USAID *Okoa Maisha Dhibiti Malaria* (OMDM) Activity

Annual Entomological Monitoring Report FY22
ZANZIBAR MALARIA ELIMINATION PROGRAMME

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USAID *Okoa Maisha Dhibiti Malaria* (OMDM)/Save Lives, End Malaria

Annual Entomological Monitoring Report
Zanzibar Malaria Elimination Programme

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List of Abbreviations

BB	blocking buffer
CS	circumsporozoite
CDC	Centers for Disease Control and Prevention
CSP	circumsporozoite protein
DNA	deoxyribonucleic acid
EIR	entomological inoculation rate
ELISA	Enzyme-linked immunosorbent assay
HLC	human landing catch
HBR	human biting rate
IRS	indoor residual spraying
ITN	insecticide-treated net
IDR	indoor resting density
kdr	knockdown resistance
LLIN	long-lasting insecticidal net
mAb	monoclonal antibody
OBR	Outdoor biting rate
PBO	piperonyl butoxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PSC	pyrethrum spray catch
USAID	United States Agency for International Development
WHO	World Health Organization
ZAMEP	Zanzibar Malaria Elimination Programme

1. Introduction

For the past 10 years, malaria in Zanzibar has remained stable, with a parasite prevalence of less than 1%. The last survey reported a further reduction in malaria prevalence to 0.2% in children under five years of age, with considerable variation between the two islands (0.4% in Unguja and 0.0% in Pemba; Tanzania Malaria Indicator Survey, 2017).[1] Malaria transmission in Zanzibar is bimodal with two peaks following rainfall patterns, heavy rain (Masika) in March–May and short rain (Vuli) in October –December.

In 2021–2022, Zanzibar Malaria Elimination Programme (ZAMEP) reported 4,315 confirmed malaria cases with an annual incidence of 2.9 cases/1,000 population compared with 9,290 cases of 2020–2021 equal to annual incidence of 5.3 cases/1,000 population.

Insecticide-treated nets (ITNs) and indoor residual spraying (IRS) are the frontline malaria vector interventions in Zanzibar. In 2021-2022, ZAMEP shifted to using reactive focal IRS after entomological investigation using clothianidin and Fludora® Fusion, whereby 21,470 (98%) of the targeted structures were sprayed protecting 98% of the population (n = 130,016) in 63 active malaria foci. IRS rapid response has shown the same value with blanket/target spraying in reducing malaria morbidity and mortality. However, it has a higher compliance rate compared with target and blanket IRS.

The recent and historical ZAMEP reports in Zanzibar show that widespread use of ITNs or IRS changes the species composition of residual vector populations by progressively diminishing densities of each species in proportion. For example, in 2005, before the intensive application of insecticide interventions, the entomological surveillance documented *Anopheles gambiae s.s.* as the predominant malaria vector with anthropophagic, endophagic, and endophilic behavior. However, the current report (2021–2022) shows that *An. arabiensis* is a dominant malaria vector that exhibits exophagic behavior. This change in vector predominance requires ZAMEP to implement complementary interventions to fight against outdoor biting vectors.

Pyrethroid resistance of *Anopheles gambiae* s.l. was described in 2010 in Pemba for the first time. However, two years later, Unguja reported the same situation as in Pemba. The strength of resistance varies across the sentinel sites, being higher in Pemba than in Unguja. The phenotypic data indicate that the pyrethroid resistance is likely because of increases in the rate of insecticide metabolism. The cytochrome P450 is the most likely candidate for conferring this resistance. The cause of resistance remained unclear. However, high long-lasting insecticidal nets (LLINs) coverage and insecticides used in agriculture and livestock within the population are suspected to be correlated to the rise in resistance.

Insecticide resistance poses challenges in insecticide-based malaria vector control interventions in many areas in sub-Saharan Africa, where pyrethroid resistance is documented. Therefore, it is crucial to understand the local malaria vector species, their behavior, disease incrimination, susceptibility to insecticides, and residual effectiveness of insecticide in treated surfaces for planning effective malaria control interventions.

[1] Ministry of Health, Community Development, Gender, Elderly and Children (MoHCDGEC) [Tanzania Mainland], Ministry of Health (MoH) [Zanzibar], National Bureau of Statistics (NBS), Office of the Chief Government Statistician (OCGS), and ICF. (2017). Tanzania Malaria Indicator Survey 2017. Dar es Salaam, Tanzania, and Rockville, Maryland, USA. https://dhsprogram.com/pubs/pdf/MIS31/MIS31.pdf

1.1 Objectives of the entomological surveillance

- Assess the quality of IRS operations and insecticide decay rate
- Determine insecticide susceptibility of malaria vectors to insecticide, intensity and mechanisms of resistance
- Assess malaria vector density, species, behavior (feeding and resting), seasonality, distribution, infection rate, and entomological inoculation rate (EIR).

2. Methodology

2.1 Sentinel sites

Entomological surveillance at sentinel sites was conducted from October 2021 to September 2022, whereas Fludora Fusion IRS quality assessment and insecticide residual efficacy studies were carried out from August 2021 to March/April 2022. Ten entomological sentinel sites are used for entomological monitoring. These sites are located in 10 districts (i.e., one site per district): six sites in Unguja; and four in Pemba (Table 1). The selection of entomological sentinel sites considered the following criteria:

- Disease incidence/prevalence
- Topography of the area
- Agricultural practices (rain fed rice, irrigation etc.)
- Urban or rural setting

Table 1. Sentinel sites in Zanzibar

Location	District	Sentinel site	Geo coordinates	Focal IRS status in 2022	Entomological parameters collected
Unguja	Mjini	Stone Town	Longitude 39.1936844 Latitude −6. 1583878	Not sprayed	Vector abundance, species distribution, seasonality, feeding time and location, and infection rate
	Kaskazini B	Bumbwini	Longitude 39.2229625 Latitude −6.1775842	Sprayed	Vector abundance, species distribution, seasonality, feeding time and location, infection rate, and insecticide decaying rate
	Kaskazini A	Donge	Longitude 39.239423 Latitude −5. 9305385	Sprayed	Vector abundance, species distribution, seasonality, feeding time and location, and infection rate
	Magharibi	Mwera	Longitude 39.4623959 Latitude −6. 3722427	sprayed	Vector abundance, species distribution, seasonality, feeding time and location, and infection rate
	Kusini	Muyuni	Longitude 39.2231848 Latitude −6. 1776179	Sprayed	Vector abundance, species distribution, seasonality, feeding time and location, and infection rate
	Kati	Cheju	Longitude 39. 3730012	Sprayed	Vector abundance, species distribution, seasonality,

Location	District	Sentinel site	Geo coordinates	Focal IRS status in 2022	Entomological parameters collected
			Latitude −6. 2223636		feeding time and location, and infection rate
Pemba	Micheweni	Tumbe	Longitude 39.7895961 Latitude −4.9473016	Sprayed	Vector abundance, species distribution, seasonality, feeding time and location, and infection rate
	Wete	Bopwe	Longitude 39.725216 Latitude −5.0487204	Sprayed	Vector abundance, species distribution, seasonality, feeding time and location, and infection rate
	Chake	Uwandani	Longitude 39.8243398 Latitude −5.1959127	Sprayed	Vector abundance, species distribution, seasonality, feeding time and location, and infection rate
	Mkoani	Wambaa	Longitude 39.7270355 Latitude −5.4011374	Sprayed	Vector abundance, species distribution, seasonality, feeding time and location, infection rate, and insecticide decaying rate

2.2 IRS quality assessment and insecticide decaying rate sites

ZAMEP, conducted a reactive focal IRS response using Fludora Fusion in early August 2021 in two selected villages in Pemba and one in Unguja after data showed an abnormal increase in local malaria cases. The operation covered over 98% of the targeted structures. Fludora Fusion is a combination of clothianidin and deltamethrin used at a dosage of 300 mg Ai/m^2. ZAMEP used Fludora Fusion for the first time in few areas to understand its residual efficacy and the killing power against susceptible malaria vectors (Table 2). Clothianidin is a slow-acting insecticide formulation. Hence, the usual World Health Organization (WHO) protocol for cone bioassays allows mosquito mortality observation after every 24 hours for one week. Deltamethrin increases the knock down to the vectors.

Table 2. Sites for Fludora Fusion residual efficacy in Zanzibar

Location	District	Shehia
Pemba	Wete	Kiungoni
	Micheweni	Mjini Wingwi
Unguja	Kusini	Kizimkazi

2.3 Vector bionomics

The objective of entomological monitoring was to assess the impact of IRS/ITNs at sentinel sites regarding potential mosquito parameters for malaria transmission. These include vector abundance, species, seasonality, density, infection rate, feeding and resting behavior and susceptibility of vectors to insecticides. Methods used for vector sampling were pyrethrum

spray catches (PSCs), human landing catches (HLC), Centers for Disease Control and Prevention (CDC) light traps and pit traps (Table 3).

2.4 Methods used for vector sampling at sentinel sites

Table 3. Vector sampling methods

Method	Purpose	Sentinel site	No. of households	Days/month	Time	Sample processing
HLC	Indoor and outdoor biting behavior	10	2 house/ site	2 days/site	1800-0600	Species, sporozoite rate
PSC/Proko pack	Indoor resting behavior	10	5 houses/site	2 days/site	0600-0800	Species, sporozoite rate, Human blood index
CDC light trap	Indoor abundance	10	2 houses/site	2 days/site	1800-0600	Species, sporozoite rate
Pit trap	Outdoor resting behavior	10	2 pits/site	2 days/site	0600-0800	Species, sporozoite rate, Human blood index

2.4.1 HLC

HLC were conducted between 18.00–06.00 hours outdoors and indoors twice per month at each site. Catches from the collections were kept in paper cup/hour/collector. Two staff collected mosquitoes outdoors and the other two collected samples indoor in two houses per site. Mosquitoes were then kept in a cool box until sorted, counted, and recorded in the following morning.

2.4.2 PSCs

PSCs were carried out in five houses during each mosquito collection morning twice per month. White sheets were laid on the entire floor and over the furniture within one room where people slept the previous night in each selected house. White sheets facilitate visibility of the knocked down mosquitoes. The doors and windows of the houses were shut, then the rooms were sprayed with pyrethrum (0.3%) synergized with piperonyl butoxide (PBO) as described by Gimnig et al. (2003)[2]. Briefly, a collector outside the house sprayed around the eaves with insecticide to prevent the mosquitoes inside the houses from escaping and another collector sprayed the roofs and the walls inside the house. The houses were then closed for 10–15 minutes. Collectors removed the white sheets from all the rooms of the houses and collected the knocked down mosquitoes using forceps. Knocked down mosquitoes for each room were recorded and then transferred onto moist filter paper inside labelled petri dishes indicating the date and house number. The same procedure was repeated for all the five houses and collected mosquitoes were put in a cool box and transported to the laboratory for further processing.

[2] Gimnig, J.E., Vulule, J.M., Lo, T.Q., Kamau, L., Kolczak, M.S., Phillips-Howard, P.A., Mathenge, E.M., ter Kuile, F.O., Nahlen, B.L., Hightower, A.W., & Hawley, W.A. (2003). Impact of permethrin-treated bed nets on entomologic indices in an area of intense year-round malaria transmission. *American Journal of Tropical Medicine and Hygiene*, *68*,16–22.

2.4.3 Pit trap collection

A rectangular pit was dug in the ground (1.5 m in depth, 1.2 m in length, and 1 m in width) within 10 m of each selected residential house. In each of the four vertical sides, about 50–60 cm and 90–100 cm from the bottom of the pit, 5-8 little cavities were dug into a depth of about 30 cm. The main pits were then shaded by an artificial framework thatched with locally available coconut palms on top to provide shade. Resting mosquitoes were sampled from 6 am to 9 am inside the cavities by using hand-held mouth aspirators or Prokopack. Collected mosquito samples were kept in paper cups before being processed accordingly in the laboratory. The collection was done twice per month at each site.

2.4.4 CDC light trap

Indoor host-seeking mosquitoes were collected using CDC light traps (John W. Hock Ltd, Gainesville, FL., USA). In every mosquito trapping night, mosquitoes were collected from two randomly selected houses from a single collection site. In each house, light traps were hung a meter off the ground and approximately 150 cm from an occupied mosquito net. A single trap was set per room and a total of two traps were set each sampling night/house. These traps were positioned near the sleeper's head. Light traps were switched on at 6 pm and switched off at 6 am the next morning. Mosquitoes were then collected in the morning. Live mosquitoes were aspirated into clearly labelled paper cups and transported to the laboratory for further processing.

2.5 Morphological identification of mosquitoes

The ZAMEP team conducted morphological identification using the taxonomic keys of Gillies and Coetzee (1987)[3] at laboratory once the samples arrived from field. Based on morphological characteristics, *Anopheles* mosquitoes were sorted to the generally known species such as *An. gambiae* s.l., *An. funestus, An. coustani, An. pharoensis*, etc.

2.6 Detection of sporozoites in mosquitoes by CSP-Enzyme-linked immunosorbent assays (ELISA) technique

ELISAs were developed to detect *Plasmodium falciparum*, *P. vivax*-210, and *P. vivax*-247 circumsporozoite proteins (CSP) in malaria-infected mosquitoes. The sensitivity and specificity of the ELISAs are based on the monoclonal antibodies (mAbs) used. ELISAs detect circumsporozoite (CS) proteins, which can be present in the developing oocysts, dissolved in hemolymph, and on sporozoites present in the hemocoel or in the salivary glands.

The ELISA assays were carried out using dried mosquitoes. The specimens were processed and preserved in Eppendorf tubes with silica gel to keep them dry. The specimens were ground using grinding solution containing blocking buffer (BB) and Igepal CA-630, combination was mixed properly so that the Igepal to dissolve in BB.

2.7 Mosquito grinding

Each mosquito sample was ground separately (one mosquito per well), only the head–thorax part was placed in a labelled 1.5-ml microcentrifuge grinding tube. Grinding buffer was then added to facilitate grinding. The pestles were rinsed with grinding solution to make sure that the rinses were held in the tube containing mosquito triturate.

[3] Gillies, M.T., & Coetzee, M. (1987). A supplement to the Anophelinae of Africa south of the Sahara (Afrotropical Region). Publications of the South African Institute for Medical Research, 55, 1–143.

The “sandwich” begun by absorption of the capture monoclonal antibodies (mAbs) to the wells of a microplate. After capturing the mAbs bound to the plate, the well contents were aspirated, and the remaining binding sites were blocked with BB containing Igepal CA-630 and an aliquot was tested. Positive and negative controls were also added to specific well at this time. If CS antigen is present, it will form AgAb complex with the capture mAbs. After a 2-hour incubation at room temperature, the mosquito triturate was aspirated, and the wells were washed. Peroxidase-linked mAbs was then added to the wells, completing the formation of the “sandwich.” After 1 hour, the well contents were aspirated, the plate was washed again, and the clear peroxidase substrate solution was added. When the peroxidase enzyme reacts with the substrate, a dark green product is formed; the intensity of the color is proportional to the amount of CS antigen present in the test sample.

2.8 Detection of bloodmeal sources by ELISA

The bloodmeal origins of freshly fed Anopheline mosquitoes collected inside houses were determined using a direct ELISA following the method of Beier et al (1988)[4] using human and bovine antibodies. Each mosquito abdomen was crushed in 50-µl phosphate buffered saline (PBS) solution (pH 7.4), which was further diluted by adding 950 µl PBS. Fifty microliters of sample were added to each well in a 96-well microtiter plate and incubated overnight at room temperature. Each well was washed twice with PBS containing Tween-20 solution, and 50 µl host specific conjugate (either human or bovine) was added to each well and incubated for one hour. After one hour, each well was washed three times with a PBS–Tween-20 solution. Finally, 100 µl of peroxidase substrate was added to each well and after 30 minutes the absorbency at 405 nm was recorded with an ELISA plate reader. Each bloodmeal sample was considered positive if the absorbance value exceeded the mean plus three times the standard deviation of the four negative controls (from a laboratory colony of *An. gambiae* Kisumu strain adults not fed with blood). Positive controls contained human and bovine blood.

2.9 Quality assurance of IRS program and insecticide decay rate

The team conducted quality assurance of IRS program of Fludora Fusion on the first seven days of the operation to assess the spray quality and sprayer performance. It was then followed by monitoring the insecticide decay rate every month for eight months. Three shehias were surveilled in both Unguja and Pemba. The shehias were purposely selected per district. The exercise involved 15 houses per shehias built up of different wall surfaces such as mud, cement, oil and water paint, and stone block. The team tested three houses per shehia each made from a different wall surface type.

2.10 Test procedures for assessment of IRS quality and insecticide decay rate

- Bioassays were conducted in accordance with WHO (2016)[5] guidelines. A susceptible colony of laboratory-reared *An. gambiae* s.s. (R-70 strain) was used for the cone bioassays on the different wall surface types.

[4] Beier, J.C., Perkins, P.V., Wirtz, J.A., Koros, J., Diggs, D., Gargan II, T.P., & Koech, J.C. (1988). Bloodmeal Identification by Direct Enzyme-Linked Immunosorbent Assay (Elisa), Tested on Anopheles (Diptera: Culicidae) in Kenya, Journal of Medical Entomology, *25*, 9–16. https://doi.org/10.1093/jmedent/25.1.9

[5] WHO (2016) Test Procedures for Insecticide Resistance Monitoring in Malaria Vector Mosquitoes, 2nd Edition. World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland

- Two to five-day-old, non-blood fed females of *An. gambiae* s.s. R-70 from the ZAMEP insectary were exposed to sprayed wall surfaces in batches of 10 mosquitoes for 30 minutes under WHO cones.
- Cones were attached to walls at two different heights in each of the two rooms sampled in a house: at a lower level (1.0 m high) and an upper level (1.5 m high).
- At the end of the test, mosquitoes were transferred to paper cups and supplied with cotton pads soaked with sugar solution.
- First knockdown was scored after 30 minutes followed by 60 minutes post-exposure; the mortality was scored and recorded after 24 hours holding period for seven days. Moribund and dead mosquitoes were counted as dead.
- Negative control bioassays were conducted on unsprayed surfaces covered with manila sheet layers. When control mortality was scored between 5% and 20%, experimental mortality was corrected using Abbott's formula [6]. If the mortality score was more than 20%, the experiment was discarded.

2.11 Insecticide susceptibility test

The entomology team assessed the susceptibility of local vectors against common insecticides. Thus, guiding the IRS campaign on insecticide to be applied. The test was carried out across the sentinel sites in 10 districts (6 in Unguja and 4 in Pemba). The tests used WHO discrimination doses of permethrin (0.75%), deltamethrin (0.05%) and bendiocarb (0.01%) alpha-cypermethrin 0.05% clothianidin 2% and pirimiphos-methyl 0.25%.

The strength/intensity of pyrethroid resistance against malaria vectors was determined at concentrations of 5x–10x of alpha-cypermethrin, deltamethrin, permethrin, and in WHO impregnated paper.

Mosquito collection

Wild *Anopheles* larvae and pupae were collected in various breeding sites from established entomological surveillance sites ranging from home yards, cultivated land, and rice fields. Therefore, mosquitoes obtained could be representative of the vectors available in the areas. Mosquito larvae were collected with a 350-ml dipper and transferred into plastic containers, which were then loosely capped to allow aeration. These were transported in cool boxes to the ZAMEP insectary in both Unguja and Pemba where they were reared at 27–30°C. Larvae collected from several breeding sites in the same village were pooled together for rearing and testing. The larvae were fed with Tetramin® fish food. The development of the larvae was monitored regularly, and all those that pupated were transferred into shallow plastic cups/small beakers using Pasteur pipettes, and then placed in appropriately labelled cages for adult emergence. Female adult mosquitoes aged 2–5 days were used for WHO susceptibility tests and PBO synergy testing.

Insecticide resistance test procedures

The susceptibility tests were carried out using the WHO test kits for adult mosquitoes [7]. Three to five-day old female F1 generation mosquitoes were tested using standard WHO

[6] Abbott, W.S. (1925). A method of computing the effectiveness of an insecticide. Journal of Economic Entomology, 18, 265-267.

[7] WHO (2016) Test Procedures for Insecticide Resistance Monitoring in Malaria Vector Mosquitoes, 2nd Edition. World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland

insecticide susceptibility procedures with four replicates of 25 wild adult female mosquitoes per test tube. Mosquitoes were exposed to papers impregnated with the WHO-recommended discriminating concentrations of deltamethrin (0.05%), bendiocarb (0.1%), permethrin (0.75%) alpha-cypermethrin (0.05%), pirimiphos-methyl (0.25%), and clothianidin (2%).

At the end of the exposure period, mosquitoes were transferred into holding tubes (lined with untreated papers) by gently blowing them through the open space between the exposure tube and the holding tubes. Cotton soaked in 10% sugar was placed on top of the holding tube as a food source to avoid death by starvation. The mortality was scored 24 hours post-exposure except for clothianidin, whereby the score and record continued for seven days. The susceptibility status was evaluated based on the WHO criteria: 98–100% mortality indicate susceptibility; 90–97% mortality required confirmation, and less than 90% mortality indicate resistance (WHO, 2016 & 2020). When the control mortality between 5% and 20% was recorded, the mean observed mortality was corrected using Abbott's formula (Abbott, 1925). All tested mosquitoes were preserved with silica gel in 1.5-ml Eppendorf tubes and transported to ZAMEP's molecular laboratory for further laboratory analyses (molecular species identification and detection of mechanisms of insecticide resistance).

We determined the strength/intensity of pyrethroid resistance against malaria vectors at three concentrations of 5x deltamethrin (0.25%), alpha-cypermethrin (0.25%), and permethrin (3.75%) in WHO impregnated paper. For the 10x, we used three concentrations, deltamethrin (0.5%), alpha-cypermethrin (0.5%), and permethrin (7.5%)

PBO – Synergist Bioassays

The synergist bioassays is used to determine the amount of mixed function oxidases in the observed phenotypic resistance. In this test, 3–5-day-old F1 adult mosquitoes were pre-exposed to 4% PBO paper for 1 hour and immediately exposed to 0.75% permethrin, 0.05% deltamethrin and 0.05% alpha-cypermethrin for 1 hour. Two controls were used during this experiment: control 1 constituted mosquitoes exposed to clean papers without insecticides or PBO; and control 2 constituted mosquitoes exposed to papers treated with PBO only. Mortalities were later assessed after exposure; the PBO synergized group was compared with the un-synergized group 24 hours after exposure. This comparison was used to evaluate the potential role of the monooxygenase enzyme in the observed resistance.

2.12 Molecular identification of *An. gambiae s.l.*

Anopheles gambiae sibling species identification was carried out according to the standard polymerase chain reaction (PCR) method [8]. Five oligonucleotide primers, GA, ME, AR, QD, and UN designed from the deoxyribonucleic acid (DNA) sequences of the intergenic spacer region of complex ribosomal DNA (rDNA) were used to amplify species-specific DNA sequences. The UN-primer is universal and anneals to the same position on the rDNA sequences of all five species, the GA anneals specifically to *An. gambiae s.s*., the ME anneals to either *An. merus* or *An. melas*, AR to *An. arabiensis* and the QD to *An. quadriannulatus.* The PCR reaction mix of 25 µl contained 1X PCR buffer (constituents), 200 µM of each of the deoxyribonucleotide triphosphates (dNTPs), 20 µM of oligonucleotide primers, 0.125 units of Taq polymerase enzyme and 0.5 µl of the extracted genomic DNA. Sterile double distilled water was added to make up the volume to 25 µl. The reaction mix

[8] Scott JA, Brogdon WG, Collins FH, 1993. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. *Am J Trop Med Hyg* 49: 520-529.

was spun down briefly at 14,000 rpm and overlaid with mineral oil to avoid evaporation and refluxing during thermo-cycling.

The amplification reactions were carried out using PTC 100 thermal cycler and the cycling parameters were as follows: 3 minutes at 94ºC (initial denaturation), followed by 35 cycles with denaturation at 94ºC for 30 seconds, annealing at 50ºC for 30 seconds and extension at 72ºC for 60 seconds and ended, with a final cycle at 94ºC for 30 seconds, annealing at 50ºC for 30s and extension at 72ºC for 10 minutes. For each reaction, a positive control containing 0.5 µl of PCR products of *An. gambiae s.s.* as template DNA and a negative control that contained no DNA template were included.

The amplified products were analyzed by agarose gel electrophoresis. Next, 10 µl of each PCR product were added to 1 µl of 10x Orange-G loading dye and electrophoresed in 2% agarose gel stained with 0.5 µg/ml of ethidium bromide. The electrophoresis was run in 1x tris acetate-EDTA buffer at 100V for one hour and were visualized and photographed over a UVP dual intensity trans-illuminator at short wavelength using a digital camera fitted with an orange filter and a hood. The amplified PCR product was identified to the sibling species based on the diagnostic band size determined by comparison with the mobility of a standard 100 bp DNA ladder.

2.13 Detection of target site resistance mechanisms

The PCR-based standard protocol used for the detection of the L1014S or L1014F knockdown resistance (*kdr*) alleles was adapted from the protocols developed [9,10] . All wild-caught *An. gambiae* s.l. from each of the above-mentioned sentinel sites were tested for KDR mutation.

Amplification was performed in a 25 µl reaction containing 2 µl of template DNA, GoTaq 5x PCR Buffer (containing 15 mM MgCl2), MgCl2 (25mM), dNTP (2-2.5 mM mix), dNTP (2-2.5 mM, and 1.5 U/µl of Go-Taq DNA polymerase, 2.5 pmol/µl for both IPCF and IPCR, 2.5 pmol/µl East primer and 8.8 pmol/µl for West primer.

The *kdr* genotyping of susceptible and resistant individuals was possible after amplifying the DNA template from mosquitoes following the PCR conditions of 95°C for 5 minutes (initial denaturation), followed by 35 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. For the East Africa *kdr* amplification, there was a final extension cycle of 72°C for 5 minutes followed by 4°C cooling. For West African *kdr* amplification, the PCR conditions involved an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds. There was a final extension cycle of 72°C for 5 minutes followed by cooling at 4°C. The products were electrophoresed through 2% agarose gel with ethidium bromide stain and visualized under UV light. Next, *kdr* genotypes of both the susceptible and resistant individuals were then recorded. All successful reactions should contain a band of 285 bp. In addition, a band of 210 bp indicates the susceptible (wildtype) allele and one of 188 bp the resistant allele.

[9] Martinez-Torres D., F. Chandre, M. S. Williamson, F. Darriet, J. B. Berge´, A. L. Devonshire, P. Guichet, N. Pasteur, and D. Pauron. 1998. Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector Anopheles gambiae s.s. Insect Mol. Biol. 7: 179 Ð184.

[10] Ranson, H., B. Jensen, J. Vulule, X.Wang, J. Hemingway, and F. Collins. 2000. IdentiÞcation of a point mutation in the voltage-gated sodium channel gene of Kenyan Anopheles gambiae associated with resistance to DDT and pyrethroids. Insect Mol. Biol. 9: 491Ð 497.

3. Results

3.1 The residual efficacy of Fludora Fusion against susceptible *Anopheles gambiae s.s.* R70 in WHO cone wall bioassays

The results indicate that the Fludora Fusion is a promising insecticide for malaria control because of quick knockdown, high toxicity to the vectors, and long residual effect in treated walls. The insecticide is potential for future use in ZAMEP (Figures 1 and 2).

Figure 1. Residual efficacy of Fludora Fusion in two selected villages in Pemba, 2021–2022

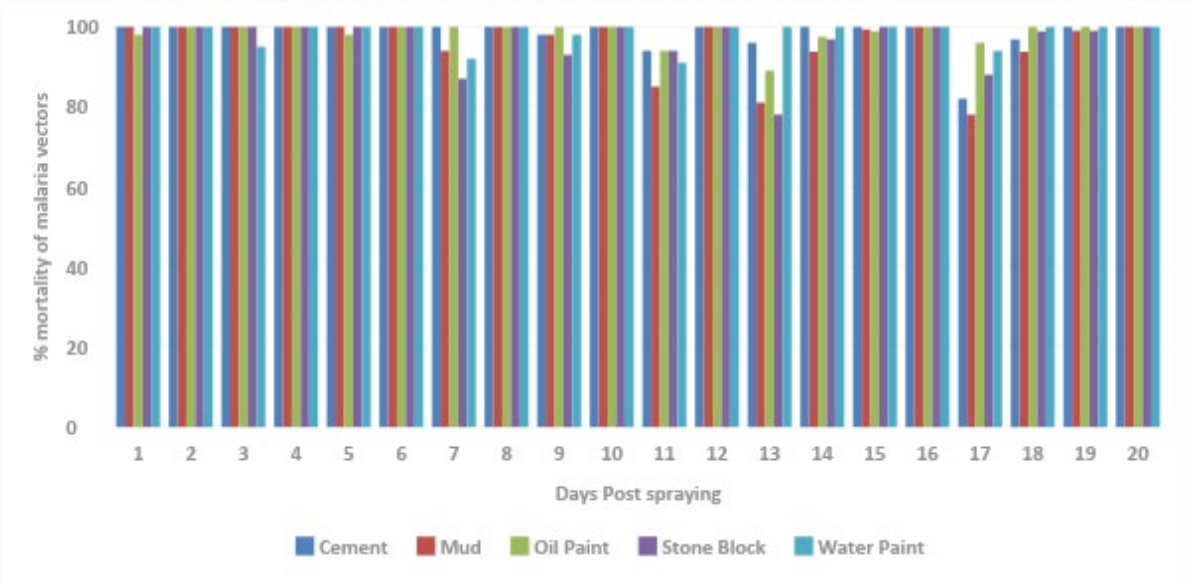
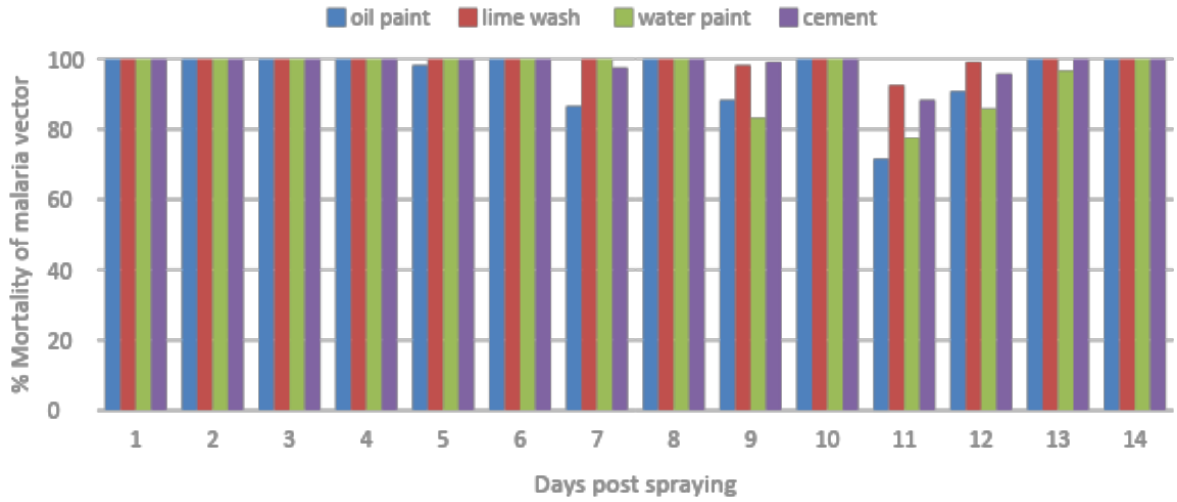


Figure 2. Residual efficacy of Fludora Fusion in Unguja 2021–2022



3.2 Malaria Vector ecology in Zanzibar

3.2.1 Malaria vector abundance, distribution, and species composition

In this reporting period, the Unguja team collected 1,628 female *Anopheles* mosquitoes from October 2021 to September 2022 across six sentinel sites. Out of the total female *Anopheles* vectors collected,1,611 (99%) were morphologically identified as *Anopheles gambiae* s.l., 17 (1%) were *An. funestus* group varying in surveillance area. Muyuni site did not collect even a single *Anopheles* mosquito vector.

In Pemba, out of the total *Anopheles* vector collected in four sentinel sites (n = 1,578; Table 5), 90% (n = 1,414) were morphologically identified as *An. gambiae s.l.* and 10% include *An. funestus* s.l., *An. rufipes*, *An. coustani*, *and An. maculipalpis*.

Human landing collection was the most efficient method for mosquito collection in Unguja and Pemba (Tables 6 and 7). In Unguja, it has collected 66% of the total catch with many of them outdoor 66% (n = 710). Pit trap was the second resourceful method followed by CDC light trap and PSC.

In Pemba, human landing collection was also the most resourceful method for mosquito collection. It has collected 72% (n = 1,135) of the total catch with many of them outdoor 82% (n = 935) followed by pit traps, pyrethrum spray catch, and CDC light traps.

Table 4. Morphological identification of malaria vectors in Unguja 2021-2022

Sentinel site	Morphological ID	Total
Bubwini	*An. gambiae* s.l.	70
Bumbwini Subtotal		**70**
Cheju	*An. funestus* group	3
	An. gambiae s.l.	760
Cheju Subtotal		**763**
Donge	*An. gambiae* s.l.	30
Donge Subtotal		**30**
Mwera	*An. funestus group*	14
	An. gambiae s.l.	701
Mwera Subtotal		**715**
Stone Town	*An. gambiae* s.l.	50
Stone Town Total		**50**
Muyuni	No collection	
Grand Total		**1,628**

Table 5. Morphological identification of malaria vectors in Pemba 2021–2022

Sentinel site	Morphological ID	Total
Bopwe	*An coustani*	11
	An. gambiae s.l.	849
	An. maculipalpis.	4
	An. rufipes	2
	An. funestus group	9

Sentinel site	Morphological ID	Total
Bopwe Subtotal		**875**
Tumbe	*An. coustani*	14
	An. gambiae s.l.	310
	An. maculipalpis	2
	An. rufipes	20
	An. funestus group	65
Tumbe Subtotal		**411**
Uwandani	*An. gambiae* s.l.	114
	An. rufipes	1
	An. funestus group	27
Uwandani Subtotal		**142**
Wambaa	*An. gambiae* s.l.	141
	An. rufipes	2
	An. funestus group	7
Wambaa Subtotal		**150**
Grand Total		**1,578**

Table 6. Mosquito trap performance in Unguja 2021–2022

Method	Biotope	Total	Percent
HLC	indoor	363	34
	outdoor	710	66
HLC Subtotal		**1,073**	**66**
LTC	indoor	8	
LTC Subtotal		**8**	**0.5**
Pit	outdoor	538	
Pit Total		**538**	**33**
PSC	indoor	9	
PSC Total		**9**	**0.5**
Grand Total		**1,628**	**100**

Table 7. Mosquito trap performance in Pemba 2021–2022

Method	Biotope	Total	Percent
HLC	indoor	200	18
	outdoor	935	82
HLC Total		**1135**	**72**
LTC	indoor	62	4
LTC Total		**62**	**4**
Pit	outdoor	235	15
Pit Total		**235**	**15**
PSC	indoor	146	9

Method	Biotope	Total	Percent
PSC Total		**146**	**9**
Grand Total		**1,578**	**100**

3.2.2 Molecular identification of An. gambiae s.l.

In Unguja, out of the 1,245 *An. gambiae* s.l. samples analyzed to the molecular species level, *An. arabiensis* was predominant by accounting 95% (n = 1,183), followed by *An. merus 4*% (n = 51), and the last one was *An. gambiae s.s. 1*% (n = 11; Table 8). However, there was variation from site to site.

In Pemba, out of the 1,303 *An. gambiae* s.l. screened for molecular identification of the sibling species, 98.7% (n = 1,287) were *An. arabiensis* and 1.3% (n = 16; Table 9) were *An. merus*. At all sites, the primary malaria vector was *An. arabiensis* and *An. merus* became a secondary vector, except in Stone Town where *An. merus* was predominant.

Table 8. PCR species identification of *An. gambiae* s.l. in Unguja 2021–2022

Sentinel site	No. of mosquitoes tested	Percent *An. arabiensis*	Percent *An. merus*	Percent *An. gambiae* s.s.
Bumbwini	60	90 (n = 54)	3 (n = 2)	7 (n = 4)
Cheju	610	99 (n = 605)	1 (n = 5)	0
Donge	27	67 (n = 18)	26 (n = 7)	7 (n = 2)
Stone Town	46	13 (n = 6)	76 (n = 35)	11 (n = 5)
Mwera	502	99.6 (n = 500)	0.4 (n = 2)	0
Total	**1,245**	**95 (n = 1,183)**	**4 (n = 51)**	**1 (n = 11)**

Table 9. PCR species identification of *An. gambiae* s.l. in Pemba 2021–2022

Sentinel site	No. of mosquitoes tested	Percent *An. arabiensis*	Percent *An. merus*	Percent *An. gambiae* s.s.
Bopwe	841	99.8 (n = 840)	0.2 (n = 1)	0
Tumbe	244	95 (n= 231)	5 (n = 13)	0
Uwandani	98	99 (n = 97)	1 (n = 1)	0
Wambaa	120	99 (n = 119)	1 (n = 1)	0
Total	**1,303**	**98.7 (n = 1,287)**	**1.3 (n = 16)**	**0**

3.2.3 Mosquito sporozoite rate

Mosquito infectivity was estimated by calculating the sporozoite rate (i.e., the proportion of mosquitoes in a population harboring sporozoite in their salivary glands). PCR facility screened 1,373 *Anopheles* vectors from Pemba for the presence of sporozoite (Table 10). Out of the tested mosquitoes, only four were found positive for *Plasmodium* infection, with an average overall sporozoite rate of 0.29%. All sporozoite positive mosquitoes were *An. arabiensis* collected from Bopwe (n = 3) and Wambaa (n = 1) using HLC.

In Unguja, out of the 1,470 *Anopheles* vectors screened for sporozoite rate,0.74% (n = 11) were found positive with *Plasmodium* infection in the salivary glands. All malaria-infected

vectors were collected from Cheju (n = 4) and Mwera (n = 7; Table 10). Out of the 11 sporozoite positive vectors, 9 were PCR analyzed as *An. arabiensis* and two are still pending, waiting for analysis. However, they were morphologically identified as *An. funestus* s.l. All sporozoite positive mosquitoes in Unguja were collected using HLC (n = 9) and Pit traps (n = 2).

Table 10. Malaria vector infection rate per sentinel site in Zanzibar 2021–2022

Location	Sentinel site	No. of vectors tested	No. of positive sporozoite	sporozoite rate %
Pemba	Tumbe	301	0	0
	Bopwe	817	3	0.37
	Wambaa	128	1	0.78
	Uwandani	127	0	0.00
Unguja	Bumbwini	70	0	0.00
	Donge	28	0	0.00
	Mwera	634	7	1.10
	Muyuni	0	0	0.00
	Stone Town	48	0	0.00
	Cheju	690	4	0.58

Further analysis in Zanzibar indicated that all infected *Anopheles gambiae s.l.* were collected during rainfall season April–July 2022 and October–December 2021.

3.2.4 Feeding location and biting time of Anopheles gambiae s.l. as expressed by human landing catch

The general feeding location of *An. gambiae s.l.* was outdoor in Unguja and Pemba (Figures 3 and 4), regardless of the spraying status of sentinel sites. Out of the 1,060 *An. gambiae s.l.* molecularly analyzed in Pemba (*An. arabiensis and An. merus*), 82% (n = 870) were found to feed outdoor with 78% (n = 681) before midnight (24 hours) varying in sentinel sites (Figure 5). In Unguja, out of the 880 of the *An. gambiae s.l.* molecularly analyzed (An. *arabiensis, An. merus*, *and An. gambiae s.s.)* after being collected using HLC, 65% (n = 576) were reported to feed outdoor with 54%of them biting before 24 hours (n = 311; Figure 6). ZAMEP conducted reactive focal IRS at sentinel sites between November 2021 and January 2022 whereby some villages of the sentinel sites were sprayed with long residual insecticide clothianidin.

Figure 3. *An. gambiae* s.l. feeding location as expressed by HLC in Pemba sites, 2021–2022

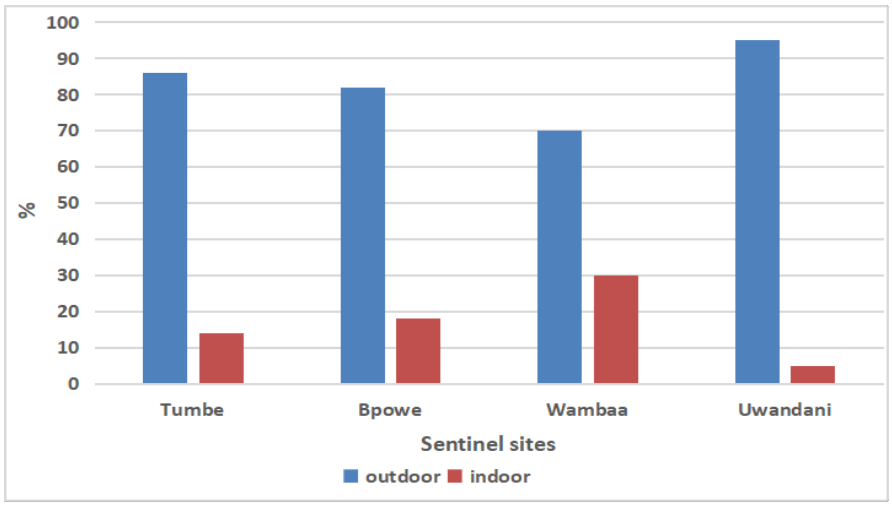


Figure 4. An. gambiae *s.l.* feeding location as expressed by HLC in Unguja sites, 2021–2022

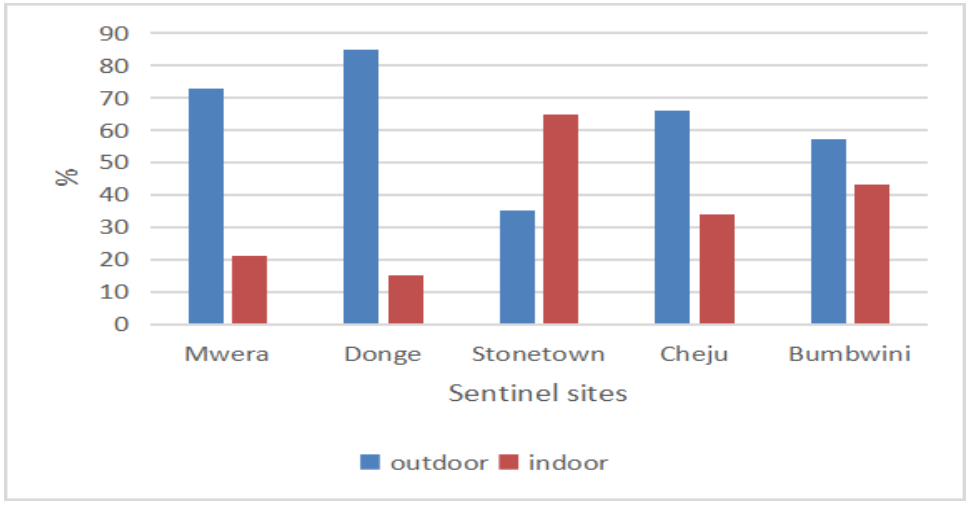


Figure 5. *An. gambiae* s.l. peak biting hours in Pemba sites, 2021–2022

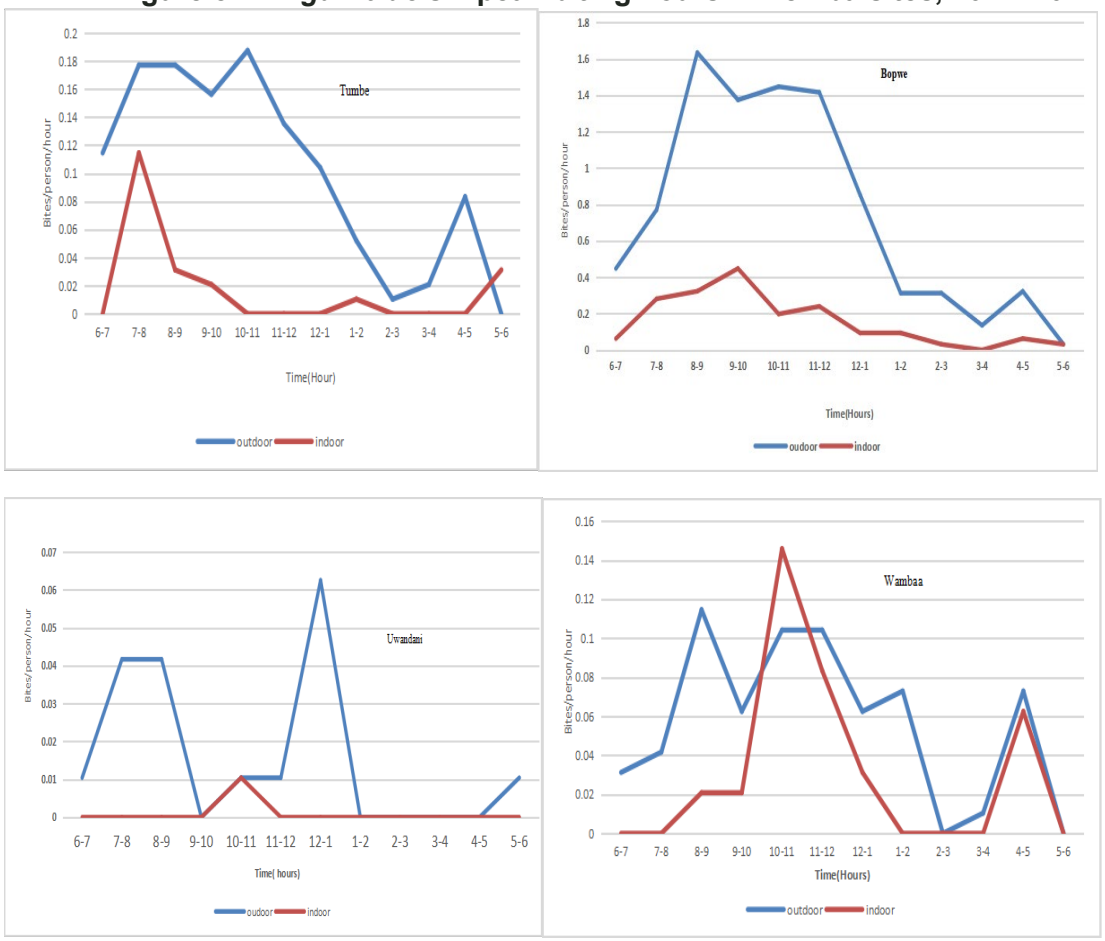
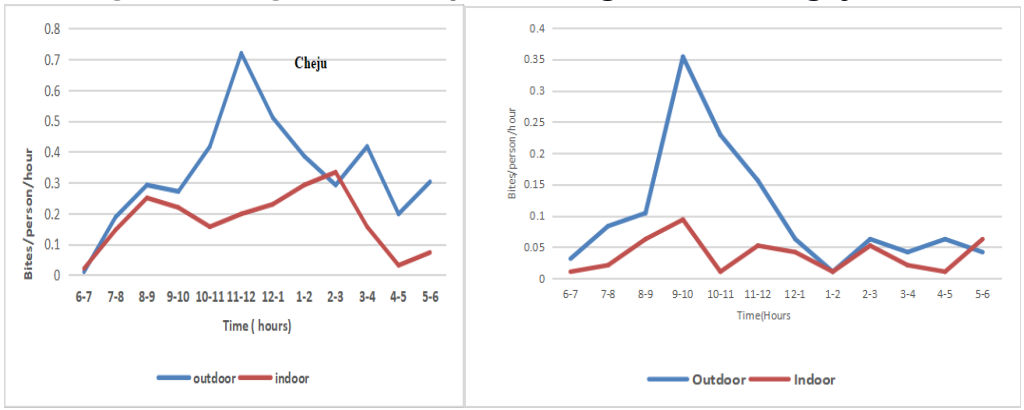
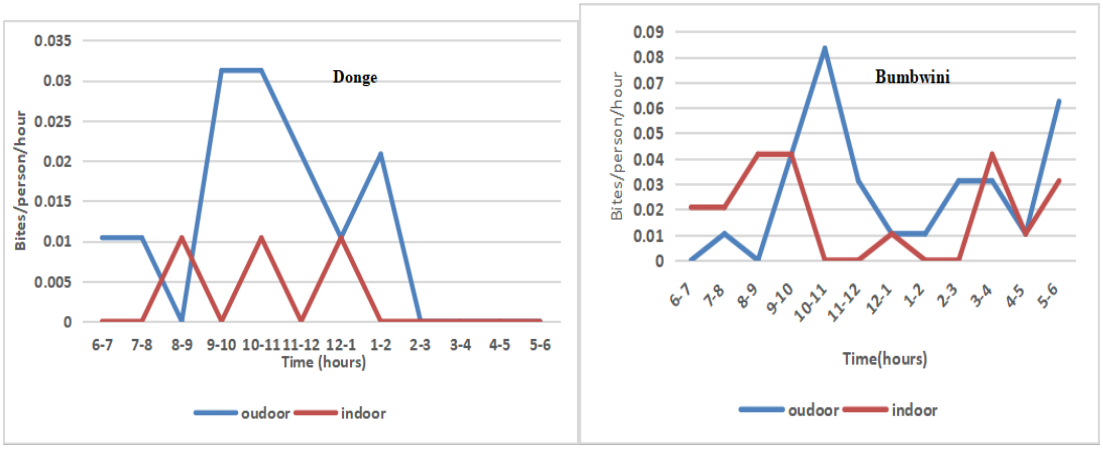


Figure 6. *An. gambiae* s.l. peak biting hours in Unguja sites, 2021–2022



Mwera



3.2.5 *Anopheles gambiae s.l.* seasonality in Zanzibar

This indicator describes how the vector population changes over time perhaps because of interventions or climatic changes. These population changes were measured using HLC to assess the average bites/person/night. The peak bites of *An. gambiae s.l.* was observed during the wet season (March–May 2022 and October–December 2021) varying in sentinel sites. Bopwe had a high biting intensity of *An. gambiae s.l.* compared with other sentinel sites (Figure 7). *An. gambiae s.l.* bites at Mwera (Figure 8) are almost perennial or continuous for the year as the site is close to irrigation scheme.

Figure 7. *Anopheles gambiae s.l.* seasonality in Pemba sites 2021–2022

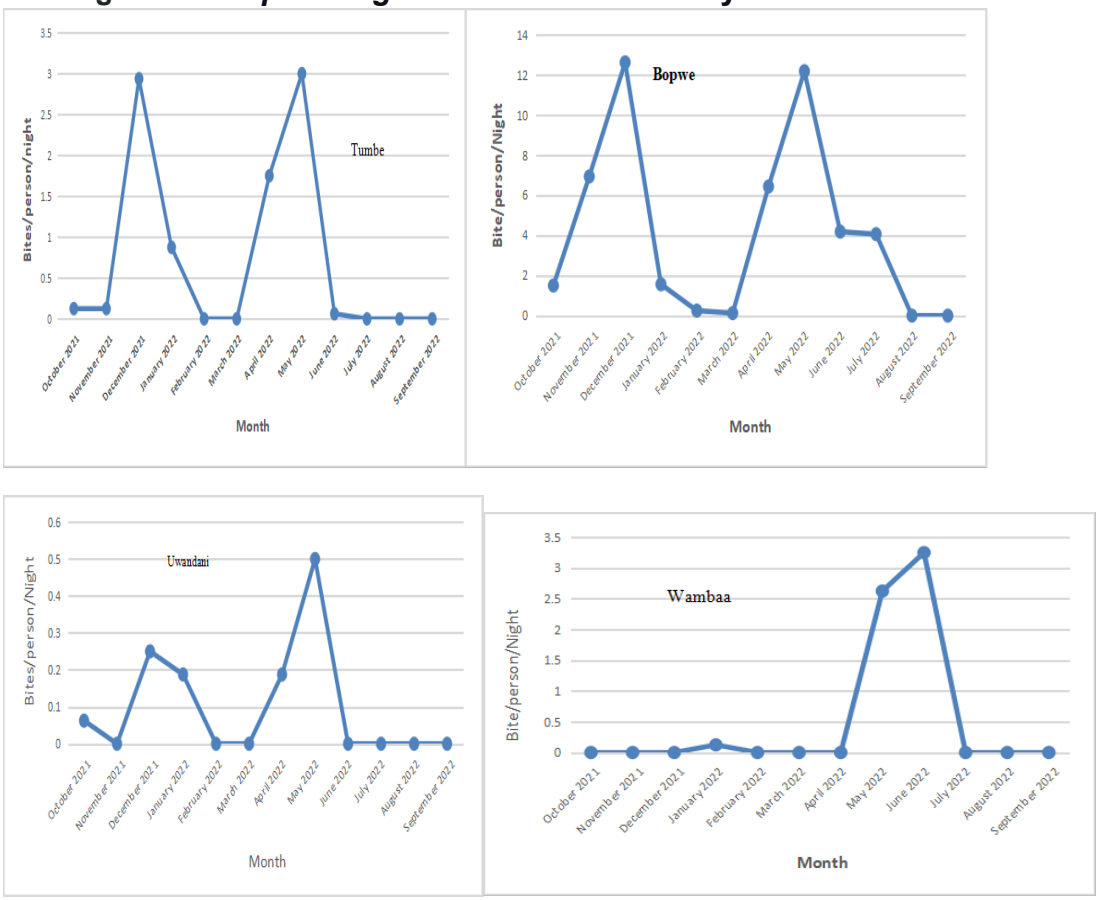
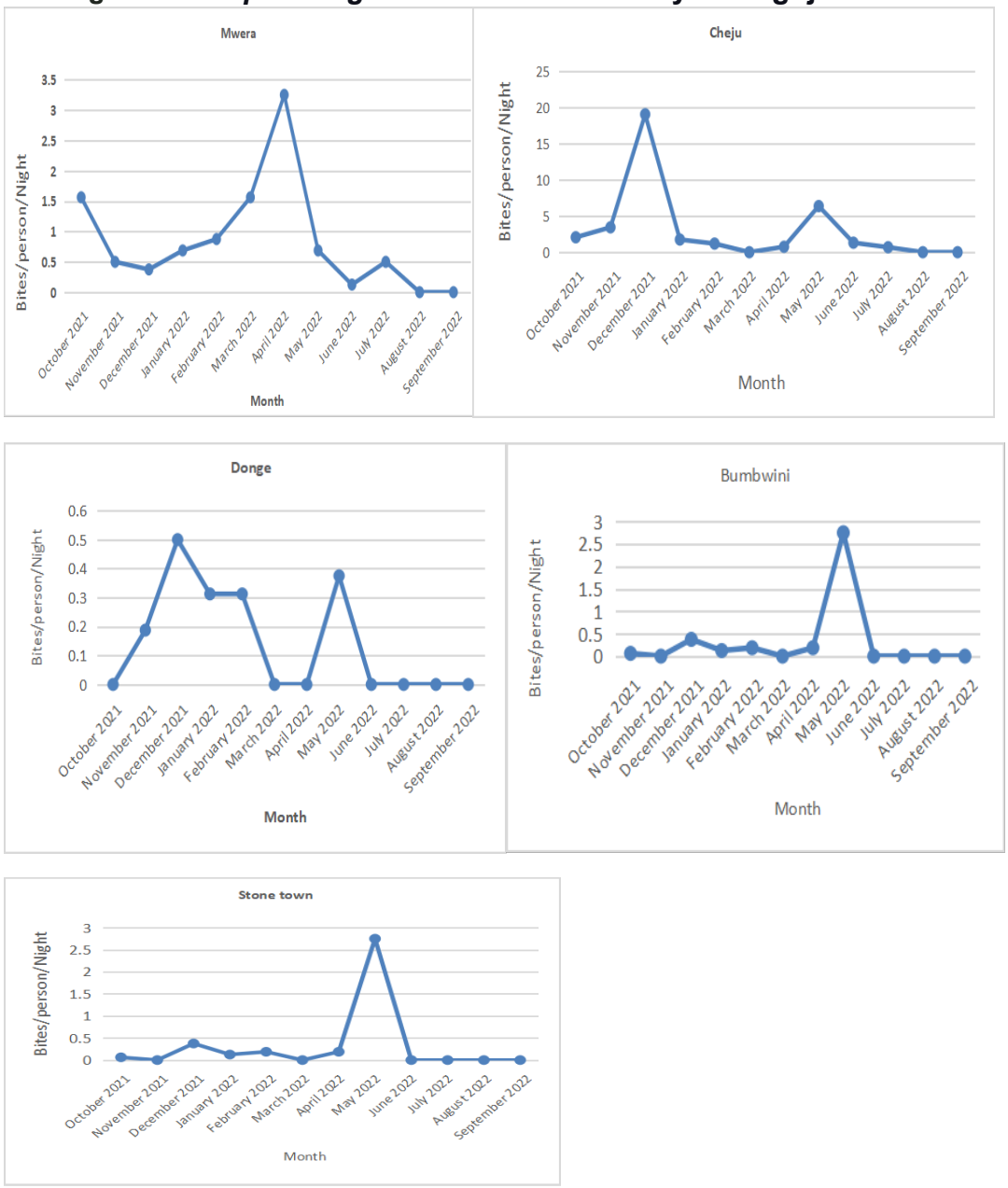


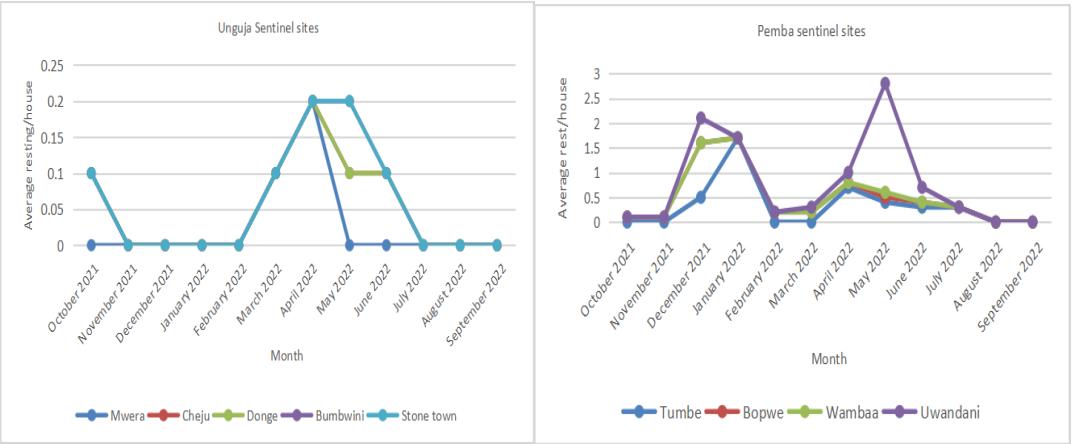
Figure 8. *Anopheles gambiae s.l.* seasonality in Unguja sites 2021–2022



3.2.6 Indoor resting density (IDR) of An. gambiae s.l.

The average IDR of *An. gambiae s.l.* was low on both islands (Figure 9). However, the mosquitoes at the Pemba sites have been shown to rest substantially more indoor than in Unguja. The data indicate that more resting was associated with rainfall patterns between April–June 2022 and October– December 2021. The low indoor resting density might be attributed to high LLIN coverage at households or the behavioral nature of the vectors . Improved housing conditions could also be the reason for low receptivity between Unguja and Pemba and the sentinel sites in rural and urban areas.

Figure 9. An. gambiae *s.l.* resting density in Zanzibar 2021–2022



3.2.7 EIR of An. gambiae s.l. *in Zanzibar 2021–2022*

EIR is used to determine mosquito infectivity by calculating the sporozoite rate (i.e., the proportion of mosquitoes in a population harboring infective sporozoites in their salivary glands). Sporozoite detection is necessary to determine the EIR, which describes the number of infectious bites an individual is exposed to in a given period (typically a year or transmission season). The average annual EIR in Pemba was **1.884** varied among the sentinel sites (Table 11). In Unguja, the average annual EIR was **2.570** (Table 12), which is twice higher than in Pemba, indicating the intensity of indigenous malaria transmission to be almost double or more.

Table 11. Annual EIR of An. gambiae s.l. per sentinel site in Pemba 2021–2022

Month	Bopwe	Wambaa	Tumbe	Uwandani
October 2021	0	0	0	0
November 2021	0	0	0	0
December 2021	0	0	0	0
January 2022	0	0	0	0
February 2022	0	0	0	0
March 2022	0	0	0	0
April 2022	0	0	0	0
May 2022	1.926	0	0	0
June 2022	3.74	1.872	0	0
July 2022	0	0	0	0
August 2022	0	0	0	0
September 2022	0	0	0	0
Annual EIR Total	**5.666**	**1.872**	**0**	**0**

Table 12. Annual EIR of An. gambiae *s.l.* per sentinel site in Unguja, 2021–2022

Month	Mwera	Cheju	Stone Town	Bumbwini	Muyuni	Donge
October 2021	0	0	0	0	0	0

Month	Mwera	Cheju	Stone Town	Bumbwini	Muyuni	Donge
November 2021	0	1.867	0	0	0	0
December 2021	1.937	3.841	0	0	0	0
January 2022	0	0	0	0	0	0
February 2022	0	0	0	0	0	0
March 2022	0	0	0	0	0	0
April 2022	4.03	0	0	0	0	0
May 2022	1.875	1.874	0	0	0	0
June 2022	0	0	0	0	0	0
July 2022	0	0	0	0	0	0
August 2022	0	0	0	0	0	0
September 2022	0	0	0	0	0	0
Annual EIR	**7.842**	**7.582**	**0**	**0**	0	**0**

3.3 Insecticide resistance monitoring of malaria vectors in Zanzibar 2022

3.3.1 Susceptibility test of An. gambiae s.l. in Zanzibar

The results of the WHO susceptibility tests indicated that *Anopheles gambiae* s.l. from sentinel sites in Pemba and Unguja were fully susceptible to pirimiphos-methyl, clothianidin, and bendiocarb. However, the vectors were found to be resistant to permethrin, deltamethrin, and alpha-cypermethrin across all the tested sites (Table 13).

Table 13. Susceptibility status (percentage mortality) of *Anopheles gambiae s.l.* to WHO discriminating concentration of insecticide

Location	District	Sentinel site	Permethrin percent mortality	alpha-Cypermethrin percent mortality	Deltamethrin percent mortality	Bendiocarb percent mortality	Pirimiphos-methyl percent mortality	Clothianidin percent mortality
Pemba	Wete	Bopwe	40	27	30	100	100	100
	Chake	Uwandani	22	15	21	100	100	100
	Mkaoni	Wambaa	60	65	71	100	100	100
	Micheweni	Tumbe	65	66	68	100	100	100
Unguja	Kati	Cheju	78	80	77	100	100	100
	Kusini	Muyuni	84	92	73	100	100	100
	Mjini	Stone Town	83	94	77	100	100	100
	Kaskazini A	Donge	76	86	78	100	100	100
	Magharibi	Mwera	86	76	93	100	99	100
	Kaskazini B	Bumbwini	50	50	50	100	100	100

Key: > 98 = Fully susceptible
90–97 = Possible resistance
<90 = Confirmed resistance

3.3.2 The strength of pyrethroid resistance against An. gambiae s.l. in Pemba

The team tested permethrin, alpha-cypermethrin, and deltamethrin as they are widely used in LLINs. The results indicated that the strength of pyrethroid resistance varies across the sites, and the vectors can survive up to 10x concentration. The intensity of resistance ranges from low, moderate, and high as shown in Figures 10 and 11.

Figure 10. Intensity of pyrethroid resistance of *An. gambiae s.l.* in Pemba sentinel sites–2022

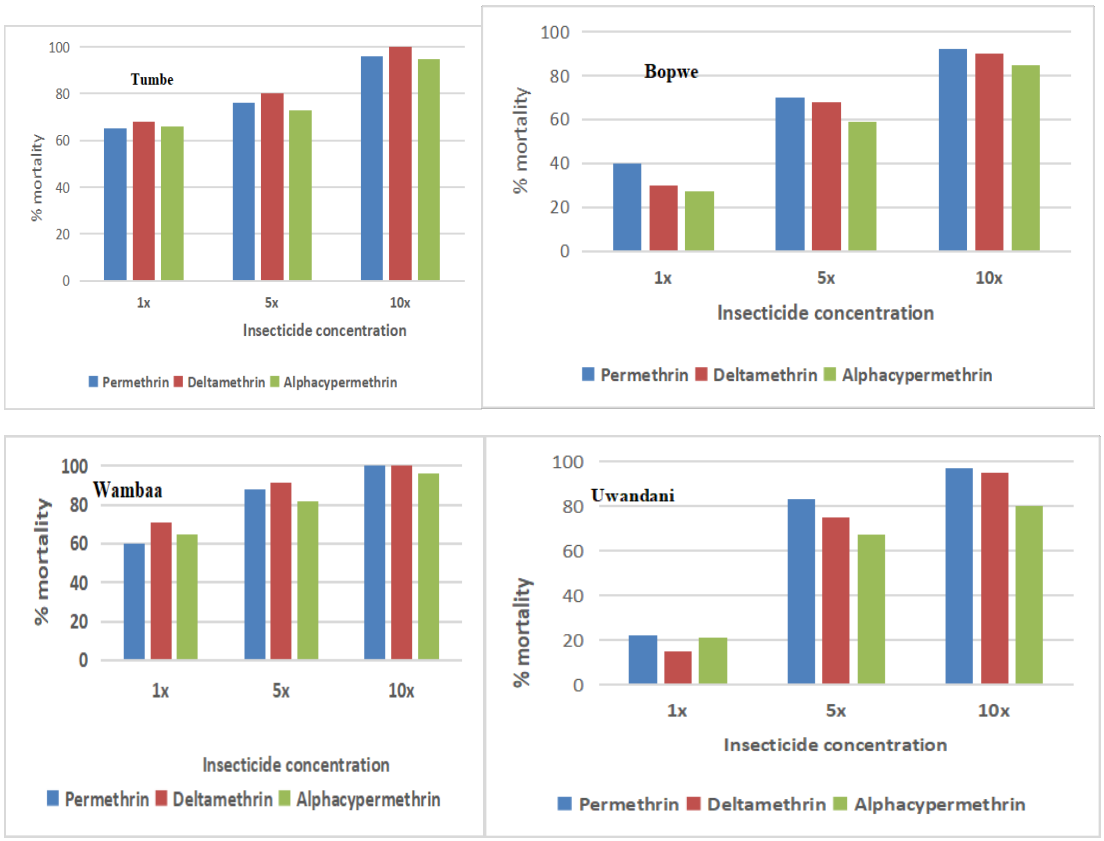
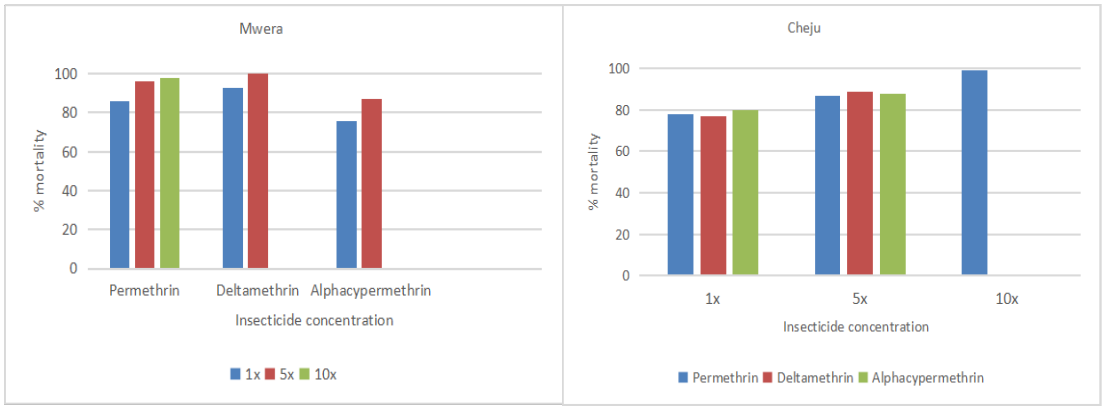
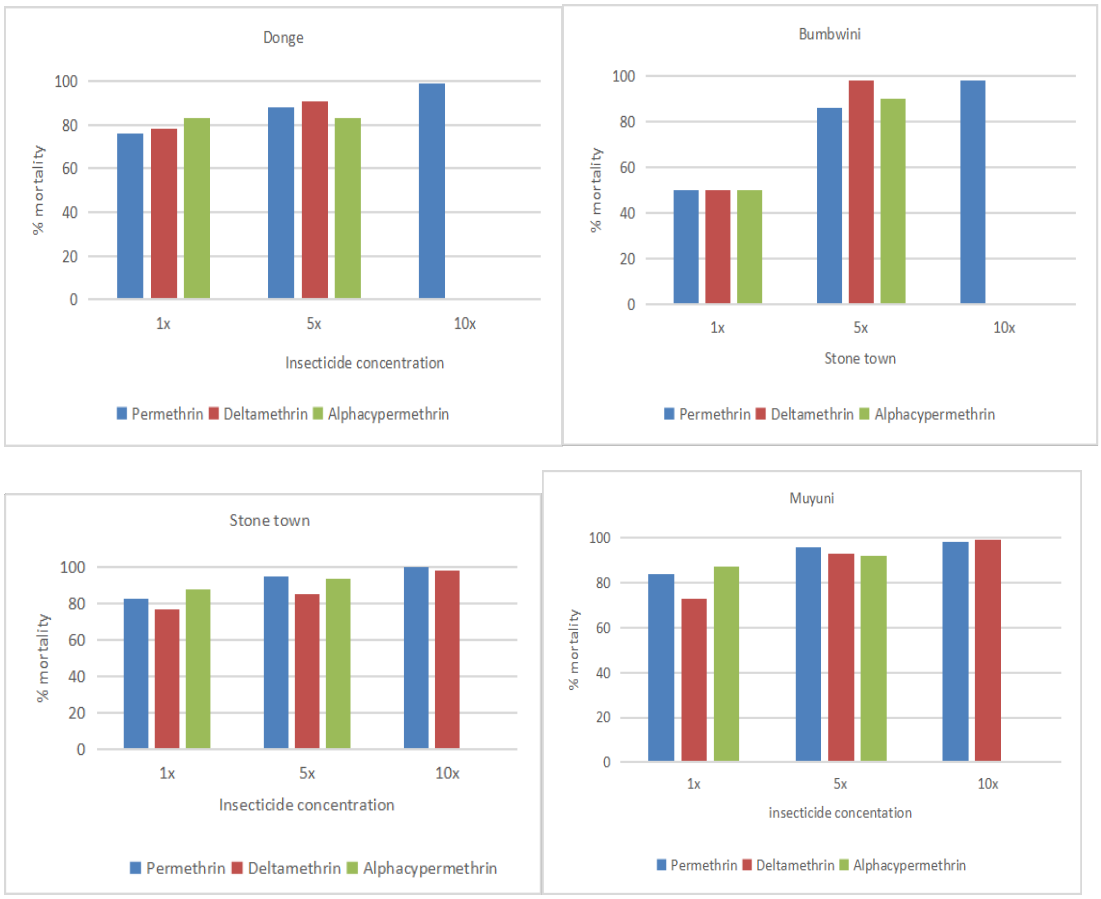


Figure 11. Intensity of pyrethroid resistance of *An. gambiae* s.l. in Unguja sentinel sites –2022

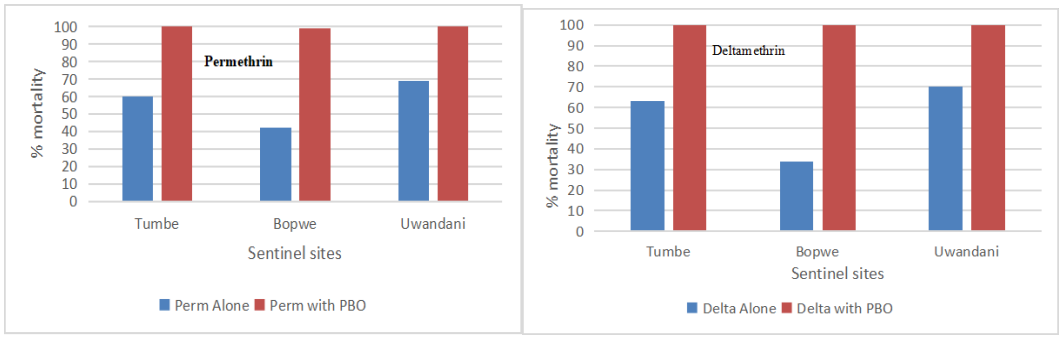




3.3.3 Mechanism of pyrethroid resistance against An. gambiae s.l. in Pemba

Malaria vectors were resistant to 1x, 5x, and even 10x of permethrin, deltamethrin, and alpha-cypermethrin. However, the resistance level went to 0%at 1x of permethrin and deltamethrin when the mosquitoes were treated with PBO and then exposed to the insecticides at the three sentinel sites (Figure 12). We did not test alpha-cypermethrin because of a shortage of mosquitoes. Relatedly, because of the shortage of mosquitoes and delay in receiving insecticide impregnated papers, the test was not conducted in Unguja.

Figure 12. Mechanism of deltamethrin and permethrin resistance against *An. gambiae* s.l. in Pemba



4. Discussion

4.1 Species composition, behavior, and vector distributions

The molecular identification of malaria vectors has identified *An. arabiensis* as the predominant malaria vector with highly exophagic behavior on both islands. However, *An. gambiae* s.s. was observed in small proportion. The An. *gambiae* s.l. abundance and biting density are dependent on mean rainfall, particularly in October–December and April–June, suggesting that the risk of malaria transmission also is dependent on rainfall patterns.

Other morphologically identified vectors were *Anopheles funestus* s.l.*, An. rufipes, An. coustani*, *and An. maculipalpis* contributed to 10% of the total catch for 2021–2022. Of these, *An. funestus s.l.* particularly *An. funestus s.s* are known to be one of the efficient malaria vectors. However, we have not confirmed their existence in Zanzibar. We did not complete molecular identification due to the breakdown of ZAMEP PCR facility. However, in 2017–2018, ZAMEP reported *An. leesoni, An. rivulorum*, *and An. parensis* as secondary vectors. Nevertheless, their roles in malaria transmission are not very clear. More investigation is needed on the presence of other species particularly *An. stephensi*, an Asian malaria vector, as they may play a silent role in driving the residual malaria transmission without being noticed.

The exophagic (outdoor feeding) behavior of *An. arabiensis* at an earlier time is likely to affect young people following social interaction activities. ZAMEP conducts a foci response following an entomology investigation last year. However, detailed information is required before the reactive focal response to determine where the vectors and humans interact to have the perfect target for interventions. There is no doubt that the outdoor biting behavior of An. *arabiensis* reduced the effectiveness of vector control interventions and, therefore, complementary methods such as larviciding to fight outdoor malaria vectors are necessary to increase efficiency towards elimination.

Indoor biting and resting density of malaria vectors are currently affected by the indoor intervention (IRS and LLINs). In addition, continuation of LLINs distribution is highly emphasized. Stopping such intervention is likely to favor the endophagic and endophilic vectors and hence increase in malaria transmission.

4.2 Sporozoite rate and EIR

The overall sporozoite rate was 0.74% in Unguja and 0.29% in Pemba, varying from sentinel sites with an average EIR of 1.884 in Pemba and 2.57 in Unguja. EIR corresponds to a few indigenous cases in Zanzibar. However, the EIR varies from sentinel sites and the higher the mosquito density, the higher the chance of getting infected mosquitoes.

4.3 Insecticide resistance monitoring, intensity, and mechanism

Pyrethroid resistance in *An. gambiae s.l. (exclusively An. arabiensis)* in Zanzibar is still stable. . However, *An. gambiae s.l.* were fully susceptible to clothianidin, pirimiphos-methyl and bendiocarb. The strength of deltamethrin, permethrin alpha-cypermethrin resistance to *An. gambiae s.l.* is not homogeneous across the sites.

Complete restoration of deltamethrin and permethrin susceptibility after exposing mosquitoes to PBO implies that a monooxygenase-based resistance mechanism fully accounts for the vector population. This finding suggests that the PBO LLINs used in the community could have additional value in malaria protection. However, the PBO LLINs must be monitored before and after distribution to households to ascertain the PBO chemical residue and their killing power against wild pyrethroid-resistant mosquitoes.

Despite the pyrethroid resistance being stable for a long time, there is no indication of disease control failure. Possibly the resistance phenotype is less frequent among the old mosquitoes, which are mature enough to transmit malaria parasites, than among younger mosquitoes. Another reason possibly is that PBO nets play a good role in reversing the effect of resistance.

4.4 Residual efficacy of Fludora Fusion in treated walls

The average mosquito mortality for the wall cone bioassays in tested areas was still effective (≥80% mortality) against susceptible Anopheles gambiae s.s. R70 at the age of eight months after IRS. The insecticide has quick knockdown, toxic to malaria vectors, and long residual effect on treated surfaces. These results from using Fludora Fusion are promising for malaria control if the mosquitoes are endophagic and endophilic.