control.

Declarations

Ethical clearance

Ethical approval for this study was obtained from the Medical Research Coordinating Committee (MRCC) of the National Institute for Medical Research (NIMR), Tanzania (Approval No.

NIMR/HQ/R.8a/Vol.IX/1584). All experimental procedures involving mosquitoes and rabbits were conducted in accordance with the approved protocol and the institutional guidelines for the care and use of laboratory animals.

*Consent for publication***:** Not applicable.

Availability of data and material: All data generated or analysed during this study are included in this published article. Additional datasets are available from the corresponding author upon reasonable request.

*Competing interests***:** The authors declare that they have no competing interests.

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Authors' contributions

EEK: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing – original draft.

MS: Methodology, Supervision, Writing – review & editing.

ILS: Investigation, Supervision, Writing – review & editing.

BA: Investigation, Supervision, Writing – review & editing.

DAH: Investigation, Writing – review & editing.

AM: Investigation, Writing – review & editing.

NA: Investigation, Writing – review & editing.

JT: Investigation, Writing – review & editing.

RB: Investigation, Writing – review & editing.

JTM: Methodology, Supervision, Writing – review & editing.

FL: Methodology, Supervision, Writing – review & editing.

SOL: Methodology, Supervision, Writing – review & editing.

TD: Conceptualization, Methodology, Supervision, Resources, Writing – review & editing.

All authors read and approved the final manuscript.

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