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MOZAMBIQUE ENTOMOLOGICAL MONITORING

ANNUAL REPORT
JULY 2019 – JUNE 2020

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ACRONYMS

Ace-1	Acetylcholinesterase 1 gene
b/p/h	Bites per Person per Hour
b/p/n	Bites per Person per Night
CDC	Centers for Disease Control and Prevention
COVID-19	Coronavirus Disease 2019
ELISA	Enzyme-linked Immunosorbent Assay
HBI	Human Blood Index
HLC	Human Landing Catch
INS	National Institute of Health (*Instituto Nacional de Saúde)*
IRS	Indoor Residual Spray
kdr	knockdown resistance gene
m/t/n	Mosquitoes per Trap per Night
NMCP	National Malaria Control Program
PBO	Piperonyl Butoxide
PCR	Polymerase Chain Reaction
PMI	President's Malaria Initiative
PSC	Pyrethrum Spray Catch
USAID	United States Agency for International Development
WHO	World Health Organization

EXECUTIVE SUMMARY

Indoor residual spraying (IRS) and insecticide-treated nets remain the primary mosquito vector control interventions in many parts of world, including sub-Saharan Africa, where malaria continues to be a major public health concern.

In Mozambique, Abt Associates implemented the U.S. President's Malaria Initiative (PMI) VectorLink Mozambique Project from July 1, 2019, to June 30, 2020. In the 2019 spray campaign, from October to December 2019, VectorLink Mozambique conducted IRS with SumiShield® 50WG in Mopeia and Morrumbala and, from November to December 2019, with Fludora® Fusion in Maganja da Costa, Milange, and Molumbo districts, in Zambezia Province. Monthly entomological monitoring was performed in three intervention districts (Maganja da Costa, Milange, and Mopeia) and one control district of Lugela, which had not received IRS. Surveillance employed several collections techniques: CDC light trap, human landing catch (HLC), pyrethrum spray catch (PSC), and Prokopack aspirator. In September 2019, the PSC method was replaced by the use of Prokopack aspiration, and in February 2020, the HLCs were interrupted in accordance with PMI guidance for ethical reason and alignment with the national entomological procedures. Cone wall bioassays were conducted to monitor the spray quality and residual life of insecticides sprayed in Maganja da Costa, Milange, and Mopeia districts. Annual insecticide susceptibility tests were carried out in the five sprayed (Maganja da Costa, Milange, Molumbo, Mopeia and Morrumbala) and one control (Lugela) district.

Mopeia district collections were conducted through October 2019 and contributed to the epidemiological study entitled "A cluster randomized trial to measure the impact of indoor residual spraying with a third-generation indoor residual spray (3GIRS) product in combination with long-lasting insecticidal nets in Zambezia, Mozambique." The study was conducted in five sentinel villages in intervention areas and five villages in control areas up to October 2018. When IRS spraying with SumiShield® 50WG was expanded into the previously unsprayed control areas, the same 10 sentinel villages were maintained in the study period extension through October 2019. CDC light trap and HLC methods were used to sample mosquitoes in the study areas. In November 2019, Prokopack collection was introduced in Mopeia and the number of sampling villages was reduced to equal numbers in other districts.

In Nampula, the Government of Mozambique with support from the Global Fund conducted IRS using Fludora® Fusion in the districts of Meconta, Murrupula, Nampula City, Rapale, and Ribaue, and SumiShield® 50WG in the districts of Angoche, Monapo, and Nacala. In addition, mass distribution campaign of insecticide-treated nets (ITN) was also conducted in all districts of Nampula province in July 2019. VectorLink Mozambique performed monthly entomological monitoring using CDC light traps, HLCs (that were phased out in February 2020), and PSCs that were replaced by Prokopack collections in September 2019 in two intervention districts, Nampula City and Monapo, and in Erati, the control district. Cone wall bioassays were also conducted in Nampula and Monapo districts. Annual insecticide susceptibility tests were carried out in the two sprayed (Nampula City and Monapo) and one control (Erati) district.

Mosquito collections using the methods described above demonstrated the presence of highly diverse species composition of anophelines, which included the two main vectors *Anopheles funestus* s.l. and *An. gambiae* s.l., and other potential vectors and non-vectors such as *An. coustani*, *An. pharoensis*, *An. pretoriensis*, *An. tenebrosus*, *An. ziemanni*, *An. caliginosus,* and *An. maculipalpis*. Our findings highlight high levels of heterogeneity and diversity in mosquito vector species composition and behavior in the monitored areas. Data on samples sent to the National Health Institute (*Instituto Nacional de Saúde*) for molecular analyses on species identification indicated that *An. funestus* s.s., *An. gambiae* s.s., and *An. arabiensis* were the malaria vectors identified.

In general, post-IRS *An. funestus* s.l. indoor resting densities were suppressed compared with pre-IRS (July to October) densities in Zambezia province. There was also a decrease in the indoor resting densities of *An. funestus* s.l. following the mass distribution campaign of nets in July and IRS in November in Nampula province.

An. gambiae s.l. densities appeared to increase, most likely because of the rapid build-up of breeding habitats due to the high level of precipitation during the post-IRS period. However, the indoor resting densities of *An. gambiae* s.l. were low at most sentinel sites.

Malaria vectors *An. gambiae* s.l. and *An. funestus* s.l. were collected both indoors and outdoors. *An. funestus* s.l. tended to bite predominantly indoors, even after IRS in most districts, whereas *An. gambiae* s.l. mainly bit outdoors. Biting activity seemed to follow human sleeping patterns, with peak indoor biting activity starting at around mid-night and extending into the morning hours mainly between 1:00-4:00 am.

Quality of IRS, assessed by cone wall bioassays, showed that spray teams might not under dose insecticide applications in all districts. The insecticide decay rate assessment showed that SumiShield® 50WG and Fludora®Fusion lasted at least 9-10 months. This is similar to the long period of residual effect (9 months) of SumiShield® 50WG reported last year in Mozambique.

Results of insecticide susceptibility tests show that local vectors are fully susceptible to pirimiphos-methyl, chlorfenapyr, clothianidin, and bendiocarb. Assays for pyrethroids (deltamethrin and permethrin) again revealed widespread vector resistance to pyrethroids. Further assays to assess the intensity of resistance in *An. gambiae* s.l. showed low, moderate, and high intensity of resistance to deltamethrin at Molumbo, Milange, and Maganja da Costa, respectively. The intensity of resistance to deltamethrin was moderate to high at Morrumbala. High intensity of resistance to permethrin was also observed at Maganja da Costa. This finding demonstrates that the current situation poses a major threat of potential intervention failure for tools dependent on pyrethroid insecticides and the importance of continued use of non-pyrethroid insecticides. Synergist assays with piperonyl butoxide (PBO) demonstrated restoration of vector's susceptibility to pyrethroids (deltamethrin and permethrin) at most of the sites in Zambezia, indicating that PBO nets may also be additional option to overcome the observed pyrethroid resistance for vector control in Zambezia. However, PBO did not restore vector's susceptibility to pyrethroid insecticides in Nampula province and the continued use of IRS with next generation insecticides is recommended for malaria vector control in the area.

Due to Coronavirus Disease 2019 (COVID-19), entomological monitoring activities were suspended from April up to July 2020 and resumed in August, this significantly affected the ability for the team to collect mosquitoes during the peak mosquito abundance season for longitudinal monitoring and collection of adult *An. funestus* s.l. for susceptibility tests.

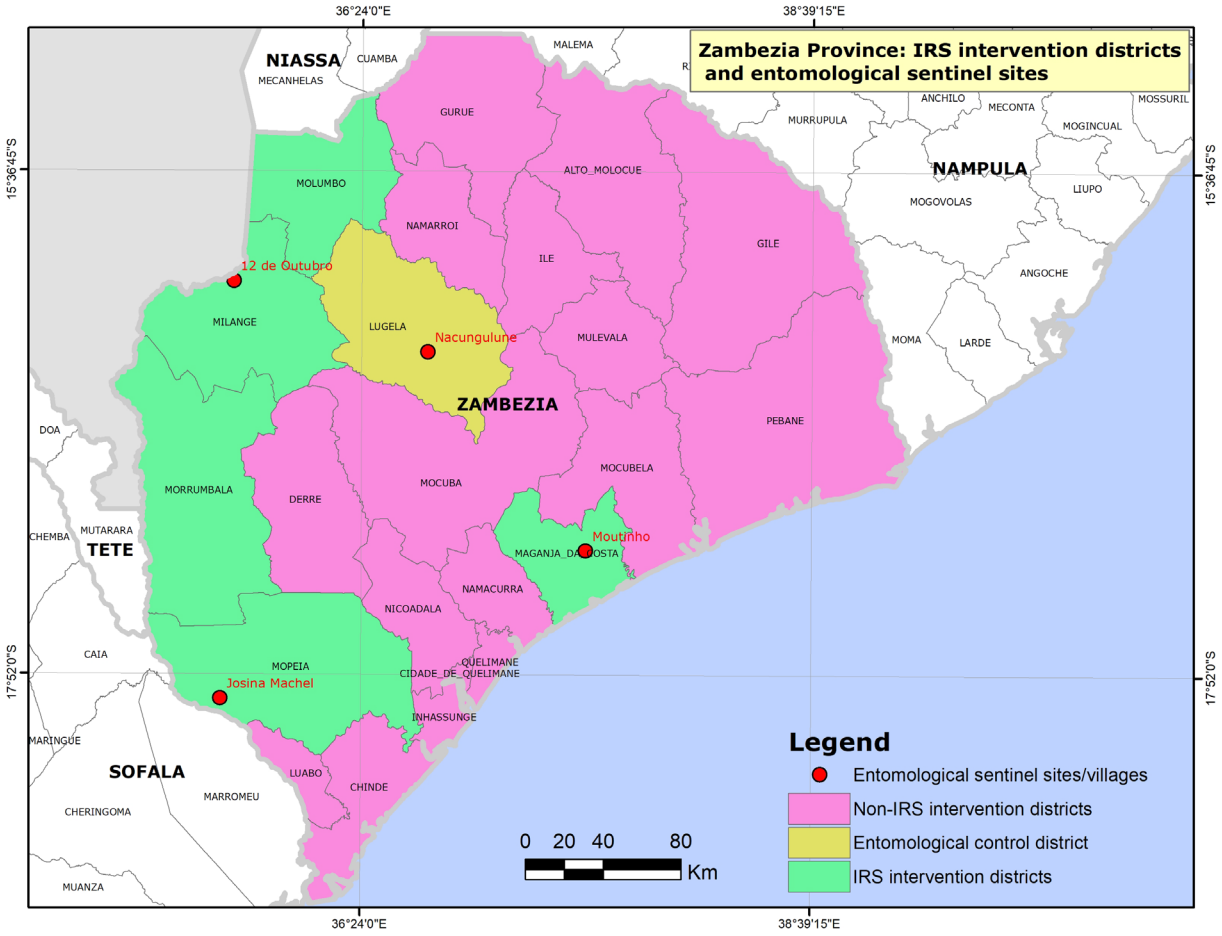
I. INTRODUCTION

Through support of the U. S. President's Malaria Initiative (PMI), Zambezia Province implemented six spray rounds of indoor residual spraying (IRS) under the PMI Africa Indoor Residual Spraying (AIRS) Project (2011–2017). In 2019, Zambezia implemented its second spray campaign under the PMI VectorLink Project. During the 2019 spray campaign, PMI VectorLink Mozambique conducted IRS in five target districts (Maganja da Costa, Milange, Molumbo, Mopeia, and Morrumbala). VectorLink Mozambique also carried out entomological monitoring activities in Zambezia, and supported the National Malaria Control Program's (NMCP's) entomological activities in Nampula and five other provinces in the north and central regions of the country to enhance in-country capacity for entomological monitoring. Having entomological monitoring data that supplements epidemiological data is essential to properly target vector control interventions; evaluate the susceptibility level of the local vectors to different insecticides and determine the underlying mechanisms; inform selection of insecticides; ensure the quality of spraying; monitor the impact of IRS and ITNs on vector density, behavior, and composition; and monitor the residual life of different insecticides on different types of wall surfaces. This entomological monitoring annual report covers the period from July 1, 2019, to June 30, 2020. However, the data in this report cover the period from July 1, 2019, to March 2020; data could not be collected in the months of April, May, and June due to restrictions related to the novel coronavirus disease 2019 (COVID-19) pandemic. On March 11, 2020, the World Health Organization (WHO) declared the global COVID-19 situation as pandemic. PMI and WHO issued guidance limiting the conduct of entomological activities on March 25 and April 10, respectively. In accordance with that guidance and based on guidance from the Mozambique National Malaria Control Program , VectorLink ceased all of its field monitoring activities (cone wall bioassay for monitoring the decay rate of insecticides sprayed; CDC light trap and Prokopack collections for longitudinal monitoring and adult *An. funestus* s.l. collections with Prokopack for susceptibility tests) between April and July 2020.

Entomological monitoring was conducted in three IRS intervention districts in Zambezia Province: Maganja da Costa, Milange, and Mopeia. Unsprayed Lugela district was used as a control district.

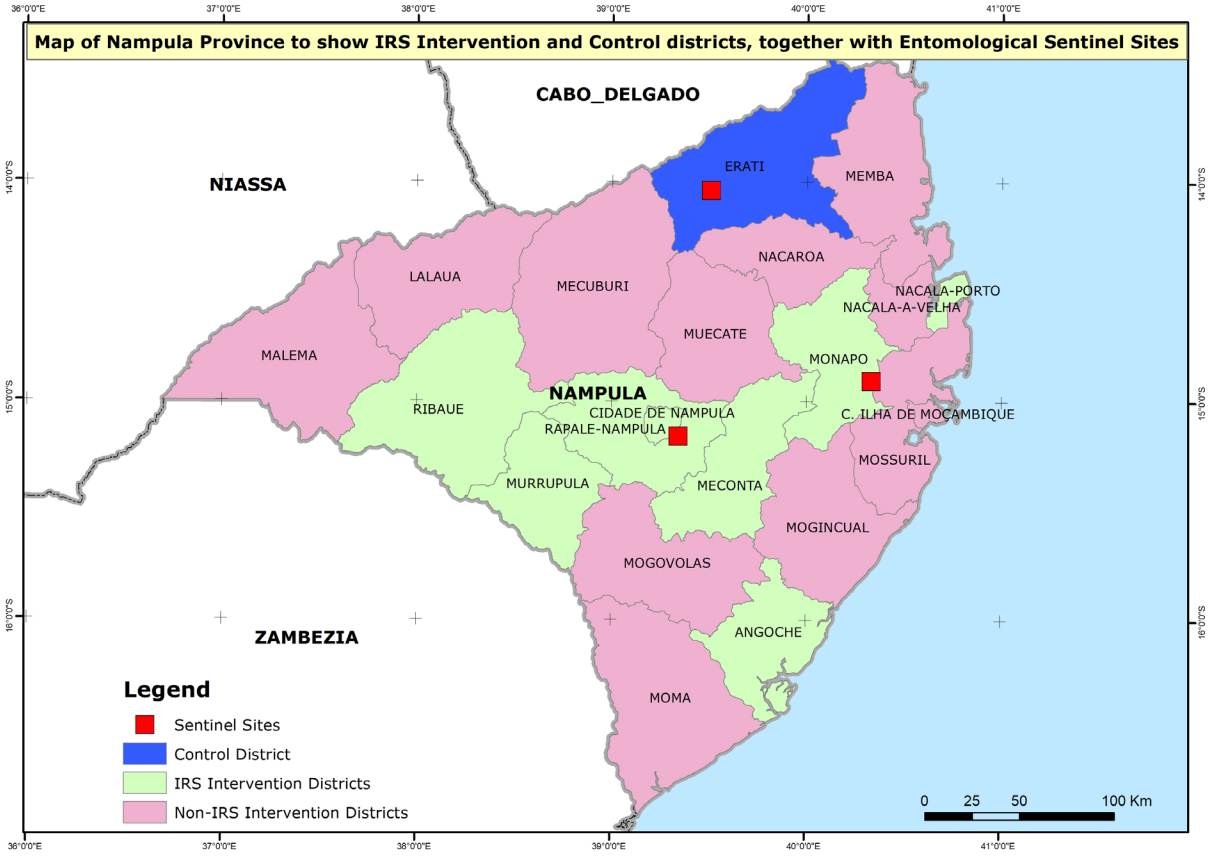
In Mopeia district, entomological monitoring data began being collected using the Centers for Disease Control and Prevention (CDC) light trap and human landing catch (HLC) collection methods; later in November 2019, Prokopack aspiration was introduced as an additional collection tool. The HLC collection method was dropped in late February 2020 due to PMI guidance, based on ethical issues with the collection method. In the other two IRS districts, Maganja da Costa and Milange, and in Lugela, the control district, entomological monitoring data were collected using the pyrethrum spray catch (PSC), CDC light trap, and HLC methods. In September 2019, PSC collection was replaced by Prokopack as per the approved work plan. Similar to Mopeia, HLC collection was also dropped in February 2020. For susceptibility tests, Prokopack aspirators were used in all districts to collect adult *An. funestus* s.l., and larval collections were conducted to collect *An. gambiae* s.l. mosquitoes. Samples collected from July 2019 up to March 2020 were selected and sent to the National Institute of Health (*Instituto Nacional de Saúde*, INS) laboratory for molecular analyses. Due to COVID-19 restrictions, only partial results are available and included in this report. Figure 1A illustrates the entomological monitoring districts and villages from the intervention and control sites in Zambezia. The susceptibility test is conducted in two different season due to abundance of the species in different season, *An. funestus* s.l., occurring mostly from June to September (dry season), while *An. gambiae* s.l. occurring mostly from January to April (rain season).

FIGURE 1A. ZAMBEZIA PROVINCE IRS INTERVENTION AND CONTROL DISTRICTS, AND ENTOMOLOGICAL SENTINEL SITES



The government of Mozambique, through the NMCP, conducted IRS in eight districts of Nampula Province: Angoche, Meconta, Monapo, Murrupula, Nacala, Nampula City, Rapale, and Ribaue and a mass ITN distribution campaign in all districts of Nampula; the campaign started on July 15 and ended in September 2019. VectorLink Mozambique provided technical support to Nampula Province for the implementation of entomological surveillance in two IRS intervention districts, Monapo and Nampula City, as well as in the control district of Erati. The map of Nampula in Figure 1B shows the province's IRS intervention and control districts, together with the entomological sentinel sites.

FIGURE 1B: NAMPULA PROVINCE IRS INTERVENTION AND CONTROL DISTRICTS, AND ENTOMOLOGICAL SENTINEL SITES



2. METHODOLOGY

2.1 LONGITUDINAL MONITORING

Data were collected from July 2019 through March 2020 using PSC, Prokopack collections, HLC, and CDC light trap collections. Data were not collected in April, May, or June due to travel restrictions imposed due to the COVID-19 pandemic.

2.1.1 PYRETHRUM SPRAY CATCH AND PROCKOPACK COLLECTIONS

The PSC method was used to collect mosquitoes and determine the indoor resting density of malaria vectors at sentinel sites in selected IRS intervention and control districts in Zambezia and Nampula provinces. In Zambezia, PSCs were conducted in the intervention districts of Maganja da Costa and Milange, and the control district of Lugela. In Nampula, PSCs were conducted in the intervention districts of Monapo and Nampula City and the control district of Erati. Ten houses in each of two villages in each district were selected for PSC, totalling 20 houses per district. PSC was conducted from 6:00 am to 8:00 am, once per month over four consecutive days in each district. The same houses were visited each month. Data were collected in five houses per day per district. The first collection was conducted three months prior to the IRS campaign and collection continued after the campaign. Baygon (commercial nomenclature) aerosol was used to knock down mosquitoes. It contains the pyrethroids deltamethrin 0.5 g/kg and imiprothrin 1.0 g/kg. In each house, one sleeping room was used for PSC. The room was closed for 10 minutes after spraying, and then knocked-down mosquitoes were collected using forceps and transferred into a labeled petri dish. The samples were identified morphologically and preserved in 1.5 ml Eppendorf tubes containing silica gel for further identification using the Polymerase Chain Reaction (PCR) technique, and a set of samples collected by this method during the July-March period were sent for PCR at the INS laboratory.

VectorLink Mozambique replaced PSC with Prokopack collections in September 2019 to estimate indoor resting densities in all collection sites per the approved work plan in alignment with the national entomological procedures. Prokopack collections were carried out in the morning between 06:00 and 08:00 am. Before the collection was performed, the team secured the household head's verbal consent, and asked all occupants to leave the house before collections. The Prokopack used a sealed, lead acid, rechargeable 12-volt battery. One team member entered the room and connected the aspirator to the battery terminals. After fitting the collection cup, the mosquitoes were aspirated systematically, starting from the door, moving on to the walls and furniture and then under beds and tables, and finishing with the roof or ceiling. Live mosquitoes in the cups were transferred first to small cages and then to paper cups. The mosquitoes were killed with chloroform, counted, and recorded their abdominal stage on the form, and placed them in a petri dish for morphological identifications and preserved in 1.5 ml Eppendorf tubes containing silica gel for further identification using the Polymerase Chain Reaction (PCR) technique.

2.1.2 HUMAN LANDING CATCHES

In Zambezia Province, HLCs were conducted in the intervention districts of Maganja da Costa, Milange, and Mopeia, and the control district of Lugela. In Nampula Province, HLCs were conducted in the intervention districts of Nampula City and Monapo and in the control district of Erati. With the exception of Mopeia, two houses were sampled in a selected village for three consecutive nights to obtain six person-nights of collection per district per month (2 houses x 3 collection nights = 6 person-nights). In Mopeia, through October 2019, one house in each village in the intervention and control areas was selected for a total of eight houses (four in intervention and four in control districts). Collections were conducted on three consecutive nights to obtain 12 person-nights per area per month (4 houses x 3 collection nights = 12 person-nights). From November 2019 forward, the HLC collection design was changed to match that of other districts. In all districts, two human

volunteers were positioned, one inside the house and the other outside, to collect mosquitoes. Collections were conducted from 6:00 pm to 6:00 am. Over each hour, collectors collected mosquitoes for 50 minutes and rested for 10 minutes, during which they exchanged positions and recorded humidity and temperature. During the time of collection, the collectors sat on a small chair and exposed their legs up to the knee; when they felt landing mosquitoes, they turned on a torch and collected the mosquitoes using a mouth aspirator. Collected mosquitoes were transferred into labeled paper cups assigned for each hourly collection. Collected mosquitoes were subsequently killed using cotton soaked in chloroform, identified, counted by species, location, and hour of collection, and preserved in 1.5 ml Eppendorf tubes with silica gel. The HLC collection was stopped in February 2019 in both provinces on PMI guidance due to ethical issues associated with the method and alignment with the national entomological procedures.

2.1.3 CDC Light Trap

In Zambezia Province, CDC light traps were installed in four houses in two of the intervention districts, Maganja da Costa and Milange, as well as in the control district of Lugela. Likewise, in Nampula Province, CDC light traps were installed in four houses in the two intervention districts of Monapo and Nampula City and the control district of Erati. Each month, the traps were set over three consecutive nights, from 6:00 pm to 6:00 am, for a total of 12 trap nights per month for each district.

In Mopeia, 10 villages were selected (five in intervention areas and five in control areas) for CDC light trapping. Eight houses were selected in each village. Data were collected on three consecutive nights, from 6:00 pm up to 6:00 am. This resulted in 240 traps nights per month, with an equal number in intervention and control areas. The described scheme of collection from Mopeia was implemented through October 2019. From November 2019 through March 2020, the Mopeia collection schedule was similar to the other Zambezia districts of Maganja da Costa, Milange, and Lugela as Mopiea's sites are now considered part of routine monitoring in Zambezia.

The traps were set up inside the house in the bedroom, at the foot of a bed with humans sleeping under untreated bed nets, about 1.5 m above the floor. After each night of collection, chloroform was used to kill the mosquitoes in the paper cups, and the mosquitoes were identified and preserved in 1.5 ml Eppendorf tubes for future species identification based on PCR. The same houses were used each month.

A subset of samples from these collections conducted in Maganja da Costa, Milange, Mopeia, and Lugela was sent to INS for PCR analyses.

2.2 IRS Quality Assays and Insecticide Decay Rate Monitoring

Standard WHO cone bioassay tests were performed in Maganja da Costa, Milange, and Mopeia districts (in the villages of Nroga, 3 de Fevereiro, and Josina Machel) from October and November 2019 through March 2020 to evaluate spray quality and residual efficacy of the insecticide used in the 2019 spray campaign. In Nampula province wall bioassays were also conducted in Nampula and Monapo districts. Wall bioassays were conducted 24 hours after spraying and then monthly up to March 2020; they had to be suspended before mortality dropped below 80% for two consecutive months due to restriction imposed by COVID-19. However, the bioassays resumed in August 2020 both in Zambezia and Nampula provinces.

In each district village, five houses were randomly selected. The same houses were used each month. Cones were placed at heights of 0.5 m, 1.0 m, and 1.5 m above the floor, arranged diagonally across a wall surface. Cones lined with self-adhesive tape were fixed on the sprayed walls for the assay. The control cone was affixed to a wall lined with a paperboard with adhesive in an unsprayed house or in the shade of a tree in the yard away from the sprayed house to avoid any potential airborne effect. Two- to five-day-old female mosquitoes were used for the tests. Susceptible *An. arabiensis* KGB strain mosquitoes were introduced into the plastic cones in batches of 10 and left exposed on the sprayed surface for 30 minutes at different heights. Numbers of mosquitoes knocked down at the 30th minute were recorded. At the end of the 30-minute exposure period, the mosquitoes were carefully collected and transferred to paper cups and provided with 10% sugar solution soaked

on cotton wool pads placed on top of the paper cups covered with net. Dead and live mosquitoes were counted at 24-hour interval up to seven days for both Fludora® Fusion and SumiShield® 50WG, and the percentage mortality was calculated in the replicates for each house and recorded according to the WHO protocol.

Tests for the airborne effect were conducted with mosquitoes placed inside of a small cage made from untreated nets and wire and hung 10 cm away from the sprayed wall surface for 30 minutes at a height of 1.5 m above the floor. The mosquitoes were transferred into clean paper cups that were kept for holding periods. Dead and live mosquitoes were counted after each 24 hours up to seven days and mortality recorded as described above.

2.3 VECTOR SUSCEPTIBILITY TESTING

From July to October 2019, adult unfed (not blood fed) *An. funestus* s.l. mosquitoes that are difficult to find at the immature stage were collected using Prokopack aspirators and directly used for susceptibility testing in Zambezia Province (Maganja da Costa, Milange, and Mopeia). Immature *An. gambiae* s.l. malaria vectors were collected from different larval habitats in Zambezia (Maganja da Costa, Milange, Mopeia, Molumbo, Morrumbala, and Lugela districts) and Nampula (Monapo, Nampula City, and Erati) from January to April 2020, and reared to adults for susceptibility tests.

Field-collected larvae of *An. gambiae* s.l. were reared in the insectary to adult stage. Batches of 25 females, sugar-fed and aged from three to five days, were subsequently subjected in four replicates to the WHO tube tests following the standard WHO 2016 protocol. The mosquitoes were exposed to pirimiphos-methyl 0.25%, deltamethrin 0.05%, permethrin 0.75%, DDT 4%, bendiocarb 0.1%, and clothianidin 2%. Chlorfenapyr (100µg/bottle) was also tested following the CDC botte assay tests. Knockdown was scored at 60 minutes immediately after the exposure period, at which time all mosquitoes were gently transferred to holding tubes and paper cups. Mortality was recorded at 24 hours after exposure and followed up to three or seven days for chlorfenapyr and clothianidin, respectively. Where control mortality scored higher than 5% but below 20%, Abbott's correction was applied to test mortalities and those above 20% led to tests being discarded (Abbott 1925). Susceptibility levels of *An. gambiae* s.l. were evaluated based on WHO criteria (WHO 2016).

Intensity assays were conducted by exposing vector mosquitoes to insecticide dosages of 5× and 10× the diagnostic concentrations of deltamethrin and permethrin, according to the standard WHO bioassay method. All exposures were for one hour, and final mortality was scored after a 24-hour holding period, during which a 10% sugar solution was made available to surviving mosquitoes.

The synergist assays were conducted using mosquitoes reared from field-collected larvae. Four bioassay exposures were done as follows: In the first group of replicates, the mosquitoes were exposed to the insecticide only (permethrin and deltamethrin), the second group was exposed to 4% piperonyl butoxide (PBO) only, the third group to 4% PBO followed by insecticide, and the last group was exposed to the solvent (control). All replicates were exposed for 60 minutes and mortality was recorded 24 hours after exposure, according to the WHO (2016) protocol. This process was repeated three times based on the standard procedure.

All the above susceptibility tests were conducted to the extent possible under the recommended optimal conditions, at temperatures around 27°C +-2°C and 70–80% relative humidity. Similar to other collections, a portion of samples from these tests were sent to the INS laboratory for PCR assays to identify sibling species and detect the presence of knockdown (*kdr)* and acetylcolinesterase-1 (*Ace-1*) genes.

2.4 STATISTICAL TESTS

The average number of mosquitoes collected by the HLC method was calculated. To compare mean indoor and outdoor biting rates, Chi-square tests were used, and *P* values less than 0.05 were considered significant.

2.5 MOLECULAR ANALYSIS

A total of 1913 *An. gambiae* s.l. and *An. funestus* s.l. mosquito samples collected by PSC, HLC and CDC light trap collections from Maganja da Costa, Milange, Mopeia and Lugela sites were processed by PCR to identify the sibling species. Additionally a total of 181 *An. gambiae* s.l. and *An. funestus* s.l. samples collected by PSC or

Prokopack collections from the same sites were also processed by PCR to identify the blood meal sources. Identification of the sibling species of *An. gambiae* complex was based on the protocol from Scott *et al.* (1993) and, for *An. funestus* group, it was based on Koekemoer *et al.* (2002), while the blood meal sources was based on Kent and Norris (2005). Sporozoite test results are not included in this report and shall be reported in an addendum once available.

3. RESULTS: ZAMBEZIA

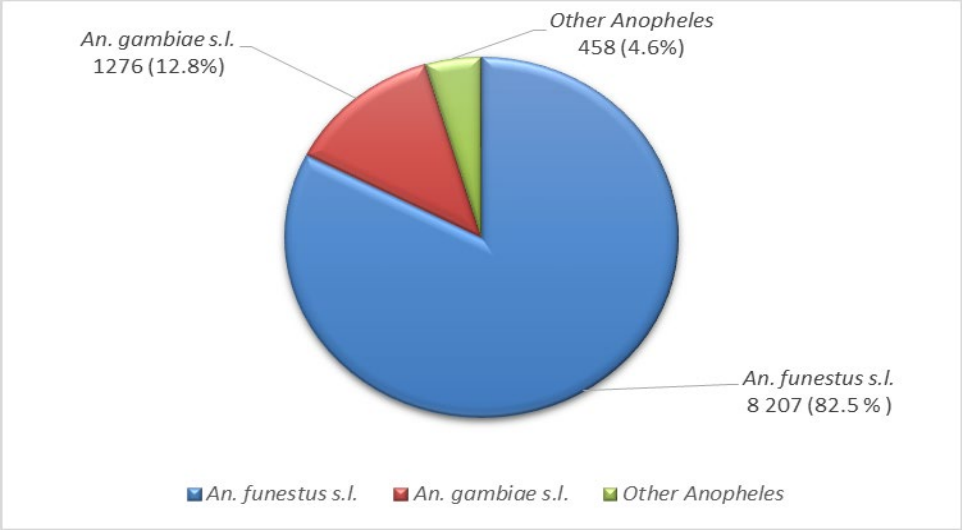
3.1 ANOPHELINE SPECIES COLLECTED BY THE DIFFERENT METHODS

During the reporting period, in the intervention districts of Maganja da Costa and Milange, the intervention and previous control arms of Mopeia, and the control district of Lugela, a total of 9,941 anopheline mosquitoes belonging to nine different species and species complexes were collected using the three collection methods (PSC plus Prokopack, CDC light trap, and HLC) and morphologically identified. The anophelines included *An. funestus* s.l., *An. gambiae* s.l., *An. coustani*, *An. pharoensis*, *An. pretoriensis*, *An. tenebrosus*, *An. ziemanni*, *An. caliginosus,* and *An. maculipalpis*. Table 1 and Figure 2 summarize the number of mosquitoes collected, by species. *An. funestus* s.l. was the most abundant anopheline collected, accounting for 82.5% of all collections, followed by *An. gambiae* s.l. at 12.8% and other anophelines at 4.6 %.

TABLE 1. NUMBER OF MOSQUITOES COLLECTED IN EACH DISTRICT BY ALL COLLECTION METHODS

Species Collected	Maganja da Costa	Milange	Mopeia	Lugela	Total per Species
An. funestus s.l.	556	279	5,353	2,019	8,207
An. gambiae s.l.	143	658	261	214	1,276
An. coustani	1	0	6	4	11
An. pretoriensis	0	0	1	0	1
An. tenebrosus	2	7	214	0	223
An. ziemanni	48	1	160	6	215
An. maculipalpis	0	0	0	4	4
An. pharoensis	3	0	0	0	0
An. caliginosus	0	0	1	0	4
Total	753	945	5,996	2,247	9,941

FIGURE 2. SPECIES COMPOSITION OF *ANOPHELES* S.L. MOSQUITOES FOR ALL SITES IN ZAMBEZIA



3.1.1 Pyrethrum Spray Catch and Prokopack Collections

PSC and Prokopack collections yielded a total of 562 *Anopheles* mosquitos (Table 2). Based on morphological identification, 534 of these belonged to *An. funestus* s.l. (95.01%) and 28 *An. gambiae* s.l. (4.98%). No other species were detected by PSC method.

Table 2. Number of Mosquitoes by Species Collected Using PSC and Prokopack in Three Intervention Districts and the Control District

Mosquito Species/District	Maganja da Costa	Milange	Mopeia	Lugela	Total
An. funestus s.l.	64	105	135	230	**534**
An. gambiae s.l.	6	11	4	7	**28**
Total	**70**	**116**	**139**	**237**	**562**

The indoor resting density of *An. funestus* s.l. and *An. gambiae* s.l. estimated from PSCs and Prokopacks was very low in both intervention and control sites. The highest indoor resting density was less than five and one per room per day for *An. funestus* s.l. and *An. gambiae* s.l., respectively (Figures 3A and 3B). These low indoor resting densities were found at most of the collection sites before and after IRS intervention during the monitoring period.

Figure 3. Mean Indoor Resting Densities of *An. funestus* s.l. and *An. gambiae* s.l. in Three Districts Before and After IRS Intervention as Estimated from the PSC and Prokopack Collections

Figure 3A. *An. funestus* s.l.

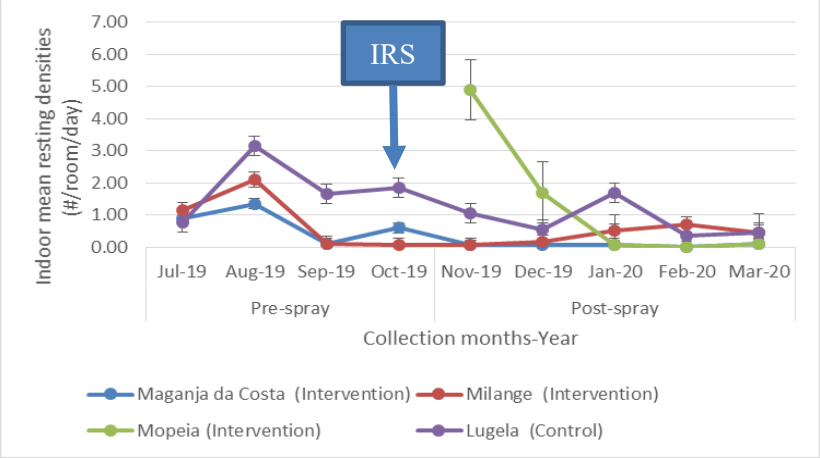
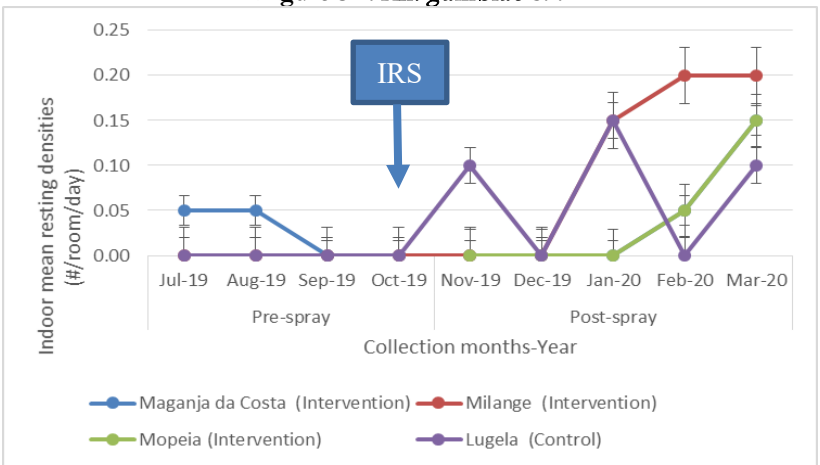


Figure 3B. *An. gambiae* s.l.



3.1.2 HUMAN LANDING CATCHES

A total of 2,606 *Anopheles* mosquitoes were collected using the HLC technique. Morphological identification showed that 1,765 were *An. funestus* s.l., 399 *An. gambiae* s.l., 213 *An. tenebrosus,* 211 *An. ziemanni,* 10 *An. coustani,* 4 *An. maculipalpis,* 3 *An. pharoensis,* and 1 *An. caliginosus*. Mopeia and Maganja da Costa had the highest anopheline diversity, followed by Lugela and Milange.

Table 3 shows a significant difference between total numbers of *An. funestus* s.l. samples collected indoors and outdoors ($p<0.05$) in Maganja da Costa, Mopeia, and Lugela (control). More *An. funestus* s.l. mosquitoes were collected indoors than outdoors in all districts including the control (Lugela).

An. gambiae s.l. mosquitoes were collected both outdoors and indoors, with relatively higher numbers collected indoors in Maganja da Costa, and higher numbers collected outdoors in the other three districts. Significantly higher numbers of *An. gambiae* s.l. samples were collected outdoors than indoors ($p<0.05$) in Milange.

TABLE 3. COMPARISON OF TOTAL NUMBER OF *AN. FUNESTUS* S.L. AND *AN. GAMBIAE* S.L. COLLECTED BY HLC INDOORS AND OUTDOORS IN FOUR DISTRICTS

District	*An. funestus* s.l.				*An. gambiae* s.l.			
	# Collected indoors	# Collected outdoors	X^2	p-value	# Collected indoors	# Collected outdoors	X^2	p-value
Maganja da Costa	153	82	21.45	$3.62\times10^{-6*}$	55	43	1.47	0.23
Milange	50	47	0.09	0.76	54	154	48.08	$4.09\times10^{-12*}$
Mopeia	337	250	12.89	0.00033*	7	11	0.89	0.35
Lugela (Control)	587	259	127.17	$1.71\times10^{-29*}$	35	40	0.33	0.56

*p-value significant

Table 4 summarizes the overall outdoor and indoor collections by species from intervention and control districts providing mean biting per person per night (b/p/n) per area; the control area experienced about twice (1.217 b/p/n) the biting rate of the intervention area (0.725 b/p/n). When observing the *An. funestus* s.l. biting rate alone, the biting rate in the control area was about three times (8.813 b/p/n) higher than the rate in the combined intervention areas (3.191 b/p/n).

Table A5 in the annex indicated that both *An. ziemanni* and *An. tenebrosus* are biting with the same proportion indoor and outdoor, or there is no significant difference between indoor and outdoor biting rate for both species in Milange, Mopeia and Lugela (control). In Maganja da Costa, *An. ziemanni* was biting mostly outdoors, while *An. tenebrosus* was biting with same proportion indoor and outdoor.

TABLE 4. MOSQUITO SPECIES COLLECTED BY HLC AND THEIR COMBINED OUTDOOR AND INDOOR MEAN BITING RATES IN INTERVENTION DISTRICTS OF MAGANJA DA COSTA, MOPEIA, AND MILANGE AND CONTROL AREA OF LUGELA

Species Collected	Intervention Area			Control Area		
	Total numbers collected	Total person nights	b/p/n	Total numbers collected	Total person nights	b/p/n
An. funestus s.l.	919	288	3.191	846	96	8.813
An. gambiae s.l.	324	288	1.125	75	96	0.781
An. coustani	6	288	0.021	4	96	0.042
An. tenebrosus	213	288	0.740	0	96	0.000
An. ziemanni	205	288	0.712	6	96	0.063
An. caliginosus	1	288	0.003	0	96	0.000
An. pharoensis	3	288	0.010	0	96	0.000
An. maculipalpis	0	288	0.000	4	96	0.042
Total	1,671	2304	0.725	935	768	1.217

Figures 4A and 4B show that *An. funestus* s.l. demonstrated a similar biting pattern throughout the period both indoors and outdoors, albeit with a biting intensity almost two times higher indoors than outdoors in Lugela. The biting rate was found to be low before IRS, ≤12 b/p/n indoors and ≤4.58 b/p/n outdoors in all districts. In the control district, where the biting rate was higher than in the interventions areas, the rates peaked in January 2020 indoors (at 30.00 b/p/n) and December 2019 outdoors (at 14.17 b/p/n). This was followed by a dramatic decrease that bottomed out in February 2020 at 9.17 b/p/n indoors and 4.83 b/p/n outdoors. In the intervention districts of Maganja da Costa, Mopeia, and Milange, indoor bites remained relatively low after IRS, at below 4.17 b/p/n up to February. A similar pattern was observed outdoors, where biting remained below 3.17 b/p/n up to February 2020.

FIGURE 4. INDOOR AND OUTDOOR MEAN HUMAN BITING RATES FOR *AN. FUNESTUS* S.L. AND *AN. GAMBIAE* S.L. IN THREE INTERVENTION DISTRICTS AND ONE CONTROL DISTRICT, BEFORE AND AFTER IRS

Figure 4A. *An. funestus* s.l. Indoor

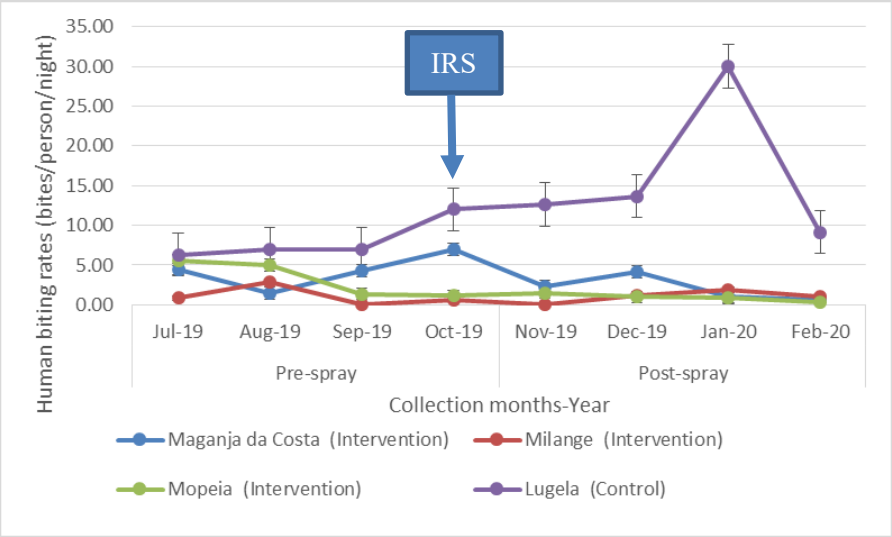


Figure 4B. *An. funestus* s.l. Outdoor

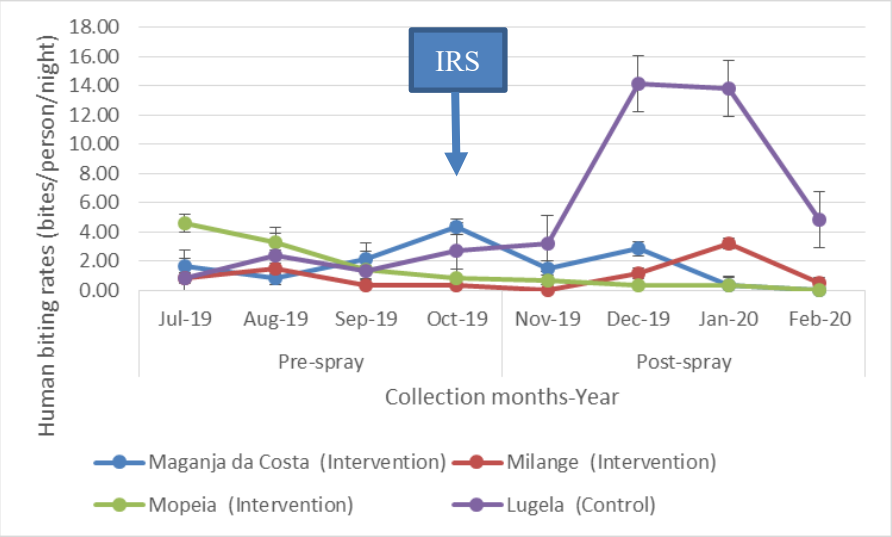


Figure 4C shows that the *An. gambiae* s.l. biting rate before IRS was observed to be low both indoors (≤1.83 b/p/n) and outdoors (≤1.50 b/p/n). The indoor biting rates dropped close to zero in the month immediately after IRS (November) in all three intervention districts. In Maganja da Costa, it started increasing again in December (1.83 b/p/n) before dropping again in January (0.33b/p/n) and February (0.67b/p/n). In Milange,

the biting rate increased from December (1b/p/n) through February (4.17 b/p/n), during the rainy season characterized by intermittent rainfall. In Mopeia, the *An. gambiae* s.l. biting rate was low throughout the monitoring period. In Lugela, the control district, the biting rates increased gradually and peaked in February (3.83b/p/n).

The *An. gambiae* s.l. outdoor biting rate was low in the three intervention districts up to December (≤2.17 b/p/n) as well as in January and February for Mopeia and Maganja da Costa (≤0.5 b/p/n) (Figure 4D). In Milange (≤13.33 b/p/n) and Lugela (≤3.67 b/p/n), it began increasing in December and continued increasing through February. This trend is similar to the indoor ones.

Figure 4C. *An. gambiae* s.l. Indoor

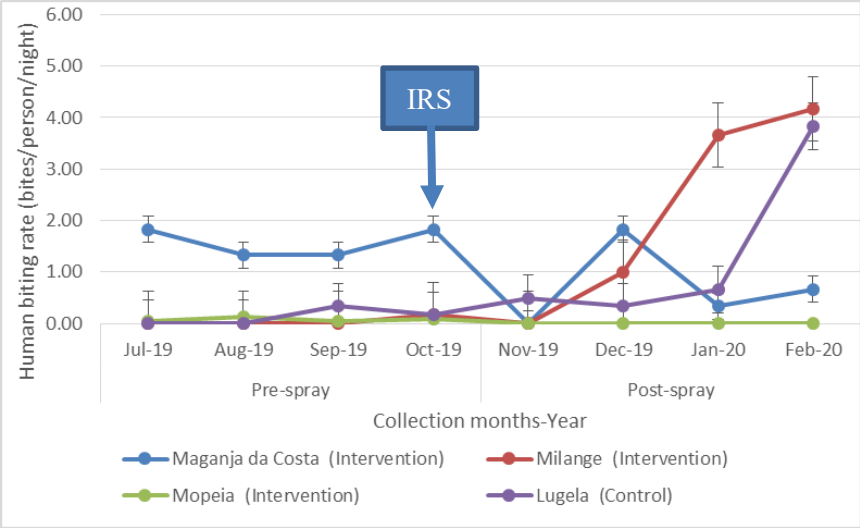


Figure 4D. *An. gambiae* s.l. Outdoor

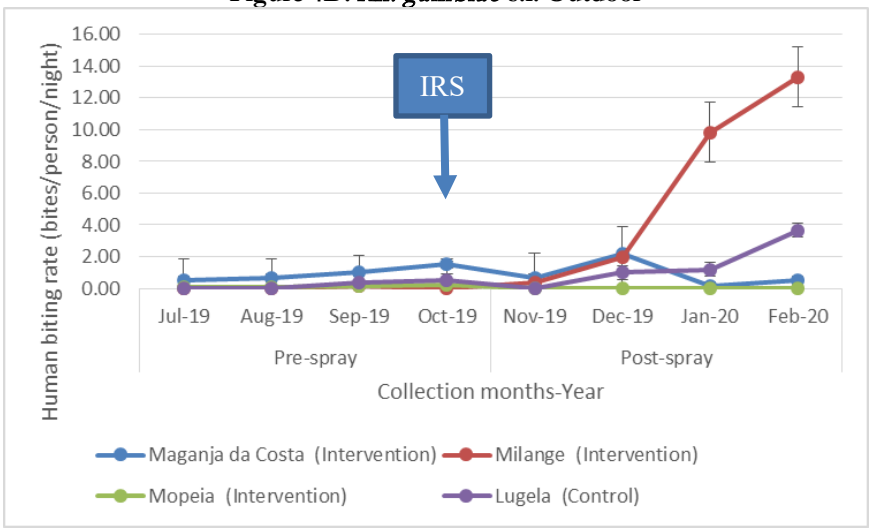


Table 5 shows the mean indoor and outdoor vector biting rates for *An. funestus* s.l. and *An. gambiae* s.l. four months before and four months after spraying. For *An. funestus* s.l. the indoor biting rate decreased after IRS in Maganja da Costa (4.33 to 2.04 b/p/n), Milange (1.08 to 1.00 b/p/n), and Mopeia (3.28 to 0.92 b/p/n); in contrast, in Lugela (control), the mean biting rate increased by around twofold (8.08 to 16.38 b/p/n), indicating the potential effect of the IRS. The trend in the outdoor mean biting rate was similar. For *An. gambiae* s.l., variation in the mean biting rate by district was observed both indoors and outdoors as well as pre- and post-spay seasons. In Maganja da Costa and Mopeia, the indoor mean biting rate decreased from pre to post spray

(1.58 to 0.71 b/p/n and 0.07 to 0.00 b/p/n, respectively), while in Milange and Lugela (control), it increase slightly (0.04 to 2.21 b/p/n and 0.13 to 1.33 b/p/n).

TABLE 5. INDOOR AND OUTDOOR MEAN BITING RATE FOR *AN. FUNESTUS* S.L. AND *AN.* GAMBIAE S.L., ESTIMATED USING HLC FROM ALL COLLECTION ROUNDS, BY DISTRICT, BEFORE AND AFTER SPRAYING

District	*An. funestus* s.l. (b/p/n)				*An. gambiae* s.l. (b/p/n)			
	Indoors		Outdoors		Indoors		Outdoors	
	Pre-spray	Post-spray	Pre-spray	Post-spray	Pre-spray	Post-spray	Pre-spray	Post-spray
Maganja	4.33	2.04	2.25	1.17	1.58	0.71	0.92	0.88
Milange	1.08	1.00	1.17	1.21	0.04	2.21	0.04	6.38
Mopeia	3.28	0.92	2.52	0.33	0.07	0.00	0.11	0.00
Lugela*	8.08	16.38	1.79	9.00	0.13	1.33	0.21	1.46

*Unsprayed control District.

The indoor and outdoor overnight biting pattern of *An. funestus* s.l., is depicted in Figures 5A and 5B respectively. Both indoor and outdoor biting activity in all districts appears to follow the same pattern. In Lugela district (control), indoor biting activity remained consistently above 8.0 bites per person per hour (b/p/h) for most of the night, with peak biting activity observed from around midnight until early morning (4-5 am) and then decreasing (4.17 b/p/h) at 5-6 am. In Milange, the highest hourly biting was observed at 1-2 am, although it was relatively low (2.17 b/p/h) throughout the monitoring period, indicating the low biting activity of *An. funestus* s.l. as compared with other intervention and control districts. In Mopeia, the highest indoor biting activity was observed at two different times, at 2-3 am and 5-6 am, at around (3.25 b/p/h) and (3.58 b/p/h), respectively. A similar biting pattern was observed in Maganja da Costa and Milange, where the highest biting activity was 2-3 am (5.50 b/p/h) and 1-2 am (2.17 b/p/h), respectively. The outdoor biting activity in Lugela (control) was high in comparison with all the intervention districts, and outdoor biting activity there was lower than the indoor biting. This indicates that *An. funestus* s.l. tends to feed mostly indoors when people are sleeping. The highest outdoor biting activity in Lugela (control district) was observed during the early morning hours, 1-2 am and 3-4 am, at 7.50 b/p/h and 6.00 b/p/h, respectively. In all intervention districts, the highest outdoor biting activity was observed during late night at 11 pm-12 am in Mopeia and early morning (2-3 am) in Maganja da Costa, reaching (2.75 b/p/h) and (2.67 b/p/h), respectively. The biting activity in Milange was less than that of Maganja da Costa and Mopeia during most of the night.

FIGURE 5. HOURLY BITING RATES OF *AN. FUNESTUS* S.L. IN MAGANJA DA COSTA, MILANGE, MOPEIA, AND LUGELA AS DETERMINED THROUGH HLCS

Figure 5A. *An. funestus* s.l. Indoor

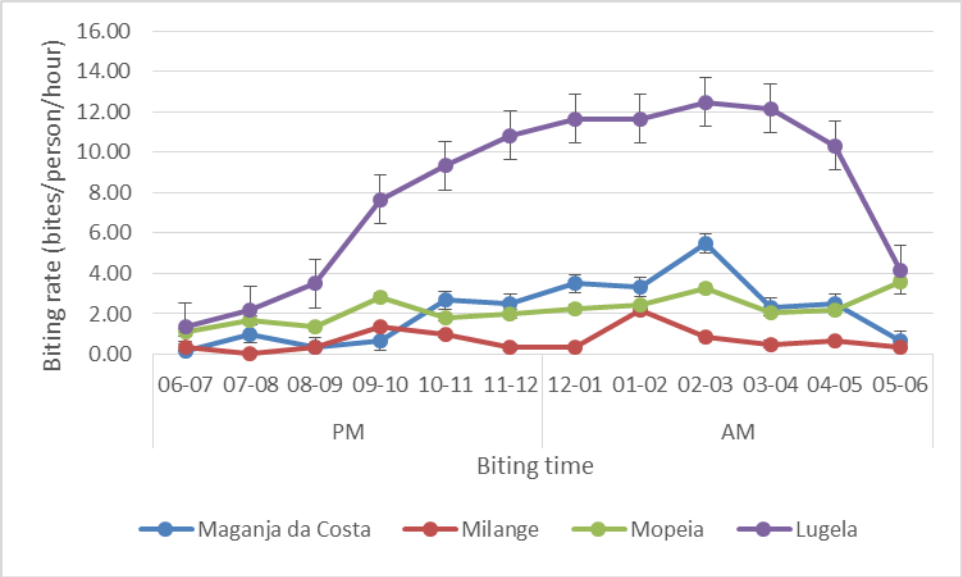
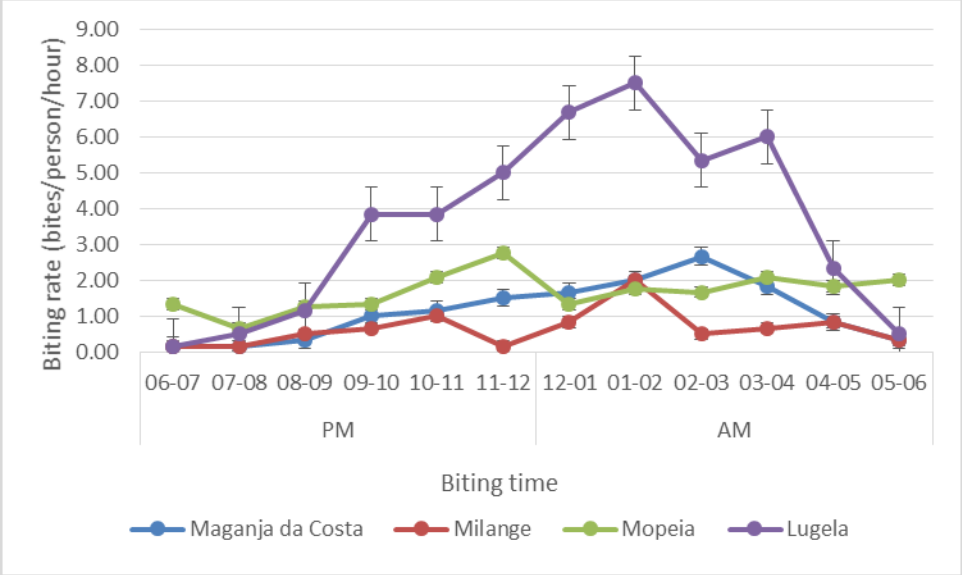


Figure 5B. *An. funestus* s.l. Outdoor



The indoor and outdoor hourly biting activity for *An. gambiae* s.l. is shown in Figures 5C and 5D, respectively. The indoor biting activity remained below 1.0 b/p/h in all districts from 6:00 pm to 10:00 pm and then it started increasing albeit with variations in the hourly biting peaks. Maganja da Costa district showed three different biting peaks, at 11 pm-12 am, 1-2 am, and 3-4 am, with the biting rates varying from 1.67 to 1.83 b/p/h. In Milange district, the biting rate was less than 1.0 b/p/h at 9-10 pm; then it fell to below 0.5 b/p/h during the next hour before increasing to the peak hourly biting of 1.67 b/p/h at around 1-2 am. Outdoors, Milange showed the highest outdoor hourly biting rate through the collection hours, with two main hourly biting peaks at 11 pm-12 am and 12-1 am at biting rates of 3.33 and 3.00 b/p/h, respectively. Maganja da Costa's outdoor biting peak occurred at around 1-2 am with a biting rate of 2.17 b/p/h. The outdoor hourly biting rate of *An. gambiae* s.l. seems to be generally higher than the indoor rate, mainly in Milange district.

Figure 5C. *An. gambiae* s.l. Indoor

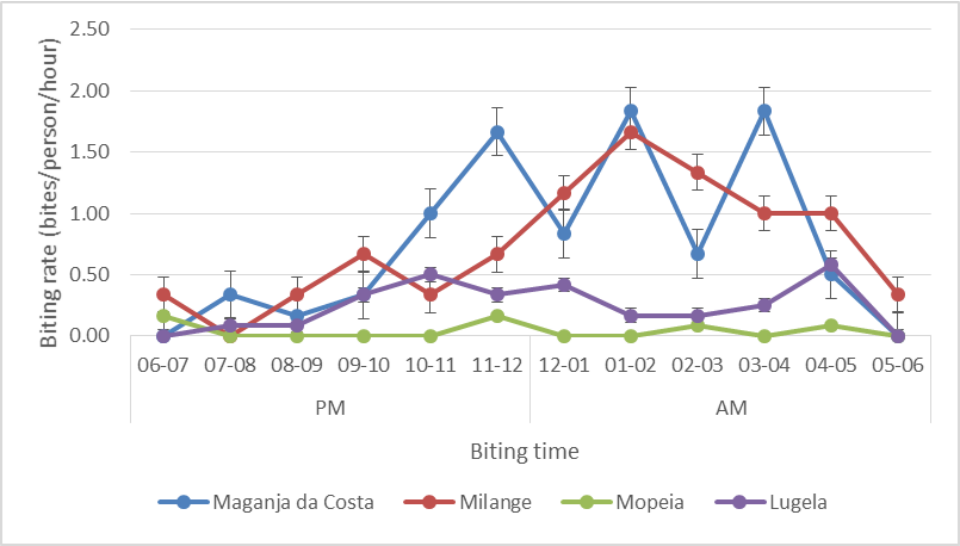
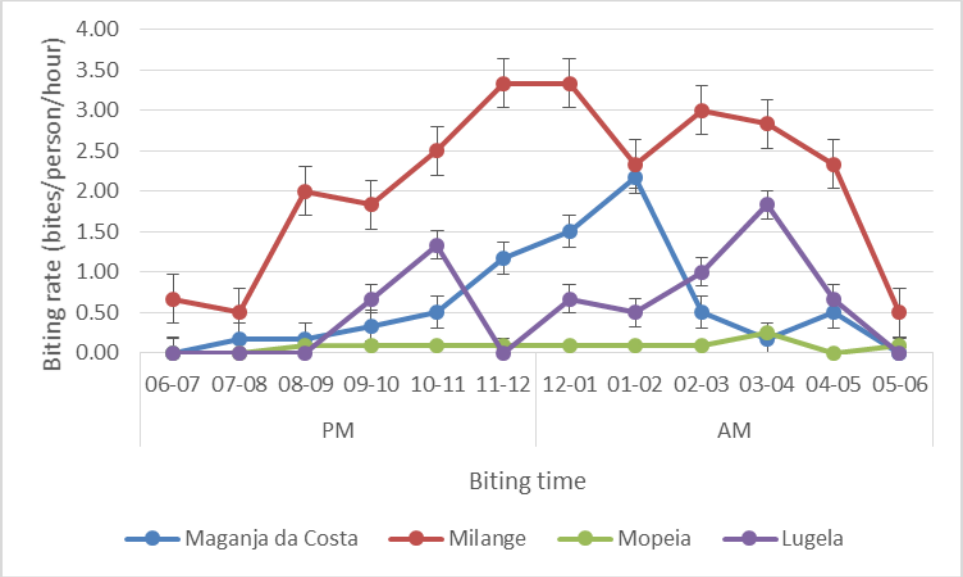


Figure 5D. *An. gambiae* s.l. Outdoor



3.1.3 CDC Light Trap

The CDC light trap collections yielded a total of 6,773 *Anopheles* mosquitoes from three intervention districts (Maganja da Costa, Milange, and Mopeia) and the control district (Lugela). Morphological identification of the mosquitoes revealed that 5,908 (87.23%) were *An. funestus* s.l., 849 (12.54%) *An. gambiae* s.l., 10 (0.15%) *An. tenebrosus,* 4 (0.06%) *An. ziemanni*, 1 (0.01%) *An. coustani,* and 1 (0.01%) *An. pretoriensis*. Lugela (control) had the second highest percentage of all *Anopheles* collected, 15.96%. The highest percentage of (78.38%) was observed in the 10 sampled villages in Mopeia compared with all other districts; in Mopeia, the collections were conducted through October 2019 and the data from the villages were aggregated. This was because the cluster randomized study ended in October 2019 and the whole of Mopeia became an IRS district.

Table 6 provides a summary of CDC light trap data from monthly collections in the four districts. Comparing mosquito densities between control and intervention districts, the data showed a significant difference in *An. funestus* s.l. between vector densities recorded in Lugela (control) and Milange (intervention) ($p<0.05$, X^2 =

6.81); no significant difference was observed between mean densities from Maganja da Costa (p=0.056) and Mopeia (p=0.79). The mean densities of *An. gambiae* s.l. were not statistically different (p≥0.05 and X^2 of 1.53, 0.47, and 0.05 for Milange, Maganja da Costa, and Mopeia, respectively) from Lugela (control).

TABLE 6. SUMMARY OF CDC LIGHT TRAP DATA FROM MONTHLY COLLECTION IN FOUR DISTRICTS: MAGANJA DA COSTA, MILANGE, MOPEIA, AND LUGELA

Districts	Species	2019						2020			Total	
		Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar		
Maganja	*An. funestus* s.l.	8	8	26	89	22	80	13	7	4	**257**	**296**
	Trap nights	12	12	12	12	12	12	12	12	12		
	Mean # Mosq/trap/night	0.67	0.67	2.17	7.42	1.83	6.67	1.08	0.58	0.33	**2.38**	
	An. gambiae s.l.	2	1	4	6	2	8	3	1	12	**39**	
	Trap nights	12	12	12	12	12	12	12	12	12		
	Mean # Mosq/trap/night	0.17	0.08	0.33	0.50	0.17	0.67	0.25	0.08	1.00	**0.36**	
Milange	*An. funestus* s.l.	3	2	1	0	0	3	32	5	31	**77**	**516**
	Trap nights	12	12	12	12	12	12	12	12	12		
	Mean # Mosq/trap/night	0.25	0.17	0.08	0.00	0.00	0.25	2.67	0.42	2.58	**0.71**	
	An. gambiae s.l.	0	2	0	0	1	4	121	156	155	**439**	
	Trap nights	12	12	12	12	12	12	12	12	12		
	Mean # Mosq/trap/night	0.00	0.17	0.00	0.00	0.08	0.33	10.08	13.00	12.92	**4.06**	
Mopeia	*An. funestus* s.l.	1501	1066	897	538	145	207	82	133	62	**4631**	**4870**
	Trap nights	240	240	240	240	12	12	12	12	12		
	Mean # Mosq/trap/night	6.25	4.44	3.74	2.24	12.08	17.25	6.83	11.08	5.17	**7.68**	
	An. gambiae s.l.	38	60	32	20	0	0	4	14	71	**239**	
	Trap nights	240	240	240	240	12	12	12	12	12		
	Mean # Mosq/trap/night	0.16	0.25	0.13	0.08	0.00	0.00	0.33	1.17	5.92	**0.89**	
Lugela	*An. funestus* s.l.	55	99	92	45	55	130	235	133	99	**943**	**1075**
	Trap nights	12	12	12	12	12	12	12	12	12		
	Mean # Mosq/trap/night	4.58	8.25	7.67	3.75	4.58	10.83	19.58	11.08	8.25	**8.73**	
	An. gambiae s.l.	2	0	1	1	1	7	5	40	75	**132**	
	Trap nights	12	12	12	12	12	12	12	12	12		
	Mean # Mosq/trap/night	0.17	0.00	0.08	0.08	0.08	0.58	0.42	3.33	6.25	**1.22**	
Total		1609	1238	1053	699	226	439	495	489	509	6757	

Table 6 also shows that, in terms of mean collections per trap per night, *An. funestus* s.l., at 8.73 mosquitoes per trap per night (m/t/n) over the 12 collection months, was most abundant in Lugela (control), and followed by 7.68 m /t /n in Mopeia.

Table 6, as well as Figure 6A and 6B, clearly show that before IRS, CDC light traps recorded low *An. gambiae* s.l. mean densities, estimated at less than 0.3 m/t/n in both intervention and control districts. *An. funestus* s.l. were more than twentyfold higher, estimated at 6.06 m/t/n in Lugela and 0.27 m/t/n in Maganja da Costa. In Milange and Maganja da Costa, both intervention districts, densities of *An. funestus* s.l. remained low (1.18 m/t/n and 2.10 m/t/n, respectively) following IRS. In Mopeia, another intervention district, the mean density of *An. funestus* s.l. increased but it was not statistically significant X^2= 2.72 and p=0.099. Mean densities of *An. gambiae* s.l., however, increased in all intervention districts and also in the control district, starting in December. The increase was not statistically significant (p>0.05) in Maganja da Costa (p=0.95), Mopeia (p=0.29), and Lugela (control) (p=0.168), but it was in Milange (X^2 = 7.16 and p=0.0074). These increases might be due to an increase in *An. gambiae* s.l. populations during the rainy season.

FIGURE 6. INDOOR CDC LIGHT TRAP DENSITY PER TRAP PER NIGHT IN MAGANJA DA COSTA, MILANGE, MOPEIA, AND LUGELA DISTRICTS

Figure 6A. *An. funestus* s.l.

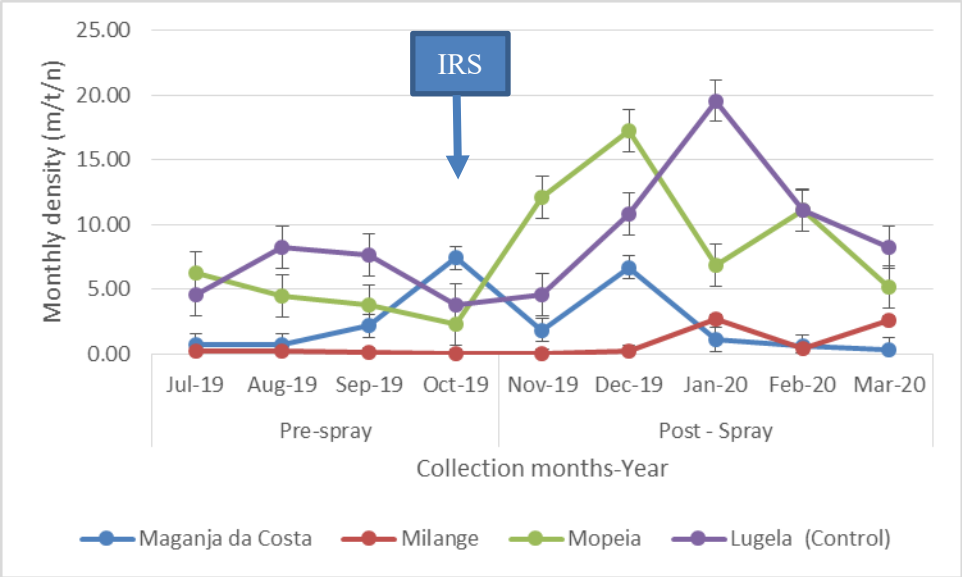
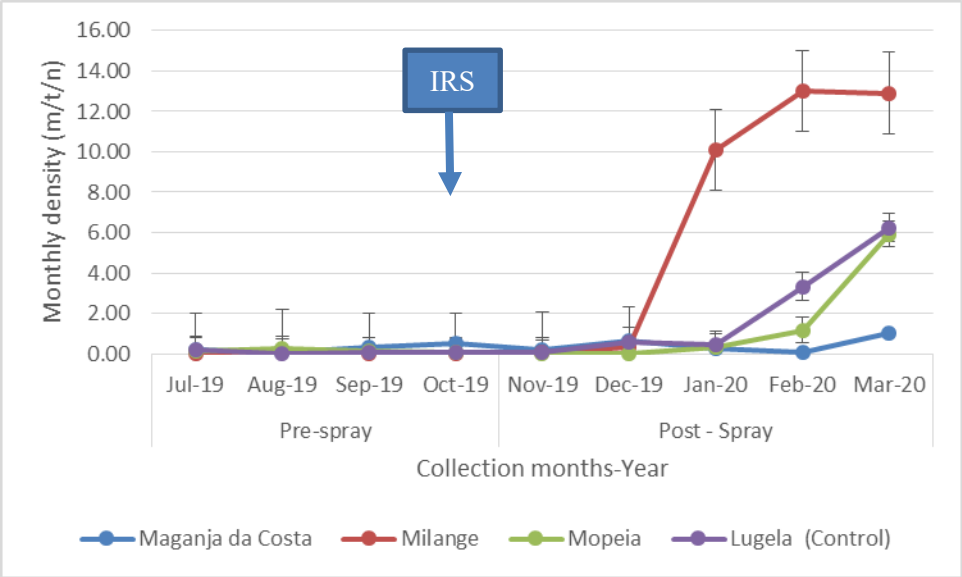


Figure 6B. *An. gambiae* s.l.



3.1.4 MOLECULAR ANALYSIS RESULTS

A total of 1590 mosquitoes morphologically identified as *An. funestus* s.l. were analyzed by PCR; of this number 1559 amplified, of which 95% were *An. funestus* s.s., 0.2% *An. leesoni,* 2.5% *An. gambiae* s.s., 0.1% *An. arabiensis*, and 31 samples (1.94%) did not amplify. For samples morphologically identified as *An. gambiae* s.l., 323 samples were analyzed by PCR and 319 were successful amplified, of which 67.1% were *An. gambiae* s.s., 4.9% *An. arabiensis*, 14.2% *An. funestus* s.s., 12.3% *An. leesoni*, and 6 samples (1.8%) were not amplified. The samples were processed at INS in Mozambique and the details are presented in Annex Tables A1, A3, and A4. The results from blood meal analyses indicated that few samples were detected carrying human blood, and the crude human blood index (HBI) among the different mosquito species for the different areas ranged from 0% to 100% (Table A2 in Annex).

3.2 CONE WALL BIOASSAY TESTS

During spray operations in October and November 2019, cone wall bioassays were conducted to measure the quality of the spray starting 24 hours after spray. Thereafter, monthly assays were performed to monitor the insecticide decay rate on various sprayed wall surfaces. Results of the quality assurance and decay rate monitoring of SumiShield® 50WG [clothianidin (50% w/w) active ingredient] in Mopeia and Fludora® Fusion [a combination of clothianidin (500 g/kg) and deltamethrin (62.5 g/kg) active ingredients] in Maganja da Costa and Milange districts are shown below in Section 3.2.2 (Figures 7 and 8, respectively).

3.2.1 QUALITY OF SPRAYING

For SumiShield® 50WG knockdown was recorded at 30 and 60 minutes post exposure, mortality scored at T_0 was 95% in all houses tested with cone wall bioassays one day (24 hours after spraying and 100% two days (48 hours) after the spray.

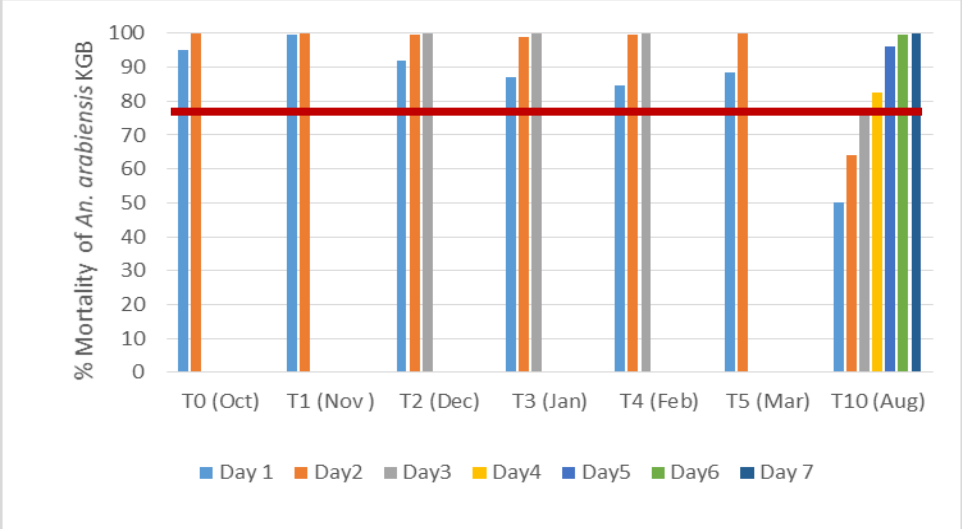
For Fludora® Fusion, knockdown was observed at 30 and 60 minutes post exposure, in all sites tested in the two intervention districts, one day (24-hour) mortality was scored at 92.5% and the final scored mortality at day 5 (120 hours) was 95.5% in Milange; for Maganja da Costa at day one (24 hours), the scored mortality was 100%.

3.2.2 INSECTICIDE DECAY RATE

SUMISHIELD® 50WG DECAY RATE

Baseline cone wall bioassays for assessing SumiShield® 50WG spray quality and subsequent monitoring of the insecticide's decay rate was conducted in Josina Machel village in Mopeia. Baseline, denoted as T_0, was conducted in October 2019, eliciting a 100% mortality by day 2 post exposure (Figure 7). Subsequent monthly cone bioassays resulted in 100% mortality up to month 10 post spray. It was also noted that scores of 100% were observed up to five months post IRS and between day 2 and 3. However, due to the COVID-19 pandemic, the data collection was interrupted at T_6 (April); it was restarted recently in month 10 (August), during which the mortality was 100% on day 7. There was a notable increase in the number of days to reach 100% mortality, from two to three days, during the first five months to seven days by the tenth month. This is presumably due to decreasing efficacy of the insecticide deposits on the sprayed surface with time. These results show that SumiShield® 50WG remained efficacious at least 10 months post spray.

FIGURE 7: SPRAY QUALITY ASSESSMENT AND RESIDUAL BIOEFFICACY OF SUMISHIELD® 50WG (CLOTHIANIDIN 50WG) IN MOPEIA



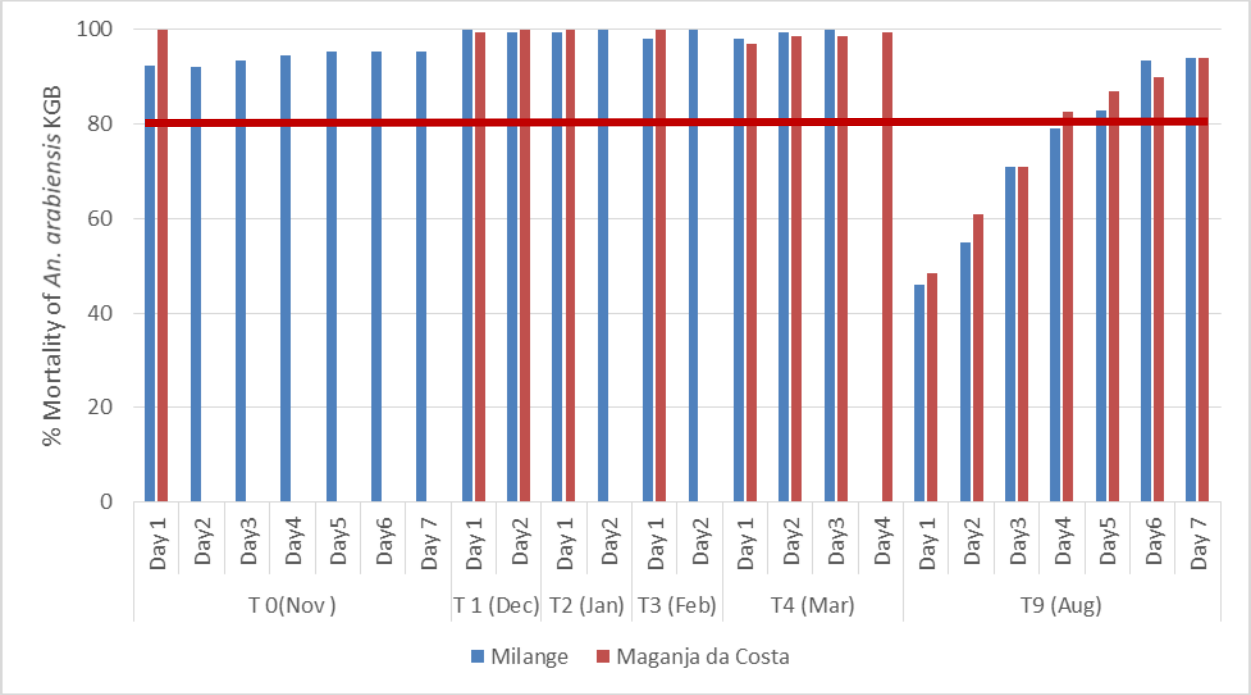
Red horizontal line indicates the 80% mortality cut-off point

FLUDORA® FUSION DECAY RATE

Baseline cone wall bioassays for assessing Fludora® Fusion IRS quality and subsequent monitoring of its decay rate were conducted in Inroga village in Maganja da Costa and 3 de Fevereiro village in Milange.

Cone bioassays data showed high mortality up to month 5 (March), after which field activities were interrupted for four months due to the COVID-19 pandemic. After the resumption of activities in month 9 (August) post IRS, assays conducted showed overall mortality of below 80% for the first five days. Mortality increased up 94% from day 6 to 7 (Figure 8). This might indicate a reduced residual effect over time.

FIGURE 8: SPRAY QUALITY ASSESSMENT AND RESIDUAL BIOEFFICACY OF FLUDORA® FUSION (CLOTHIANIDIN AND DELTAMETHRIN) IN MILANGE AND MAGANJA DA COSTA

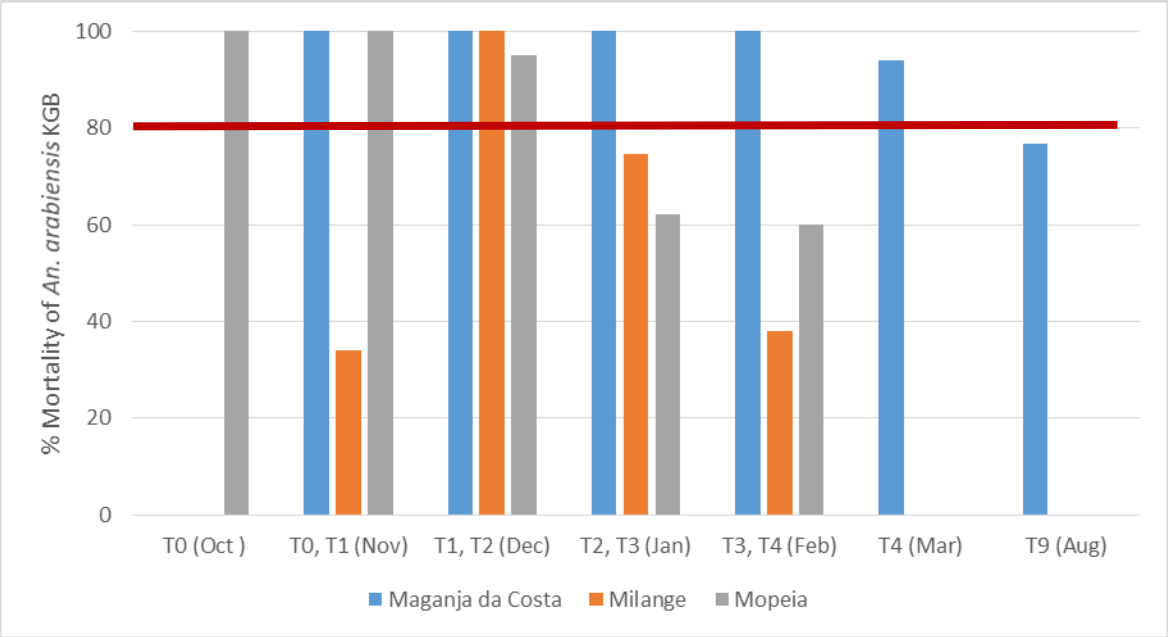


Red horizontal line indicates the 80% mortality cut-off point

3.2.3 THE AIRBORNE EFFECT

Figure 9 shows bioassay data to illustrate the airborne effect of the insecticides. The airborne fumigant effect of SumiShield® 50WG was found to be high in Mopeia in the first three months, after which it dropped to below 80%. The effect of Fludora® Fusion in Milange was low at the beginning, but was 100% about one month after spray and then dropped below 80% mortality. This contrasts with what was observed in Maganja da Costa, where the fumigant effect of Fludora® Fusion dropped below 80% around nine months post spray.

FIGURE 9. PERCENT MORTALITY OF *AN. ARABIENSIS* KGB SUSCEPTIBLE STRAIN ON AIRBORNE FUMIGANT EFFECT TEST AGAINST SUMISHIELD® 50WG IN MOPEIA AND FLUDORA® FUSION IN MILANGE AND MAGANJA DA COSTA



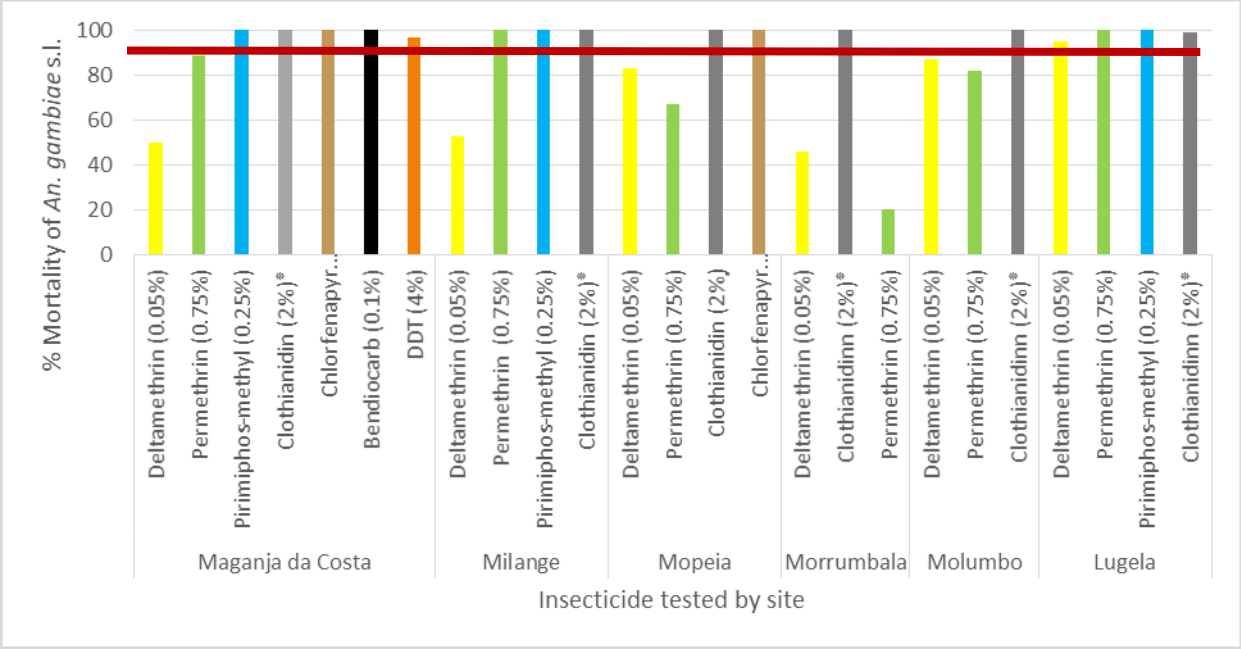
Red horizontal line indicates the 80% mortality cut-off point

3.3 INSECTICIDE SUSCEPTIBILITY TESTS

Susceptibility tests against *An. gambiae* s.l. were conducted from January through April 2020 in Lugela, Maganja da Costa, Milange, Molumbo, Mopeia, and Morrumbala, by exposing the *An. gambiae* s.l. to diagnostic dosages of deltamethrin (0.05%), permethrin (0.75%), pirimiphos-methyl (0.25%), clothianidin (2%), chlorfenapyr (100 µg/bottle), bendiocarb (0.1%), and DDT (4%). More tests were conducted to explore the intensity of resistance to deltamethrin (5X), deltamethrin (10X), permethrin (5X), and permethrin (10X). Synergist assays were also conducted with PBO for permethrin and deltamethrin insecticides.

The mortality results presented in Figure 10 show that *An. gambiae* s.l. was fully susceptible to pirimiphos-methyl, clothianidin, chlorfenapyr, and bendiocarb in all districts where the insecticides were tested. Resistance to deltamethrin (0.05%) was observed in all districts except for possible resistance in Lugela. For permethrin (0.75%), resistance was noted in all districts except Milange and Lugela. DDT (4%) was tested only in Maganja da Costa, and the results indicated possible resistance. Some insecticides were not tested in certain districts due to low number of larvae collected to cover all insecticides.

FIGURE 10. PERCENTAGE MORTALITY OF ADULT *AN. GAMBIAE* S.L. RAISED FROM LARVAL COLLECTIONS EXPOSED TO A RANGE OF INSECTICIDES AT RESPECTIVE DIAGNOSTIC CONCENTRATIONS AND HOLDING PERIODS



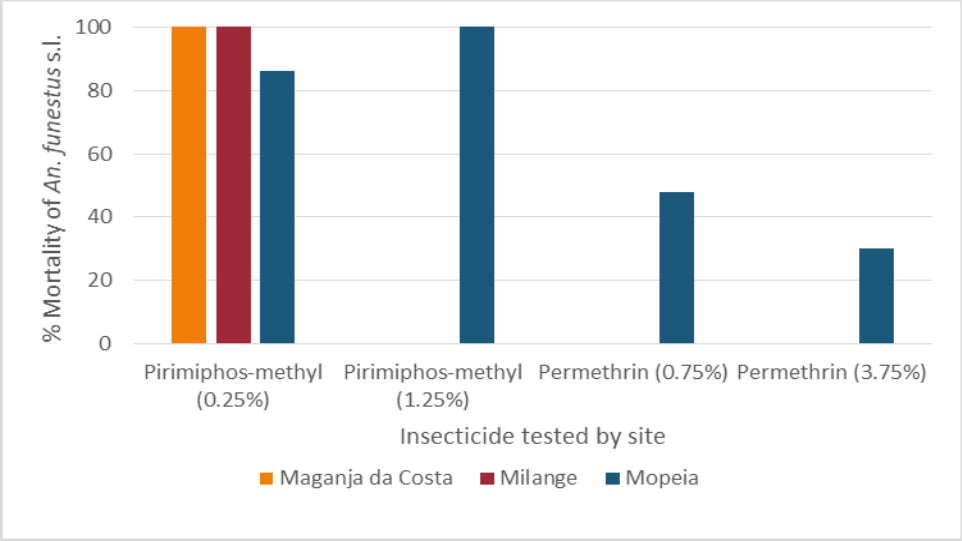
Note: clothianidin and chlorfenapyr holding periods were up to 3 and 7 days, respectively. The mortality for other insecticides was at 24 hr holding period

Red horizontal line indicates the 98% mortality cut-off point for susceptibility

Susceptibility testing was conducted with *An. funestus* s.l. samples collected from June to October 2019 in Maganja da Costa, Milange, and Mopeia. A shortage of mosquitoes in the field meant the tests could not be conducted in all districts with all planned insecticides.

Adult *An. funestus* s.l. collected indoors using Prokopack aspirators were directly tested in Maganja da Costa and Milange; some of the tests conducted with samples from Mopeia were F1 progeny from the forced oviposition of the adult gravid females. The *An. funestus* s.l. exposed to diagnostic dosages of pirimiphos-methyl in Maganja da Costa and Milange were found to be fully susceptible. The strain from Mopeia showed signs of resistance to diagnostic dosages of pirimiphos-methyl although tests at 5X intensity concentration of pirimiphos-methyl resulted in 100% mortality indicating the status as low intensity of resistance (Figure 11). Resistance of the same population from Mopeia also was detected to permethrin at the diagnostic concentration and at the 5X concentration.

FIGURE 11. 24-HOUR MORTALITY FROM THE WHO TUBE TESTS OF ADULT *AN. FUNESTUS* S.L. COLLECTED BY PROKOPACK

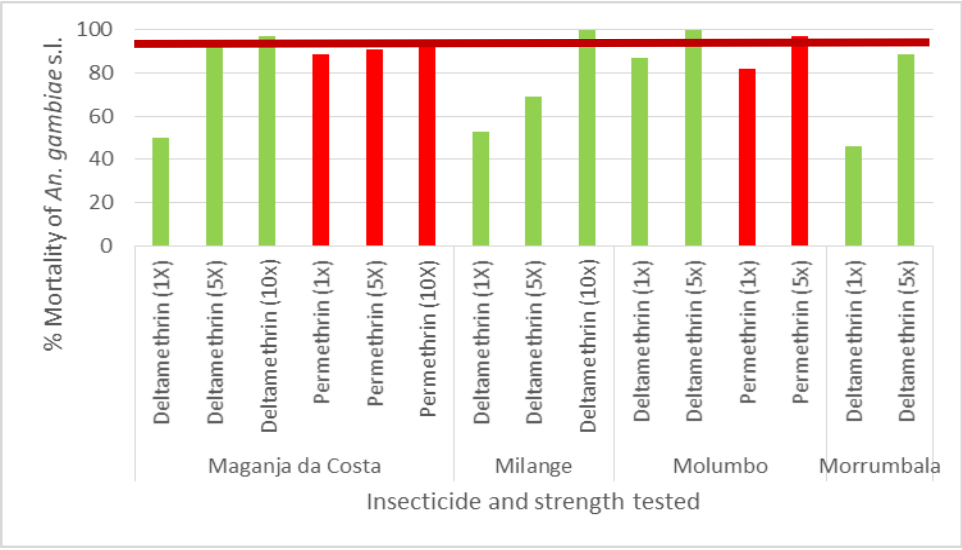


3.3.1 DETERMINATION OF THE INTENSITY OF RESISTANCE AND SYNERGIST ASSAYS USING WHO TUBE TESTS ON *AN. GAMBIAE* S.L.

Bioassays for intensity of resistance were conducted where *An. gambiae* s.l. was resistant to discriminating concentrations (24 hrs mortality <90%) of pyrethroid insecticides.

Figure 12 shows results of exposure to deltamethrin and permethrin at diagnostic doses and 5X and 10X intensities. Low, moderate, and high intensity of resistance to deltamethrin was observed at Molumbo, Milange, and Maganja da Costa, respectively. The intensity of resistance to deltamethrin was moderate to high at Morrumbala. High intensity of resistance to permethrin was also observed at Maganja da Costa. The presence of medium to high intensity resistance in these areas suggests the need to continue with the next generation IRS insecticides in the areas.

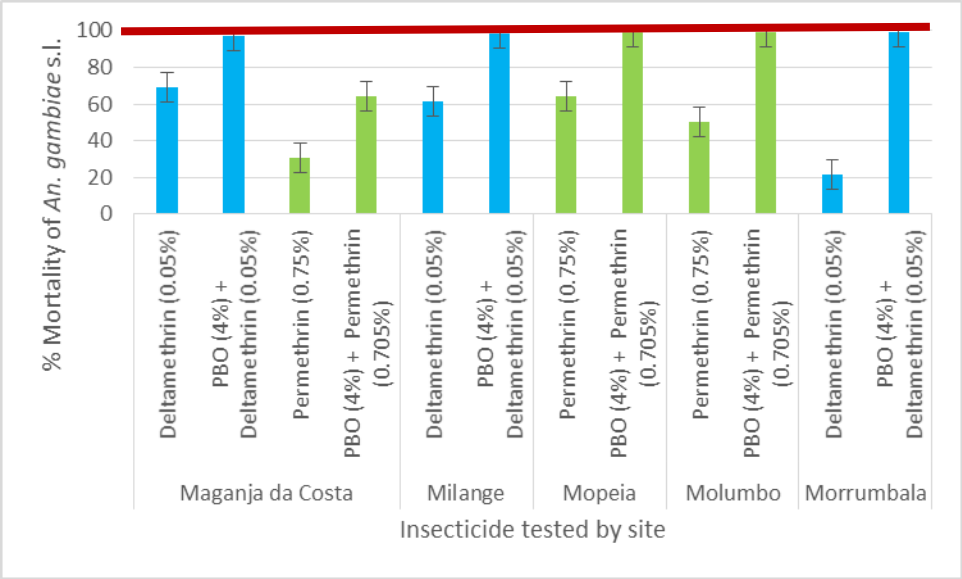
FIGURE 12. 24-HOUR MORTALITY OF ADULT *AN. GAMBIAE* S.L. TO DIAGNOSTIC AND INTENSITY ASSAY CONCENTRATIONS



Red horizontal line indicates the 98% mortality cut-off point for susceptibility

Figure 13 summarizes the results of synergist assays on *An. gambiae* s.l. from Maganja da Costa, Milange, Mopeia, Morrumbala, and Molumbo. The synergist PBO restored full susceptibility to deltamethrin, estimated as ≥98% mortality, up from 61% to 99% in Milange and from 21% to 99% in Morrumbala. In Maganja da Costa, one of the assays showed restored susceptibility from 69% to 97%, indicating partial involvement of monoxygenases as the mechanism of resistance. Full restoration of susceptibility was observed in Mopeia and Molumbo for permethrin with PBO synergist where mortality increased from 64% to 99%, and 51% to 99%, respectively. Similar to deltamethrin, partial involvement of monoxygenases was detected in Maganja da Costa when permethrin was tested with PBO, with mortality increasing from 31% to 65%. This observation suggests that monoxygenases were involved as one of the mechanisms, but other mechanisms might also be involved.

FIGURE 13. SYNERGIST ASSAY MORTALITY RESULTS IN *AN. GAMBIAE* S.L. FROM FIVE INTERVENTION DISTRICTS.



4. RESULTS: NAMPULA

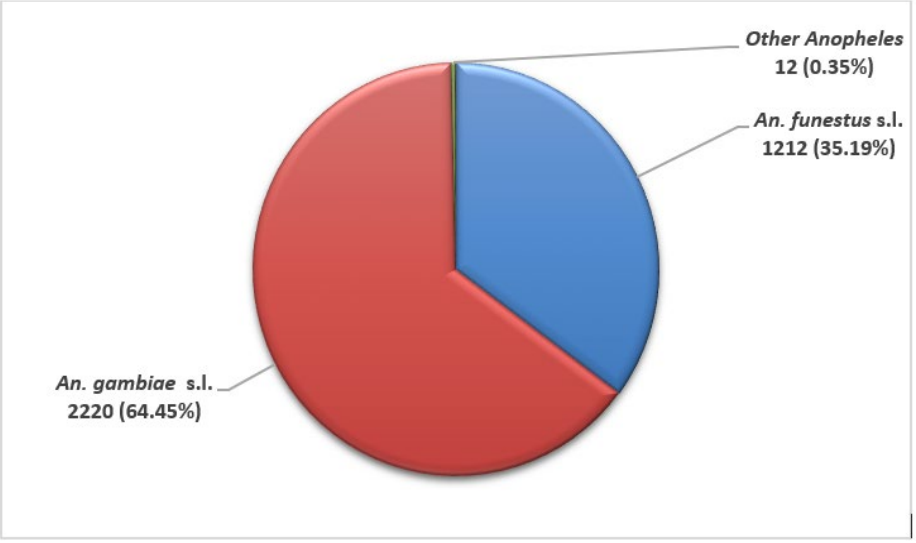
4.1 ANOPHELINE SPECIES COLLECTED BY THE DIFFERENT METHODS

During the reporting period, in the intervention districts of Monapo and Nampula City, and the control district of Erati, a total of 3,442 anopheline mosquitoes belonging to eight different species and species complexes were collected using the three collection methods (PSC plus Prokopack, CDC light trap, and HLC) and morphologically identified. The anophelines were *An. funestus* s.l., *An. gambiae* s.l., *An. ziemanni*, *An. rufipes*, *An. coustani*, *An. pretoriensis*, *An. tenebrosus*, and *An. maculipalpis*. Table 7 and Figure 14 summarize the number of mosquitoes collected, by species. *An. gambiae* s.l. was the most abundant anopheline, accounting for 64.45% of all collections, followed by *An. funestus* s.l. at 35.19%, and the other anophelines at 0.35 %.

TABLE 7. NUMBER OF MOSQUITOES COLLECTED IN EACH DISTRICT BY ALL COLLECTION METHODS

Species Collected	Monapo	Nampula City	Erati	Total per Species
An. funestus s.l.	72	834	306	**1,212**
An. gambiae s.l.	106	1227	887	**2,220**
An. coustani	0	1	0	**1**
An. pretoriensis	0	0	1	**1**
An. rufipes	0	0	5	**5**
An. ziemanni	0	0	1	**1**
An. tenebrosus	0	0	1	**1**
An. maculipalpis	0	0	1	**1**
Total	**178**	**2,062**	**1,202**	**3,442**

FIGURE 14. SPECIES COMPOSITION OF *ANOPHELES* MOSQUITOES FOR ALL SITES IN NAMPULA PROVINCE



A total of 2,220 *Anopheles gambiae* s.l. mosquitoes were collected: 82 from PSCs and Prokopack (3.69%), 1,014 using CDC light traps (45.68%), and 1,124 (50.63%) using HLCs. A total of 1,212 *An. funestus* s.l. were collected: 122 from PSCs and Prokopack (10.07%), 543 using CDC light traps (44.80%), and 547 (45.13%) using HLCs.

4.2 Pyrethrum Spray Catch and Prokopack Aspirator Collections

PSC and Prokopack collections yielded a total of 204 *Anopheles* mosquitoes (Table 8). Based on morphological identification, 122 of these belonged to *An. funestus* s.l. (59.80%) and 82 to *An. gambiae* s.l. (40.20%). No other species were detected by PSC and Prokopack collection methods.

Table 8. Number of *An. funestus* s.l. and *An. gambiae* s.l. Mosquitoes Collected Using PSC and Prokopack, by District

Species	District			Total
	Monapo	Nampula	Erati	
An. funestus s.l.	19	63	40	122
An. gambiae s.l.	14	33	45	82
Total	**23**	**96**	**85**	**204**

The mean indoor resting densities of *An. funestus* s.l. peaked in July in Nampula City with 1.75 mosquitoes per room per day and in August for Monapo and Erati (control) with 0.55 and 1.45 *An. funestus* s.l. mosquitoes per room per day, respectively (Figure 15). In Nampula, indoor resting density declined from 1.75 *An. funestus* s.l./ room/day in July to 0.0 *An. funestus* s.l./ room/day in October, and it remained low through February 2020. There was a decline in the indoor resting density in Monapo, from 0.55 *An. funestus* s.l./room/day in August to 0.0 *An. funestus* s.l./ room/day from September to February 2020, and in Erati, from 1.45 *An. funestus* s.l. / room/ day in August to 0.0 *An. funestus* s.l./ room/ day in March 2020. This was after the distribution of insecticide-treated nets with permethrin (Nampula and Erati) and alphacypermethrin (Monapo) in July 2019, and IRS in both Nampula City (Fludora®Fusion) and Monapo districts (Sumishield®50WG).

Over the monitoring period, the *An. gambiae* s.l. resting densities were consistently low (<1 mosquito/ room/day) in all districts over most months during the reporting period. The highest mean indoor resting density of *An. gambiae* s.l., estimated at 0.5 mosquitoes per room per day, was observed in Nampula in July 2019 and Erati in September 2019.

FIGURE 15. INDOOR RESTING DENSITIES OF *AN. FUNESTUS* S.L. AND *AN. GAMBIAE* S.L. IN IRS DISTRICTS MONAPO (SUMISHIELD® 50WG) AND NAMPULA CITY (FLUDORA® FUSION), AND CONTROL DISTRICT ERATI USING PSC AND PROKOPACK METHOD, JULY 2019–MARCH 2020

Figure 15A: *An. funestus* s.l.

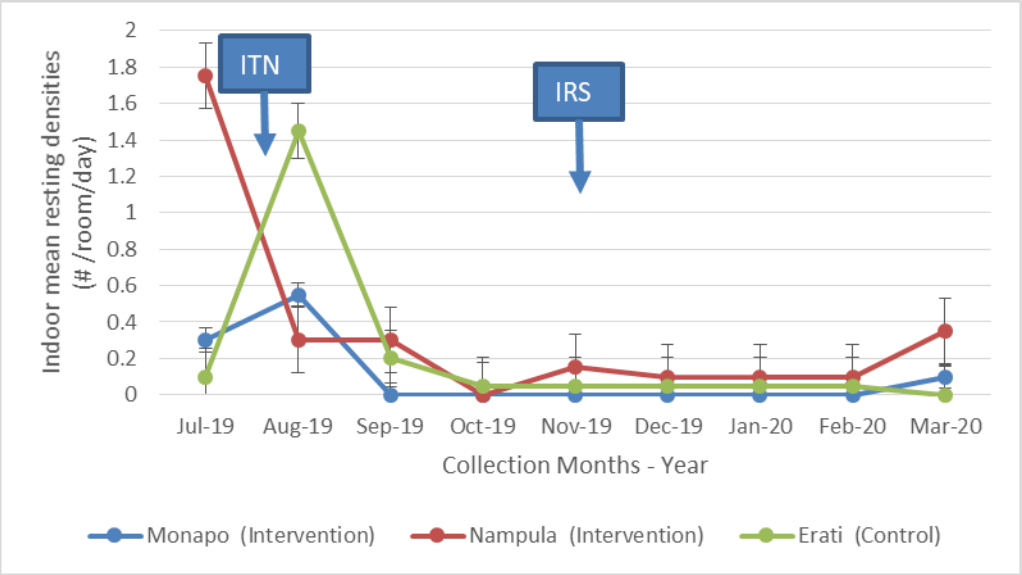
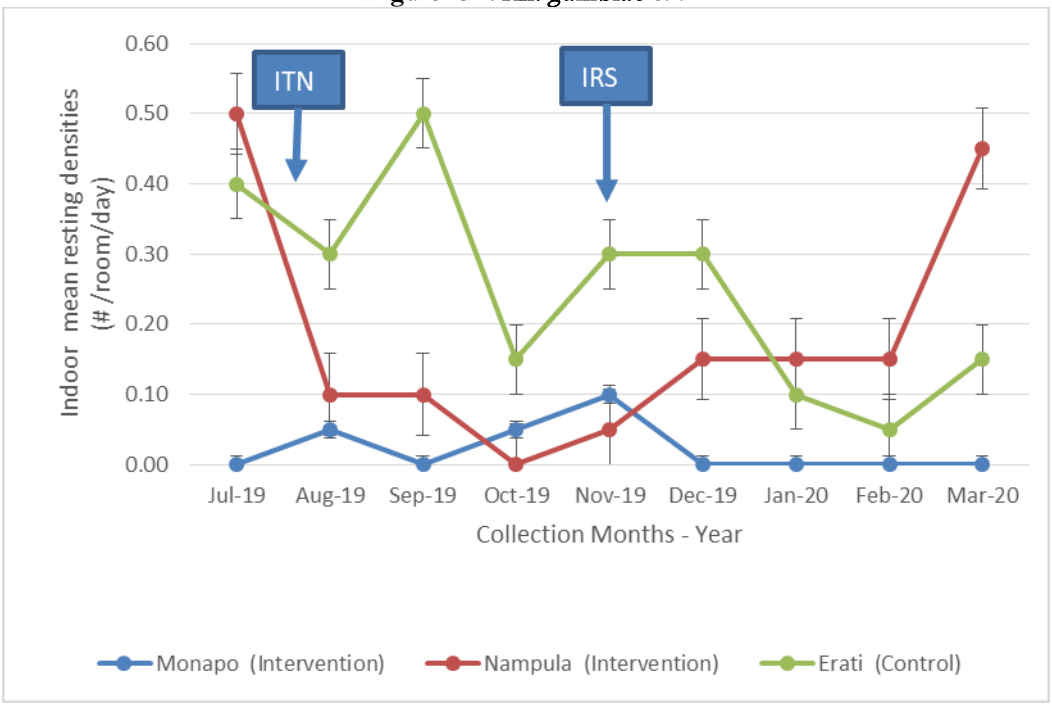


Figure 15B: *An. gambiae* s.l.



4.2.1 HUMAN LANDING CATCHES

A total of 1,682 *Anopheles* mosquitoes were collected using the HLC technique from July 2019 to February 2020. The species identified morphologically from this collection were found to belong to *An. gambiae* s.l. (1,124), *An. funestus* s.l., (546), *An. rufipes* (5), *An. ziemanni* (3), and *An. coustani*, *An. tenebrosus*, *An. maculipalpis* and *An. pretoriensis* (1 each). Erati was the district with highest diversity of *Anopheles* mosquitoes collected.

Table 9 shows there is no significant difference between total numbers of *An. funestus* s.l. samples collected indoors and outdoors (p>0.05) in Nampula City, Monapo, and Erati (control) sites.

Significantly higher numbers of *An. gambiae* s.l. samples were collected outdoors as compared with indoors (p<0.05) in Nampula and Erati sites. The differences were not significant for the Monapo site, but overall the number of mosquitoes collected from this site was low.

TABLE 9. COMPARISON OF TOTAL NUMBER OF *AN. FUNESTUS* S.L. AND *AN. GAMBIAE* S.L. COLLECTED BY HLC INDOORS AND OUTDOORS IN THREE DISTRICTS

District	*An funestus* s.l.				*An. gambiae* s.l.			
	# Collected indoors	# Collect outdoors	X^2	p-value	# Collected indoors	# Collect outdoors	X^2	p-value
Monapo	19	10	2.79	0.095	31	42	1.66	0.198
Nampula	180	201	1.16	0.282	277	341	6.63	0.010
Erati (Control)	75	61	1.44	0.230	183	250	10.37	0.001*

*p – value significant at 0.05 level

Table 10 summarizes the combined outdoor and indoor collections from the intervention and control districts, providing mean biting rates per night (b/p/n) for each species. The control area experienced a similar overall biting rate (0.50 b/p/n) to that of the intervention areas (0.48 b/p/n). For *An. funestus* s.l. alone, a higher biting rate (1.42 b/p/n) was observed in the intervention areas than in the control area (0.94 b/p/n) but the difference was not significant (p> 0.05). For *An. gambiae* s.l. alone, the control area showed higher biting rates (3.00 b/p/n) than the intervention areas (2.40 b/p/n), but the difference was not significant (p>0.05).

TABLE 10. MOSQUITO SPECIES COLLECTED BY HLC AND THEIR COMBINED OUTDOOR AND INDOOR MEAN BITING RATES IN INTERVENTION DISTRICTS OF NAMPULA CITY AND MONAPO AND CONTROL AREA OF ERATI

Species Collected	Intervention Area			Control Area		
	Total numbers collected	Total person nights	b/p/n	Total numbers collected	Total person nights	b/p/n
An. funestus s.l.	410	288	1.42	136	144	0.94
An. gambiae s.l.	691	288	2.40	433	144	3.00
An. coustani	0	288	0.00	1	144	0.01
An. pretoriensis	0	288	0.00	1	144	0.01
An. tenebrosus	0	288	0.00	1	144	0.01
An. ziemanni	0	288	0.00	3	144	0.02
An. maculipalpis	0	288	0.00	1	144	0.01
An. rufipes	0	288	0.00	5	144	0.03
Total	**1,101**	**2,304**	**0.48**	**581**	**1,152**	**0.50**

Figures 16A-16D provide a summary of data from monthly HLCs performed concurrently both indoors and outdoors for the two vectors *An. funestus* s.l. and *An. gambiae* s.l. The data, covering the period from July 2019 to February 2020, are from the intervention districts of Nampula City and Monapo, and from Erati, the control.

Figures 16A and 16B show that *An. funestus* s.l. demonstrated a similar biting pattern throughout the year both indoors and outdoors. The rate was found to be low before IRS, ≤4.50 b/p/n indoors and ≤4.17 b/p/n outdoors in all districts. In Nampula City (intervention), where the biting rate was higher than Monapo (intervention) and Erati (control), biting rates peaked in January 2020 indoors (at 6.83 b/p/n) and in February 2020 outdoors (at 6.5 b/p/n). The indoor biting rate decreased in February 2020 to 4.33 b/p/n. In Monapo

and Erati, indoor and outdoor biting rates remained relatively low after IRS, below 0.67 b/p/n through February.

FIGURE 16. INDOOR AND OUTDOOR BITING RATES FOR *AN. FUNESTUS* S.L. AND *AN. GAMBIAE* S.L. IN INTERVENTION AND CONTROL DISTRICTS, BEFORE AND AFTER IRS

Figure 16A: *An. funestus* s.l. Indoor

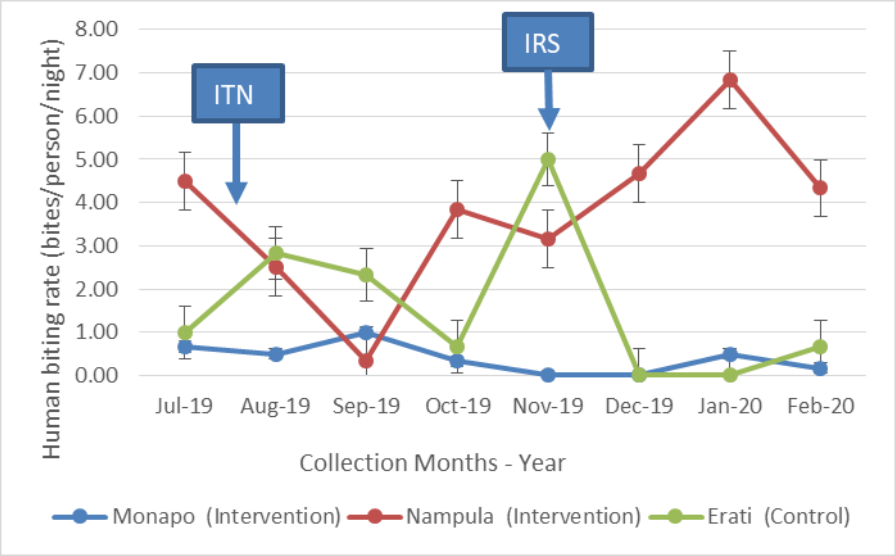


Figure 16B: *An. funestus* s.l. Outdoor

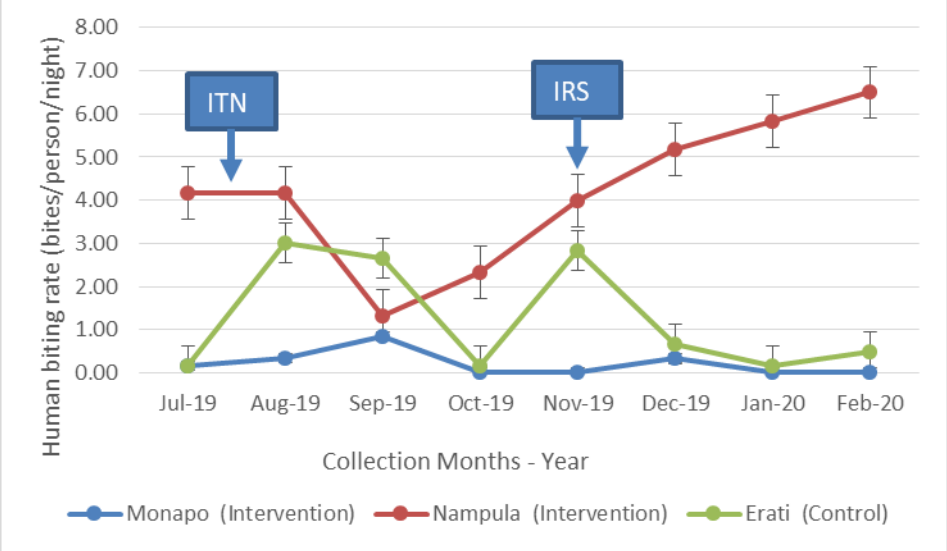


Figure 16C shows that the *An. gambiae* s.l. biting rate before IRS was observed to be low both indoors (≤3.83 b/p/n) and outdoors (≤5.33 b/p/n). The indoor biting rates dropped in the month immediately after IRS (November) at Erati. In Erati, the rate increased again in January then dropped in February. In Monapo the biting rate increased in January and February, months after the rain. In Nampula, in general, the *An. gambiae* s.l. biting rate was high and the trend remained almost the same indoors and outdoors through the monitoring period. In Monapo, the *An. gambiae* s.l. outdoor biting rate was low, less than 2.0 b/p/n, throughout the monitoring period (Figure 16D), but it was higher at Erati (≤13.83 b/p/n) and Nampula (≤20.33 b/p/n).

Figure 16C: *An. gambiae* s.l. Indoor

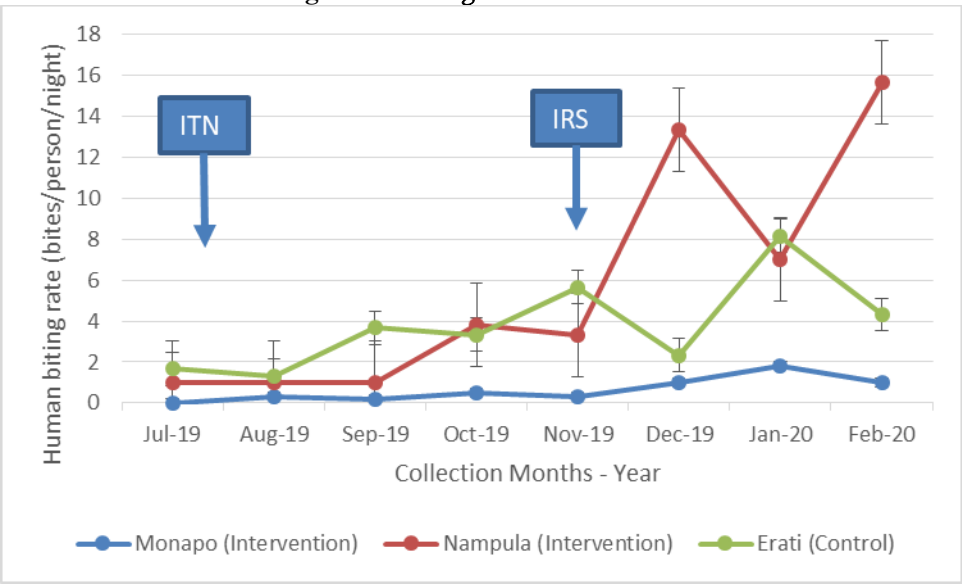


Figure 16D: *An. gambiae* s.l. Outdoor

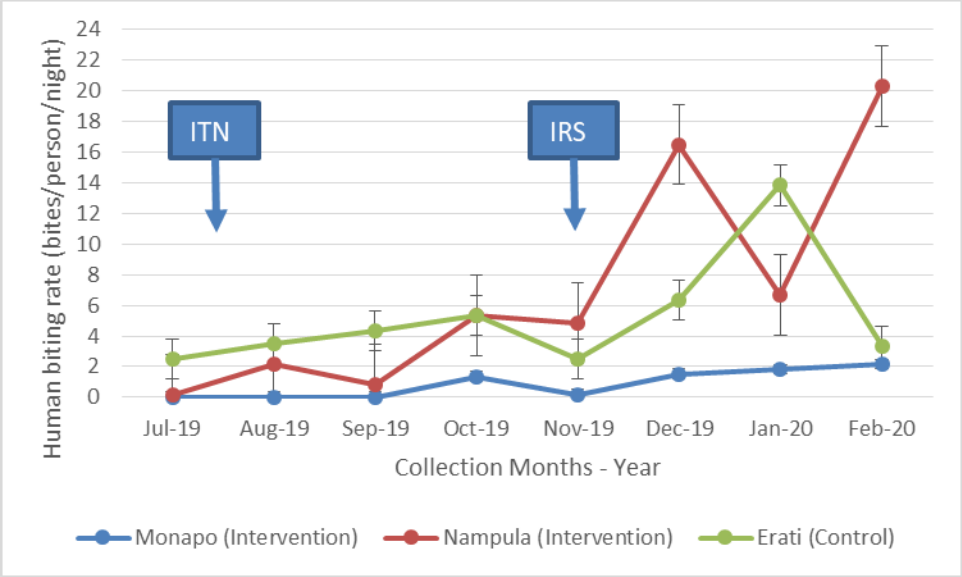


Table 11 shows that after IRS, the *An. funestus* s.l. biting rate both indoors and outdoors, increased in Nampula City and Erati; in Monapo, it decreased. The *An. gambiae* s.l. biting rates, indoors and outdoors, showed a notable increase after spraying at all sites. These observations were as expected because they reflect the natural increase in vector populations after the rainy season.

TABLE 11. INDOOR AND OUTDOOR MEAN BITING RATE FOR *AN. GAMBIAE* S.L. AND *AN. FUNESTUS* S.L., ESTIMATED USING HLC, BY DISTRICT, BEFORE AND AFTER SPRAYING

District	*Anopheles funestus* s.l. (b/p/n)				*Anopheles gambiae* s.l. (b/p/n)			
	indoors		outdoors		indoors		outdoors	
	Pre-spray	Post-spray	Pre-spray	Post-spray	Pre-spray	Post-spray	Pre-spray	Post-spray
Monapo	0.63	0.17	0.33	0.08	0.25	1.04	0.33	1.42
Nampula	2.75	4.75	3.00	5.38	1.71	9..83	2.13	12.08
Erati (Control)	3.38	4.92	1.50	1.04	1.67	5.93	3.92	6.50

Figures 17A and 17B show the overnight biting pattern of *An. funestus* s.l. Both indoors and outdoors, biting activities were relatively low during the evening hours, 6:00 pm–12:00 am, except in Erati, where the peak indoor biting occurs between 7:00 and 8:00 pm; after midnight, there was a steady increase in biting activity for several hours, both indoors and outdoors. Most indoor bites took place from 1:00 am to 4:00 am, peaking at 3.67 b/p/h in Nampula City at 12:00–1:00 am and 3:00-4:00 am, at 2.0 b/p/h in Erati at 7:00–08:00 pm, and at 0.5 b/p/h in Monapo at 1:00–2:00 am. Most outdoor bites took place at 12:00–2:00 am, peaking at 1:00–2:00 am for Nampula City (5.50 b/p/h), Erati (1.83 b/p/h), and Monapo (0.5b/p/h). The outdoor biting in Monapo remained low (<0.5 b/p/h) throughout the night, whereas in Nampula it continued until 6:00 am.

FIGURE 17. HOURLY BITING RATES OF *AN. FUNESTUS* S.L. AND *AN. GAMBIAE* S.L. INDOOR AND OUTDOOR DETERMINED THROUGH HLCS

Figure 17A. *An. funestus* s.l. Indoor

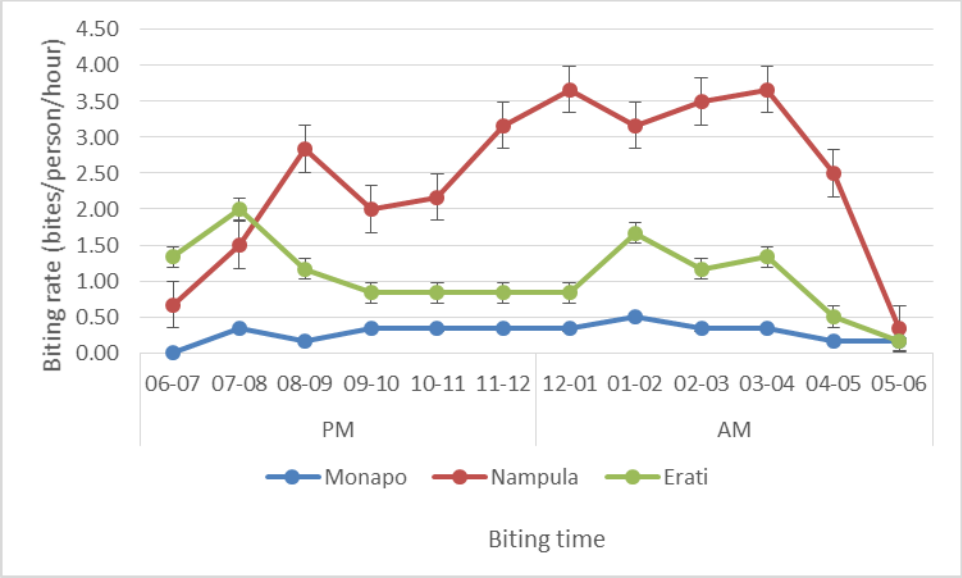
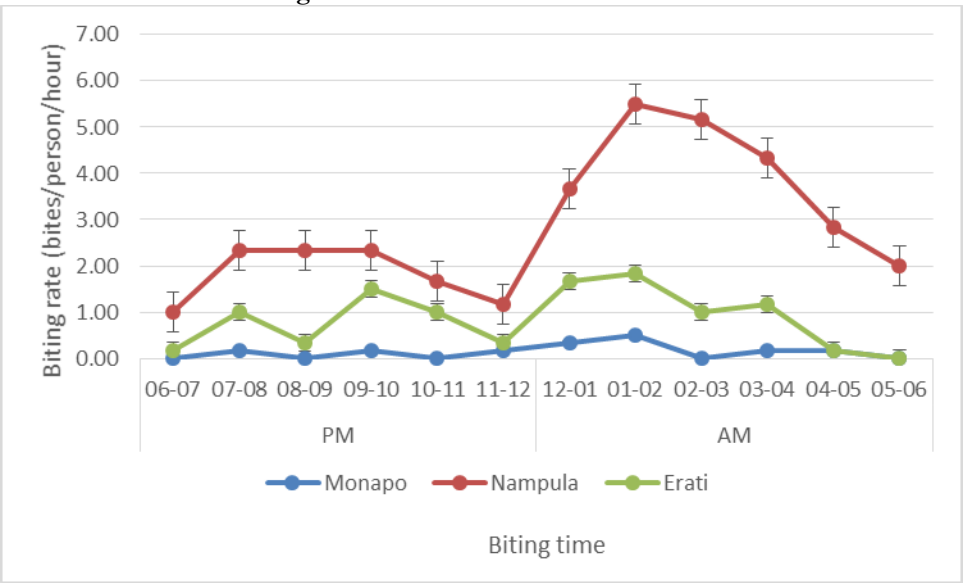


Figure 17B. *An. funestus* s.l. Outdoor



Figures 17C and 17D show the overnight biting pattern of *An. gambiae* s.l. indoors and outdoors. Both indoor and outdoor biting activity in Erati and Nampula City (1.33 and 2.33 b/p/h) started at a higher level than in Monapo (0.17 b/p/h). Most *An. gambiae* s.l. bites were observed to take place between 12:00 am and 3:00 am both indoors and outdoors. The peak biting, an estimated 7.0 b/p/h, was recorded outdoors in Nampula. Indoor and outdoor biting activity in Monapo continued at a low level through the night, at less than 1.0 b/p/h with occasional, and slight spikes not exceeding 2.0 b/p/h occurring mainly outdoors at 8:00 pm–9:00 pm.

Figure 17C. *An. gambiae* s.l. Indoor

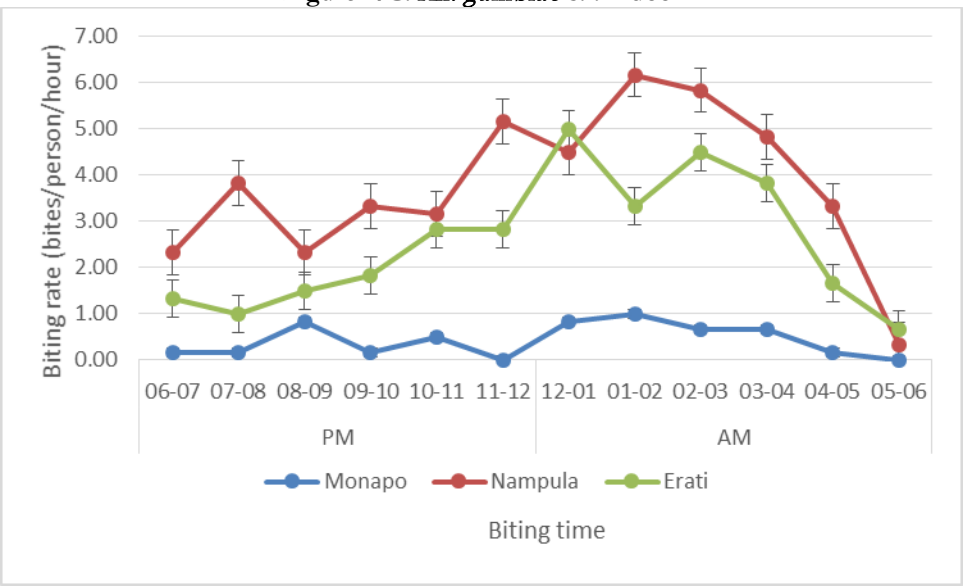
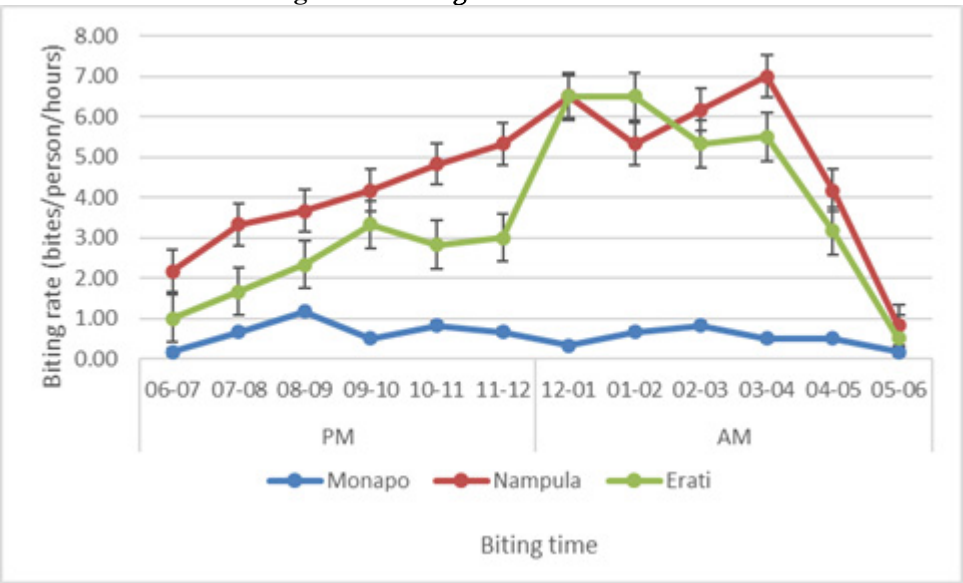


Figure 17D. *An. gambiae* s.l. Outdoor



4.2.2 CDC Light Trap Collections

The CDC light traps collected a total of 1,557 vector mosquitoes from the three districts. Table 12 shows the major vector species identified morphologically from these collections: 1,014 (65.13 %) were *An. gambiae* s.l., and 543 (34.97 %) were *An. funestus* s.l. Nampula City and Erati were the districts where the most anopheline mosquitos were collected, 965 (61.98 %) and 539 (39.31 %), respectively. In Monapo, 53 (3.40 %) were collected.

TABLE 12. CDC LIGHT TRAP DATA FOR MONTHLY COLLECTION OF *AN. FUNESTUS* S.L. AND *AN. GAMBIAE* S.L. COLLECTED IN NAMPULA PROVINCE

Districts	Species	2019						2020			Total & Average Densities	
		Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar		
Monapo	*An. funestus* s.l.	3	3	11	1	0	0	0	5	1	**24**	**53**
	Trap nights	12	12	12	12	12	12	12	12	12		
	Mean # Mosq/trap/night	0.25	0.25	0.92	0.08	0	0	0	0.4	0.08	**0.17**	
	An. gambiae s.l.	0	2	0	4	0	6	1	7	9	**29**	
	Trap nights	12	12	12	12	12	12	12	12	12		
	Mean # Mosq/trap/night	0	0.17	0	0.33	0	0.5	0.08	0.6	0.75	**0.2**	
Nampula	*An. funestus* s.l.	44	40	33	20	6	14	68	44	120	**389**	**965**
	Trap nights	12	12	12	12	12	12	12	12	12		
	Mean # Mosq/trap/night	3.67	3.33	2.75	1.67	0.5	1.17	5.67	3.7	10	**2.7**	
	An. gambiae s.l.	2	20	9	8	8	42	65	117	305	**576**	
	Trap nights	12	12	12	12	12	12	12	12	12		
	Mean # Mosq/trap/night	0.17	1.67	0.75	0.67	0.67	3.5	5.42	9.8	25.4	**4.0**	
Erati	*An. funestus* s.l.	38	22	22	5	4	6	0	10	23	**130**	**539**
	Trap nights	12	12	12	12	12	12	12	12	12		
	Mean # Mosq/trap/night	3.17	1.83	1.83	0.42	0.33	0.5	0	0.8	1.92	**0.9**	
	An. gambiae s.l.	51	30	41	18	40	91	72	21	45	**409**	
	Trap nights	12	12	12	12	12	12	12	12	12		
	Mean # Mosq/trap/night	4.25	2.5	3.42	1.5	3.33	7.58	6	1.8	3.75	**2.84**	
Total		**138**	**117**	**116**	**56**	**58**	**159**	**206**	**204**	**503**	**1,557**	

As shown in Figures 18A and 18B, over the reporting period, *An. funestus* s.l. was most abundant in Nampula City (mean collection of 2.7 m/t/n), followed by Erati (mean collection of 0.9 m/t/n). *An. gambiae* s.l. was the most abundant species in Nampula City (2.41 m/t/n) and Erati (2.84 m/t/n). Monapo had the lowest mean collection for both *An. funestus* s.l. (0.17 m/t/n) and *An. gambiae* s.l. (0.20 m/t/n). The highest densities of *An. funestus* and *An. gambiae* s.l. were recorded in March, both in Nampula City.

FIGURE 18. *AN. FUNESTUS* S.L. AND *AN. GAMBIAE* S.L. DENSITIES ESTIMATED FROM CDC LIGHT TRAP COLLECTIONS

Figure 18A: *An. funestus* s.l.

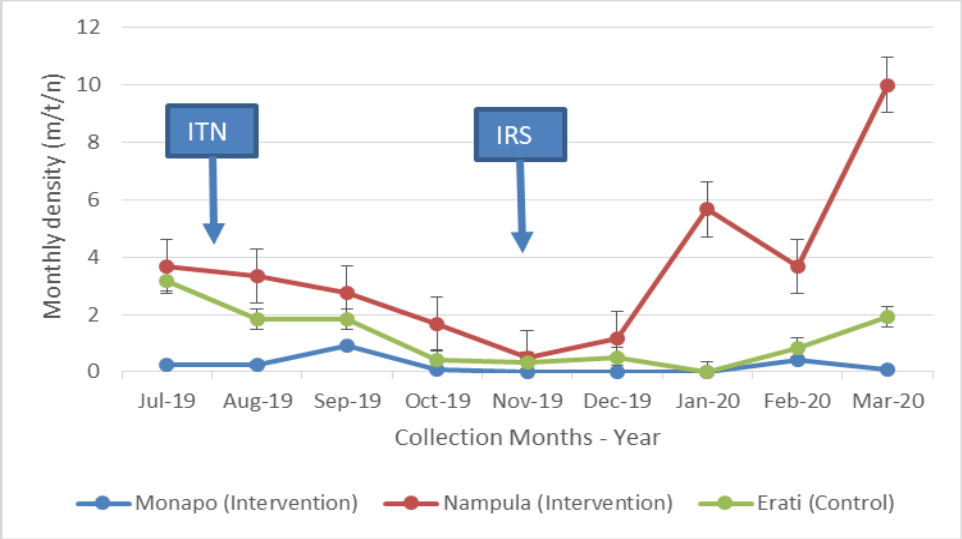
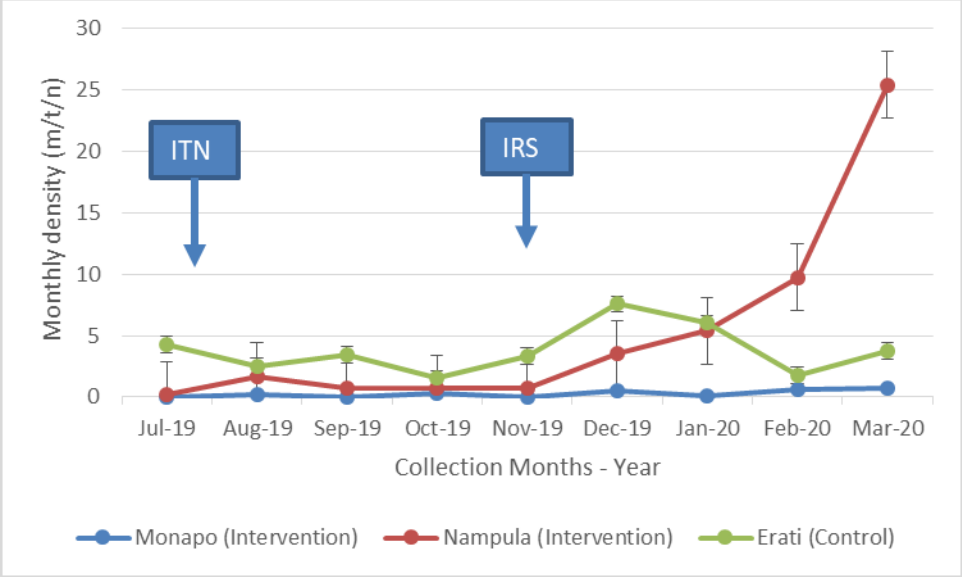


Figure 18B: *An. gambiae* s.l.



4.3 CONE WALL BIOASSAYS

During spray operations in November 2019, cone wall bioassays were conducted to measure the quality of the spray starting 24 hours after spray. Thereafter, monthly assays were performed to monitor the insecticide decay rate on various sprayed wall surfaces. Results of the quality assurance and decay rate monitoring of SumiShield® 50WG in Monapo and Fludora® Fusion in Nampula City are summarized in Figures 19 and 20.

4.3.1 Quality of Spray

For SumiShield® 50WG (Monapo), mortality scored at T_0 was 100% in all houses tested with cone wall bioassays in three days (72h) after spraying. For Fludora® Fusion (Nampula City), mortality scored at T_0 was 100% in all houses tested with cone wall bioassays in 24h after spraying.

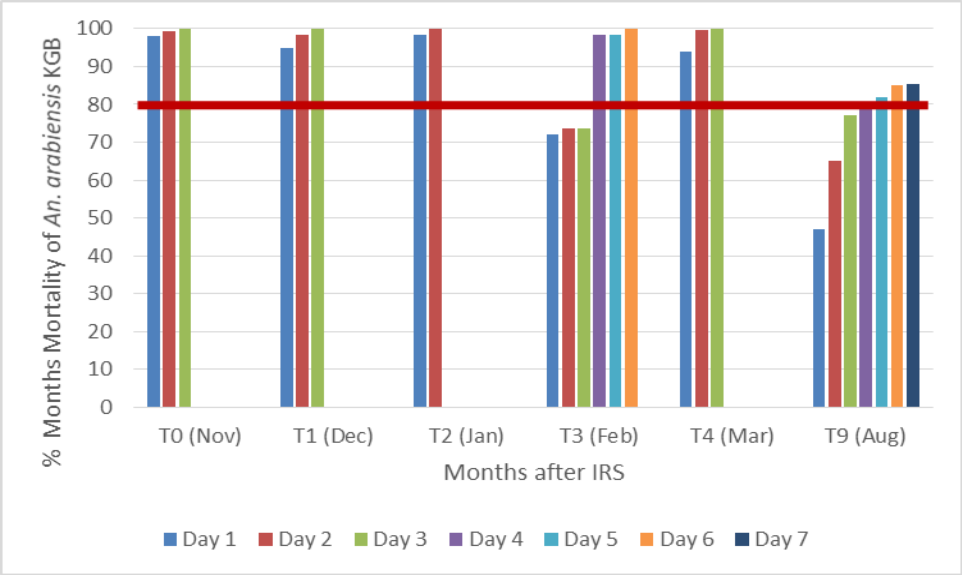
Bioassay results for assessing the quality of spraying exhibited high mortalities of 100% of female *An. arabiensis* KGB strain upon exposure to all three types of sprayed surfaces. As expected for SumiShield® 50WG, low levels of knockdown were observed 30 minutes post-exposure to almost all sprayed substrates, whereas Fludora® Fusion elicited high knockdowns at 30 minutes post-exposure. SumiShield®50WG demonstrated its typical slow-acting characteristic where mosquitoes were observed to survive up to 72 hours after exposure, at which 100% mortality was recorded. The results obtained from these wall assays strongly suggest that the spray teams were skilled in applying the insecticide uniformly, resulting in high 24- and 72-hour mortalities for Fludora® Fusion and SumiShield® 50WG, respectively.

4.3.2 Insecticide Decay Rate

SumiShield® 50WG decay rate

Baseline cone wall bioassays for assessing SumiShield® 50WG IRS quality and subsequent monitoring of its decay rate was conducted in Nachicuva village in Monapo. The baseline, denoted as T_0, was conducted in November 2019, and elicited a 100% mortality by day 3 post exposure (Figure 19). Subsequent monthly cone bioassays resulted in more than 80% mortality up to 9 months. Overall mortality below 80% was not observed in these months. It was also noted that scores of 100% were observed up to four months post IRS. However, there was a notable increase in the number of days when 100% mortality was achieved, from three days during the first month to six days by the fourth month and up to seven days by the ninth month. This is presumably due to decreasing efficacy of the insecticide deposits on the sprayed surfaces. These results show that SumiShield® 50WG remained efficacious at least nine months post spray. Data collection was interrupted by travel restrictions of the COVID-19 pandemic from March to July 2020.

Figure 19. Results of Cone Wall Bioassays on Walls Sprayed with SumiShield® 50WG in Monapo District

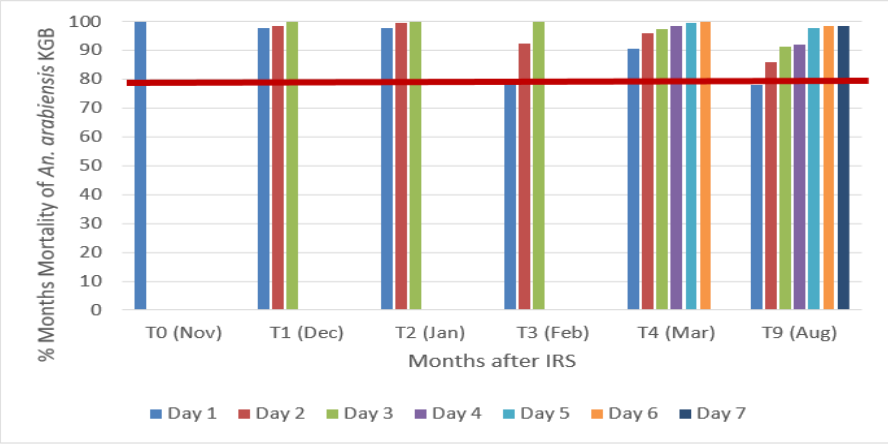


Red line indicates 80% mortality cut-off point

FLUDORA® FUSION DECAY RATE

Baseline cone wall bioassays for assessing Fludora® Fusion IRS quality and subsequent monitoring of its decay rate was conducted in Muriaze village in Nampula City. Baseline, denoted as T_0, was conducted in November 2019, and elicited a 100% mortality by day 1 post exposure (Figure 20). Subsequent monthly cone bioassays resulted in more than 80% mortality up to month 9. Overall mortality below 80% was not observed in these months. It was also noted that scores of 100% were observed up to four months post IRS. However, there was a notable increase in the number of days when 100% mortality was achieved, from three days during the first three months to six days by the fourth month and up to seven days by the ninth month. This is presumably due to decreasing efficacy of the insecticide deposits on the sprayed surface. These results show that Fludora® Fusion remained efficacious up to nine months post spray. Data collection was interrupted by COVID-19 pandemic from March to July 2020.

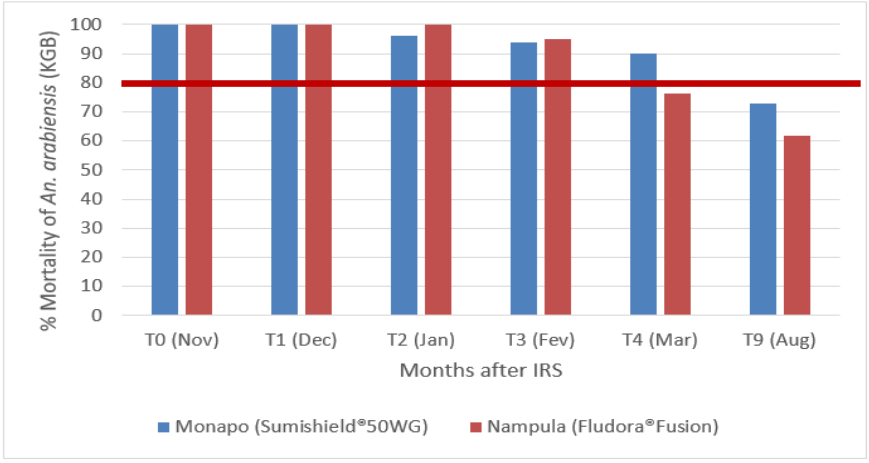
FIGURE 20. RESULTS OF CONE WALL BIOASSAYS ON WALLS SPRAYED WITH FLUDORA® FUSION IN NAMPULA CITY



4.3.3 THE AIRBORNE EFFECT

Figure 21 shows bioassay data to illustrate the airborne effect of the insecticides. The airborne fumigant effect of SumiShield® 50WG was found to be high in the first four months, then it dropped to below 80% in Monapo. In Nampula, the fumigant effect of Fludora® Fusion dropped below 80% around four months post spray.

FIGURE 21. PERCENT MORTALITY OF *AN. ARABIENSIS* KGB SUSCEPTIBLE STRAIN ON AIRBORNE FUMIGANT EFFECT TEST AGAINST SUMISHIELD® 50WG IN MONAPO AND FLUDORA® FUSION IN NAMPULA

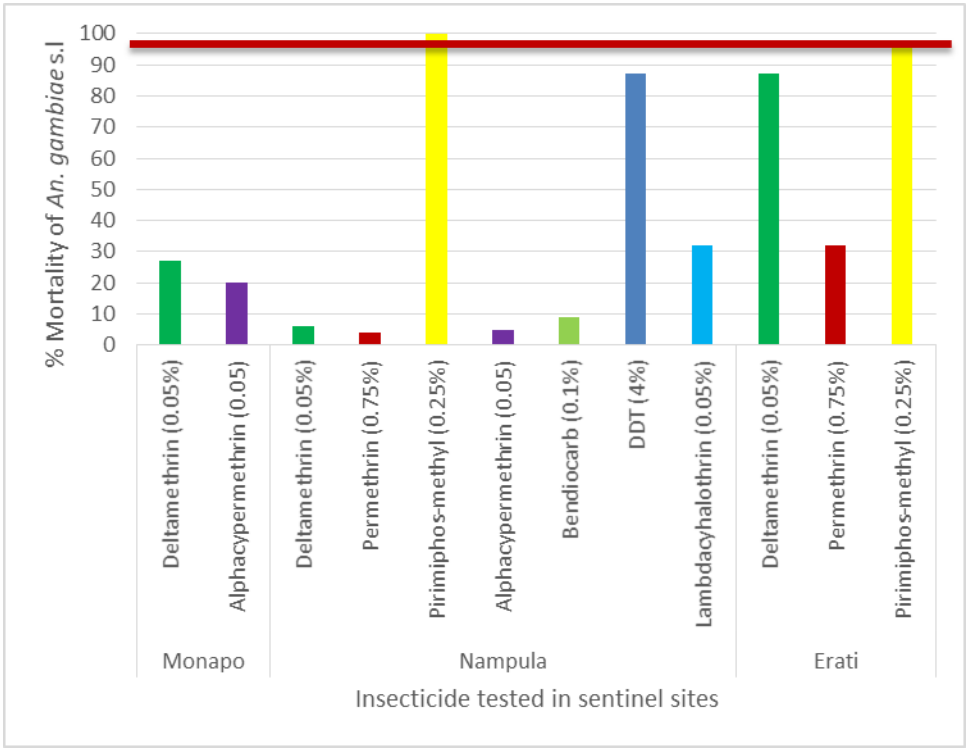


4.4 WHO SUSCEPTIBILITY TESTING

Susceptibility tests on *An. gambiae* s.l. started in February 2020 and stopped in March because of restrictions due to COVID-19. Tests were conducted on clothianidin, deltamethrin, permethrin, pirimiphos-methyl, alpha-cypermethrin, lambda-cyhalothrin, DDT, and bendiocarb (Figures 22 and 23). Insecticides used for IRS and found on the type of nets distributed in the areas were prioritized for insecticide susceptibility tests. Synergist assays with PBO were also conducted (Figure 26).

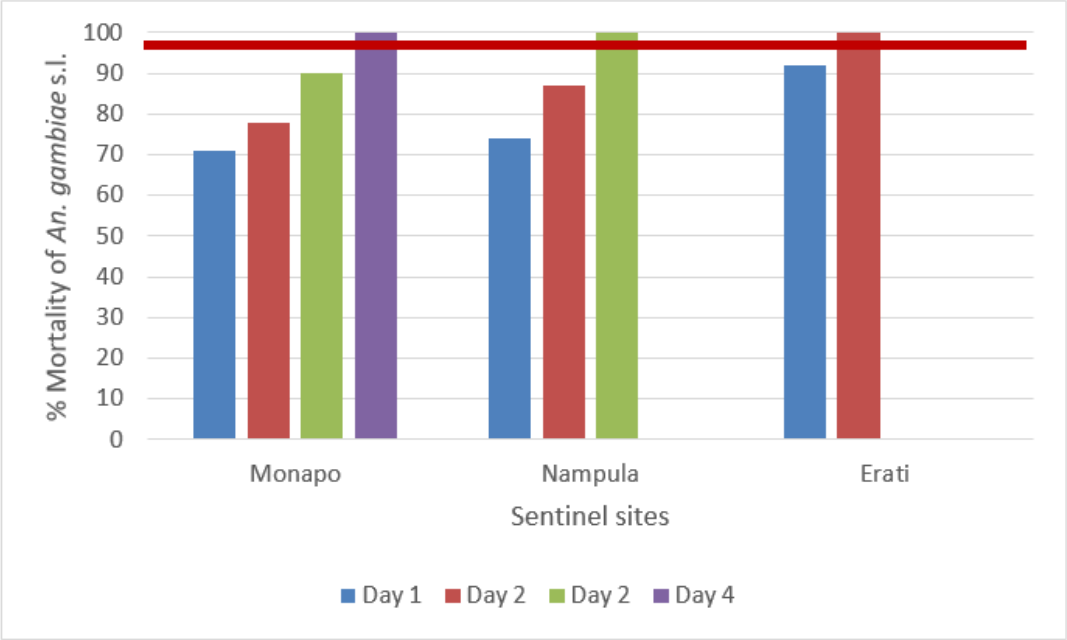
WHO susceptibility tests indicated that *An. gambiae* s.l. mosquitoes from Erati and Nampula districts were susceptible to pirimiphos-methyl (with mortalities ranging between 98% and 100%). *An. gambiae* s.l. was resistant to deltamethrin across all the sites, resistant to permethrin in Nampula and Erati, and resistant to alpha-cypermethrin in Nampula and Monapo (Figure 22). In Nampula there was resistance at diagnostic concentrations to lambda-cyhalothrin, DDT, and bendiocarb. *An. gambiae* s.l. from across all sites tested were fully susceptible to clothianidin within four days post-exposure (Figure 23).

FIGURE 22. 24-HOUR MORTALITY OF ADULT *AN. GAMBIAE* S.L. RAISED FROM LARVAL COLLECTIONS EXPOSED TO A RANGE OF INSECTICIDES AT RESPECTIVE DIAGNOSTIC CONCENTRATIONS, BY WHO TUBE TEST



Red horizontal line indicates the 98% mortality cut-off point for susceptibility

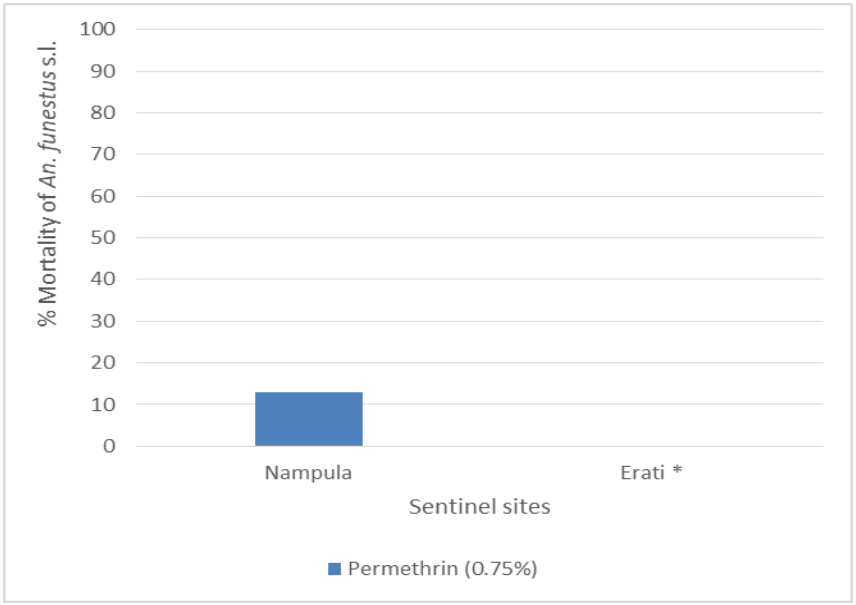
FIGURE 23. CLOTHIANIDIN SUSCEPTIBILITY OF *AN. GAMBIAE* S.L., REARED FROM LARVAL COLLECTIONS IN NAMPULA (JANUARY–MARCH 2020), BY WHO TUBE TEST



Red horizontal line indicates the 98% mortality cut-off point for susceptibility

In Nampula Province, adult *An. funestus* s.l. females were collected indoors using Prokopack aspirators for susceptibility tests. The blood-fed females underwent forced oviposition in the insectary and the eggs were reared to adults. The WHO tests were conducted with samples from Nampula City and Erati districts on permethrin 0.75%. Figure 24 showed that *An. funestus* s.l. is resistant (13% mortality at Nampula and 0% mortality at Erati after the 24 hrs holding period) to permethrin in both Nampula City and Erati.

FIGURE 24. 24-HOUR MORTALITY FROM THE WHO TUBE TESTS OF ADULT *AN. FUNESTUS* S.L. COLLECTED BY PROKOPACK ASPIRATORS IN NAMPULA CITY AND ERATI DISTRICTS (JULY–DECEMBER 2019)



*These tests were conducted with fewer mosquitos than the standard WHO-recommended numbers, some replicates with 16 mosquitos and other replicates with 15 mosquitos. In Erati, all mosquitos exposed survived.

4.4.1 Determination of the Intensity of Resistance and Synergist Assays using WHO Tube Tests

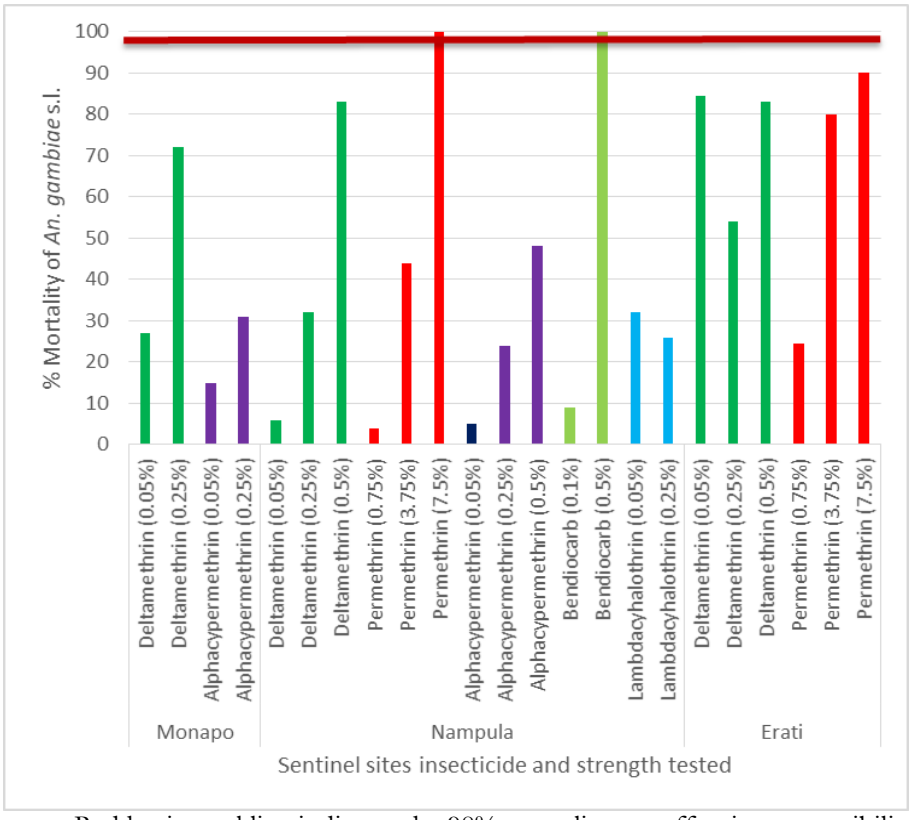
In Nampula City district, *An. gambiae* s.l., was resistant to deltamethrin and intensity assay results indicated that there was high intensity of resistance (32% mortality at 5x, and 83% mortality at 10x). In Monapo, moderate intensity of resistance (72% mortality at 5x) and in Erati high intensity of resistance (83% mortality at 10x) was detected to deltamethrin (Figure 25).

Resistance intensity to permethrin was moderate (44% mortality at 5x, but susceptible at 10x) at Nampula and high intensity (80% mortality at 5x and 90% mortality at 10x) at Erati.

For alpha-cypermethrin, in Nampula district high intensity of resistance (48% mortality at 10x) was detected. In Monapo, there was moderate intensity of resistance (31% mortality at 5x), but tests were not conducted at 10x.

In Nampula, resistance to lambda-cyhalothrin was detected at 1x (32% mortality) and moderate intensity of resistance at 5x (26% mortality) but tests have not yet been conducted at 10x. For bendiocarb, resistance was detected at 1X (9% mortality) and low intensity (100% mortality at 5x).

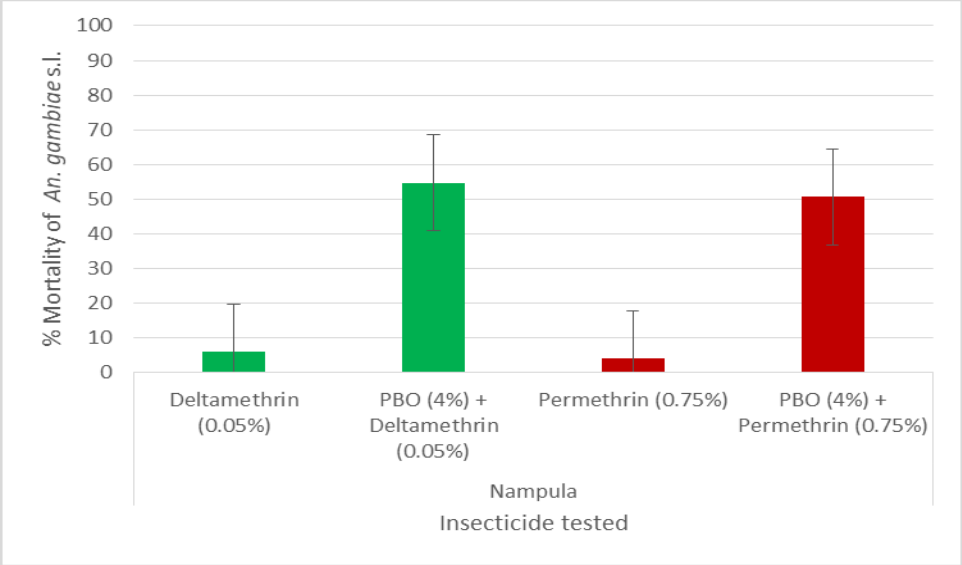
Figure 25. 24-hour Mortality of Adult *An. gambiae* s.l. to Diagnostic and Intensity Assay Concentrations



Red horizontal line indicates the 98% mortality cut-off point susceptibility

Pre-exposure of *An. gambiae* s.l. to PBO seems to increase susceptibility to deltamethrin and permethrin, but did not restore full susceptibility (Figure 26). The mortality rates increased significantly from 6% with deltamethrin alone to 54.7% with deltamethrin + PBO, and from 4% with permethrin alone to 50.7% with permethrin + PBO. However, for both insecticides, the mortality after pre-exposure to PBO was below 98%, indicating that monoxygenases are not the only form of metabolic resistance in the area.

FIGURE 26. MORTALITY OF *AN. GAMBIAE* S.L. EXPOSED TO DELTAMETHRIN AND PERMETHRIN WITH AND WITHOUT PBO IN NAMPULA (JANUARY–MARCH 2020).



5. Discussion and Lessons Learned

5.1 Zambezia Province

The entomological surveillance conducted in Zambezia initially employed three main collection methods, PSC/Prokopack aspirator, HLC, and CDC light traps. In September 2019, Prokopacks replaced PSC, and in February 2020, we stopped using HLCs. Anophelines collected by all these methods were identified using the morphological identification key, revealing the presence of nine anopheline species, with *An. funestus* s.l. being the most abundant (82.4%), followed by *An. gambiae* s.l. (12.83%) and other species, which accounted for 4.60%. Together, the two dominant vectors constituted 95.32% of the anopheline population collected. Coincidentally, the two species are known to be the most efficient malaria vectors in Africa. The greatest diversity of anopheline species was collected using HLCs, eight species out of the total nine collected, followed by CDC-light traps at six species and PSC/Prokopacks at only two species.

Unlike the other three districts, in Milange *An. gambiae* s.l. is more abundant than *An. funestus* s.l. during the reporting period. This is in contrast to the previous annual reports that indicated the dominance of *An. funestus* s.l. in all other sentinel sites in Zambezia. The team will further assess during the current collection season if there is any significant shift in species dominance and determine if this is in response to the IRS intervention in the area. A previous study (Bayoh *et al* 2010) showed a marked decline of *An. gambiae* s.s. occurred as household ownership of bed nets increased in western Nyanza province in Kenya. But, there was proportinate increase in *An. arabiensis* compared to *An. gambiae* s.s. for the same period.

Low levels of indoor resting mosquitoes were recorded in both intervention and control districts for the two main vector species, though more *An. funestus* s.l. than *An. gambiae* s.l. were recorded in most districts. Monthly indoor resting patterns show *An. funestus* s.l. to be more abundant during the dry season, between July and September, with a peak in August. This is followed by *An. gambiae* s.l. during the rainy season, with intermittent peaks in different districts between October and March. This observation shows how species succession reflects the seasonal variations driven by the annual climatic cycle.

Our findings show that IRS intervention reduced both the indoor resting density and biting rates of *An. funestus* s.l. from almost all districts. The most reduced biting rate was in Maganja da Costa, which fell by half from 4.33 b/p/n in the pre-spray period to 2.04 b/p/n in the post-spray period. Regarding bites by *An. funestus* s.l., the leading malaria vector, people in the control districts are apt to receive about two times (8.08 b/p/n to 16.38b/p/n) more bites than those in the intervention areas from pre-spray to post-spray periods indicating effect due to IRS. However, for the *An. gambiae* s.l. there was increase in Milange from 0.04 to 2.21 b/p/n, and in the control Lugela from 0.13 to 1.33 b/p/n. However, the indoor biting rates decreased in Milange from 1.58 to 0.71 b/p/n, and Mopeia from 0.07 to 0 b/p/n. This variability might have been associated with the rain fall patterns and availability of suitable breeding habitats of *An. gambiae* s.l during the rainy seasons in the areas.

In most districts, even intervention districts, biting activity was found to be higher indoors than outdoors. In contrast in Milange, the outdoor biting activity by *An. gambiae* s.l. post spray was higher (0.04 b/p/n in the pre spray and 6.38 b/p/n in the post spray), and this difference was statistically significant. Over all the data from mean indoor and outdoor biting rates of *An. funestus* s.l. in most districts, suggest a strong endophagic tendency of this species.

Overnight biting patterns for both *An. funes*tus s.l. and *An. gambia*e s.l. show that most biting occurs around midnight and in the early morning hours, when most people are expected to be sleeping in thmo houses under

a treated net. This finding shows the potential for sprayed houses and treated nets in protecting communities against infective bites from the two major vectors.

The molecular results of samples collected by PSC/Prokopack, HLC, and CDC light trap show that *An. funestus* s.s. and *An. gambiae* s.s. were the most abundant malaria vectors in the monitored sites, and the accuracy on these morphological identification ranges from 42.11% to 100%. The blood meal analysis was conducted only with samples collected with PSC/Prokopack and the only blood meal source tested was human. *An. funestus* s.s. and *An. gambiae* s.s. were the only known malaria vectors of the área positive with human blood meals. Surprisingly, *An. leesoni* was found to be positive for human blood. Although the simple size is generally low, the over all HBI varies from 0% to 100% for the different areas.

The quality of IRS assessed by cone wall bioassays showed that spray teams might not underdose the spraying in all districts, demonstrating appreciable skills in consistent, uniform application of the insecticides across districts. Subsequent monthly cone wall bioassays to monitor insecticide decay rates found that SumiShield® 50WG remains active at least 10 months post spray in Mopeia, while Fludora® Fusion remains effective at least 9 month. However, one more month data was collected for Sumishield to date due to the early start of the spray campaign in Mopeia. This long period of residual efficacy was also observed last year. The airborne fumigant effect of SumiShield® 50WG in Milange was found to be low for around two months; the same was observed with Fludora® Fusion where the effect lasted only one month. In Maganja da Costa, the effect of Fludora® Fusion lasted around four months.

Insecticide susceptibility test results show that local vectors are fully susceptible to pirimiphos-methyl, chlorfenapyr, clothianidin, and bendiocarb, however in Mopeia, posible resistance to pirimiphos-methyl was observed at diagnostic dose. Assays for pyrethroids again revealed widespread *An. gambiae* s.l. and *An. funestus* s.l. resistance to these insecticides. Further assays to assess the strength of the observed resistance in *An. gambiae* s.l. and *An. funestus* s.l. showed the presence of moderate to high intensity resistance to pyrethroids in five districts tested in Zambezia. The results may suggest to continue IRS with next generation insecticides for malaria vector control. Synergist assays with PBO demonstrated restoration of susceptibility at most of the sites, indicating involvement of oxidase-mediated resistance mechanisms. This shows the potential for PBO nets to effectively overcome the observed pyrethroid resistance threat in the area.

5.2 NAMPULA PROVINCE

A total of 3,442 anophelines were collected in surveyed districts of Nampula Province, using PSC and Prokopack, CDC light trap, and HLC techniques. The anopheline mosquitoes were found to belong to eight species and species complexes: *An. gambiae* s.l., *An. funestus* s.l., *An. coustani*, *An. pretoriensis, An. maculipalpis*, *An. ziemanni*, *An. tenebrosus,* and *An. rufipes*. *An. gambiae* s.l. and *An. funestus* s.l. were the major vectors, making up 64.45% and 35.19%, respectively, of the mosquitoes caught.

Lower indoor resting densities of *An. funestus* s.l. were observed after IRS in all intervention and control districts. The densities recorded in the control site were almost similar to Nampula City (intervention) for both *An. funestus* s.l. and *An. gambiae* s.l. before and after spraying. Other collections using CDC-LTs show that indoor densities in Erati were lower than in Nampula City, but much higher than in Monapo. HLC data for *An. funestus* s.l. biting activity show that while there was an increase in biting rate in Nampula (intervention) district, a drop was recorded in Monapo (intervention) district. *An. gambiae* s.l. biting activity demonstrated an increase in both control and intervention districts. These findings could potentially be an outcome of seasonal abundance of vector species during the rainy season.

Although the peak outdoor biting time of *An. funestus* s.l. in Nampula was between 1 to 2 am, biting continued outdoor in the morning hours of 5 to 6 am when residents might be awake and moved to outdoor for the daily routine activities. This might not be a favorable situation for any indoor based malaria vector control intervention. It is important to extend the collection period in the morning at least up to 7 am to better understand the extent of day time outdoor biting by this species in the area. Previous study in Senegal showed a behavioral change of *An. funestus* after introduction of ITNs, remaining anthrophophilic and endophilic, while adopting, diurnal feeding (Sougoufara *et al* 2014). The study from Senegal indicated that there were six times

more *An. funestus* captured in broad daylight than at night. Significant biting also occurred between 6 to 7 pm both indoor and outdoor by *An. gambiae* s.l. in Nampula. It is important to start collections at 5 pm to better understand potential exposure to mosquito bites in the early evening hours.

The quality of IRS assessed by cone wall bioassays showed that spray teams were able to achieve optimal insecticide application in all districts, demonstrating appreciable skills in consistent uniform application of insecticides across districts. Subsequent monthly cone wall bioassays to monitor insecticide decay rates found that SumiShield® 50WG sprayed in Monapo and Fludora® Fusion in Nampula were found to remain effective on sprayed walls at least nine months.

An. gambiae s.l. remained susceptible to both clothianidin and pirimiphos-methyl in Nampula province. There is however, high intensity of resistance to pyrethroid insecticides at most sites in Nampula province, and synergist assays showed that PBO did not restore susceptibility to pyrethroid insecticides in the area. This might indicate that PBO nets with deltamethrin and permethrin insecticides may not be a future option for malaria vector control in the areas, and signifies the importance of continued application of next generation insecticides with indoor residual spraying.

Longitudinal monitoring, wall bioassay tests and insecticide susceptibility tests were suspended from April to July 2020 in both Zambezia and Nampula provinces due to the COVID-19 pandemic. All activities resumed in August 2020 with mitigation measures in place as per the project wide operating guidelines to reduce COVID transmission. This interruption significantly affected the ability of the team to collect mosquitoes during part of the peak mosquito abundance season for longitudinal monitoring and collection of adult *An. funestus* s.l. mosquitoes for susceptibility tests.

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ANNEX

TABLE A1. PCR SPECIES IDENTIFICATION OF SAMPLES COLLECTED BY PSC/PROKOPACK IN MAGANJA DA COSTA, MILANGE, MOPEIA, AND LUGELA

Districts	Identified by field team	# identified by field team	PCR species identified by INS					
			An. funestus s.s.	*An. gambiae* s.s.	*An. arabiensis*	*An. leesoni*	Not amplified	% Accuracy
Maganja da Costa	*An. funestus* s.l.	42	38	3	0	1	0	90.48
	An. gambiae s.l.	2	0	0	0	2	0	0
Milange	*An. funestus* s.l.	33	17	16	0	0	0	51.52
	An. gambiae s.l.	0	0	0	0	0	0	0
Mopeia	*An. funestus* s.l.	26	19	2	0	0	5	73.08
	An. gambiae s.l.	0	0	0	0	0	0	0
Lugela	*An. funestus* s.l.	76	75	0	0	1	0	98.68
	An. gambiae s.l.	2	0	2	0	0	0	0
Total		**181**	**149**	**23**	**0**	**4**	**5**	

TABLE A2. BLOOD MEAL SOURCE OF VECTOR SPECIES COLLECTED BY PSC/PROKOPACK IN MAGANJA DA COSTA, MILANGE, MOPEIA, AND LUGELA

Districts	Identified by field team	# identified by field team	Human blood meal positivity of species identified by PCR at the INS					% human blood
			An. funestus s.s.	*An. gambiae* s.s.	*An. arabiensis*	*An. leesoni*	Not amplified	
Maganja da Costa	*An. funestus* s.l.	42	10	2	0	0	30	28.57
	An. gambiae s.l.	2	0	0	0	2	0	100
Milange	*An. funestus* s.l.	33	0	0	0	0	33	0
	An. gambiae s.l.	0	0	0	0	0	0	0
Mopeia	*An. funestus* s.l.	26	0	0	0	0	26	0
	An. gambiae s.l.	0	0	0	0	0	0	0
Lugela	*An. funestus* s.l.	76	6	0	0	0	70	7.89
	An. gambiae s.l.	2	0	0	0	0	2	0
Total		**181**	**16**	**2**	**0**	**2**	**161**	

TABLE A3. PCR SPECIES IDENTIFICATION OF SAMPLES COLLECTED BY HLC IN MAGANJA DA COSTA, MILANGE, MOPEIA, AND LUGELA

Districts	Identified by field team	# identified by field team	PCR species identified by INS					% Accuracy
			An. funestus s.s.	*An. gambiae* s.s.	*An. arabiensis*	*An. leesoni*	Not amplified	
Maganja da Costa	*An. funestus* s.l.	100	99	1	0	0	0	99
	An. gambiae s.l.	77	9	66	0	0	2	85.71
Milange	*An. funestus* s.l.	49	47	0	0	2	0	100
	An. gambiae s.l.	18	1	17	0	0	0	94.44
Mopeia	*An. funestus* s.l.	238	233	5	0	0	0	97.90
	An. gambiae s.l.	19	10	8	0	0	1	42.11
Lugela	*An. funestus* s.l.	98	98	0	0	0	0	100
	An. gambiae s.l.	19	8	11	0	0	0	57.89
Total		**618**	**505**	**108**	**0**	**2**	**3**	

TABLE A4. PCR SPECIES IDENTIFICATION OF SAMPLES COLLECTED BY CDC LIGHT TRAP IN MAGANJA DA COSTA, MILANGE, MOPEIA, AND LUGELA

Districts	Identified by field team	# identified by field team	PCR species identified by INS					% Accuracy
			An. funestus s.s.	*An. gambiae* s.s.	*An. arabiensis*	*An. leesoni*	Not amplified	
Maganja da Costa	*An. funestus* s.l.	43	34	4	0	0	5	79.07
	An. gambiae s.l.	21	7	14	0	0	0	66.67
Milange	*An. funestus* s.l.	9	5	0	1	0	3	55.56
	An. gambiae s.l.	7	3	1	3	0	0	57.14
Mopeia	*An. funestus* s.l.	834	812	4	2	0	16	97.36
	An. gambiae s.l.	148	2	96	11	38	1	72.30
Lugela	*An. funestus* s.l.	42	35	5	0	0	2	83.33
	An. gambiae s.l.	10	4	4	0	0	2	40.00
Total		**1114**	**902**	**128**	**17**	**38**	**29**	

TABLE A5. COMPARISON OF TOTAL NUMBER OF *AN. ZIEMANNI* AND *AN. TENEBROSUS* COLLECTED BY HLC INDOORS AND OUTDOORS IN FOUR DISTRICTS

District	*An. ziemanni*				*An. tenebrosus*			
	# Collected indoors	**# Collected outdoors**	**X^2**	**p-value**	**# Collected indoors**	**# Collected outdoors**	**X^2**	**p-value**
Maganja da Costa	14	34	8.33	0.0038	2	0	2	0.157
Milange	1	0	1	0.317	2	5	1.29	0.256
Mopeia	80	76	0.1	0.748	114	90	2.82	0.093
Lugela (Control)	2	4	0.67	0.4142	0	0		