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THE PMI VECTORLINK PROJECT
UGANDA

ANNUAL ENTOMOLOGY REPORT

JANUARY 1 2021 – DECEMBER 31, 2021

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THE PMI VECTORLINK PROJECT

UGANDA ANNUAL ENTOMOLOGY REPORT JANUARY 1, 2021 – DECEMBER 31, 2021

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ACRONYMS AND ABBREVIATIONS

Ace-1	Acetylcholinesterase 1 gene
CDC	Centers for Disease Control and Prevention
COVID-19	Coronavirus disease of 2019
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked Immunosorbent Assay
HBR	Human Biting Rate
HLC	Human Landing Catch
IDRC	Infectious Disease Research Collaboration
IRS	Indoor Residual Spray
Kdr	Knockdown resistance gene
LLIN	Long-Lasting Insecticidal Net
PBO	Piperonyl butoxide
PCR	Polymerase Chain Reaction
PMI	President's Malaria Initiative
PSC	Pyrethrum Spray Catch
SOP	Standard Operating Procedure
VCO	Vector Control Officer
WHO	World Health Organization

EXECUTIVE SUMMARY

During the 2021 spray campaign, the President's Malaria Initiative VectorLink Uganda Project conducted IRS using only one insecticide, Fludora Fusion 56.25 WP-SB, in 14 districts. To assess the quality and impact of vector control interventions, the project conducted monthly entomological monitoring using human landing catches indoors and outdoors, and pyrethrum spray catches in six districts: Bugiri, Lira, and Tororo (IRS districts), and Apac, Otuke, and Soroti (non-IRS districts). All bionomics study districts received pyrethroid-only insecticide-treated nets (PermaNet 2.0 and Yorkool LN), in the 2020 mass campaign with the exception of Apac, which received piperonyl butoxide (PBO) insecticide-treated nets (Permanet 3.0) and pyriproxyfen insecticide-treated nets (Royal Guard). Cone wall bioassays for quality assurance of the IRS were conducted in seven districts, and for monthly residual efficacy in four districts (Bugiri, Lira, Tororo, and Serere). Insecticide susceptibility tests were carried out to assess the susceptibility of *An. gambiae* s.l. to insecticides used in public health vector control (pyrethroids (alpha-cypermethrin, deltamethrin, and permethrin), carbamates (bendiocarb), organophosphates (pirimiphos-methyl), pyrroles (chlorfenapyr), and neonicotinoids (clothianidin)) in eight geographically dispersed districts: three IRS districts (Bugiri, Lira, and Tororo) and five non-IRS districts (Apac, Arua, Kanungu, Mityana, and Moroto).

The two longitudinal sampling methods (human landing catches and pyrethrum spray catches) combined yielded a total of 30,949 *Anopheles* mosquitoes from the six sentinel sites. 23,721 (76.6%) were *An. funestus* s.l., 7,197 (23.3%) were *An. gambiae* s.l., and 31 (0.1%) were other *Anopheles* mosquitoes, namely *An. coustani* and *An. ziemanni*. Vector distribution differed by study site: *An. gambiae* s.l. was dominant in Bugiri and Tororo districts while *An. funestus* s.l. was the predominant species complex in Soroti, Apac, Lira, and Otuke. Soroti (non-IRS site) had the highest percentage of all *An. funestus* s.l. collected, 73.8% (n=17,503). In Lira and Otuke, *An. funestus* was found at higher densities indoors compared to *An. gambiae* s.l., but *An. gambiae* s.l. was found at higher densities outdoors compared to *An. funestus* s.s.No *An. funestus* s.l. were caught either indoors or outdoors in Tororo.

The impact of the IRS was mixed – as anticipated, the proportion of indoor resting gravid and half-gravid *An. gambiae* s.l. was smaller in the IRS districts than in the non-IRS districts, but this was not true for *An. funestus* s.l. Indoor resting densities of both species complexes was initially suppressed by IRS in Tororo and Bugiri, though densities started to return to pre-IRS levels toward the end of the year. In Lira, mosquito densities had already increased in April before IRS was applied. More encouragingly, the endophagic rate in the IRS districts was higher than that in the non-IRS districts possibly suggesting that repeated applications of IRS are not resulting in exophagic behavior. However, the endophagic rate was higher for *An. funestus* s.l. than for *An. gambiae* s.l., and as the latter remains the more prevalent species complex in Tororo and Bugiri continued IRS in those districts may be having a diminishing impact. Surprisingly, human biting rates for *An. gambiae* s.l. increased initially after IRS in both Tororo and Lira, though the increases were not as marked as those in non-IRS districts and densities were much lower. Tororo experienced its highest April rainfall in the last five years, which may account for the increase in densities observed in that month. Worryingly, a rebound in the density of *An. gambiae* s.l. in Otuke has occurred following withdrawal of IRS in 2021.

In all districts where tests were performed, *An. gambiae* s.l. remained susceptible to pirimiphos-methyl, an insecticide used in the 2019 IRS campaign and in part in the 2020 IRS campaign. Susceptibility to chlorfenapyr and clothianidin, one of the active ingredients of Fludora Fusion, was also reported from all districts where susceptibility tests were performed. *An. gambiae* s.l. was resistant to the three pyrethroids (alpha-cypermethrin, deltamethrin, and permethrin) at all sites, though resistance intensity was highly variable. Synergist tests performed with piperonyl butoxide partly or fully restored pyrethroid susceptibility, indicating at minimum a partial role of mixed function oxidases in the resistance that was phenotypically expressed. Encouragingly, pre-exposure to PBO fully restored susceptibility in Apac, where PBO-nets have been distributed. No susceptibility

tests were conducted with *An. funestus* s.l. due to difficulties collecting and rearing larvae in all the eight study districts.

Cone wall bioassays performed for IRS quality assurance resulted in 100% mortality for all wall surface types sprayed with Fludora Fusion within 48 hours after exposure, indicating that the spraying was performed well. The residual efficacy bioassays demonstrated that the Fludora Fusion remained efficacious for at least six months for all substrates in all districts, but there was some variation between districts, and this efficacy does not seem to correlate with reduced human biting rates for *An. gambiae* s.l. soon after the IRS was sprayed in both Tororo and Lira, In Tororo, the mortality from the cone wall bioassays remained above 80% for eight months on all substrates, and at least nine months for the painted cement wall surfaces. In contrast, in Serere, the mortality dropped below 80% for mud and brick surfaces in month seven, and for the painted cement surface in month eight.

Molecular assays are ongoing for identification of sibling species within species complexes, sporozoite infection rate determination, and detection and identification of genetic resistance markers (knockdown resistance and Acetylcholinesterase-1 genes); these results will be shared in a supplementary addendum once available.

In conclusion, the insecticides selected for IRS remain relatively efficacious when measured purely by the residual efficacy indicator , but it is concerning that the protection that IRS provides does not seem to last long enough to prevent a resurgence in mosquito densities in October–November 2021. This is different from what was seen in 2020, when October-November densities did not exceed pre-IRS levels. In addition, attention should be given to the timing of IRS campaigns, especially for the northern districts, so that spraying is conducted prior to any increase in mosquito densities.

1. INTRODUCTION

Indoor residual spraying (IRS) and long-lasting insecticide-treated nets (ITNs) 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. During the 2021 spray campaign, the President's Malaria Initiative VectorLink Uganda Project conducted IRS in two phases, with Phase I conducted on March 1–March 27, 2021, and Phase II on April 26–May 22, 2021. The project used only one insecticide, Fludora Fusion 56.25 WP-SB, for the 2021 spray campaign in 14 targeted districts. Phase I comprised eight districts in eastern Uganda (Budaka, Bugiri, Butaleja, Butebo, Kibuku, Namutumba, Pallisa, and Tororo) and Phase II comprised six northern districts (Amolatar, Dokolo, Kaberamaido, Kalaki, Lira, and Serere). IRS in Amolatar, Dokolo, Kaberamaido, and Kalaki was funded by the Department for International Development, United Kingdom, now the Foreign, Commonwealth and Development Office; spraying in the remaining 10 districts was funded by the United States Agency for International Development/ President's Malaria Initiative.

During the reporting period, the President's Malaria Initiative (PMI) VectorLink Project carried out entomological monitoring activities in 11 districts (Figure 1) and supported the entomological surveillance activities of the National Malaria Control Division (NMCD) in other districts. The entomological data generated inform the selection of insecticides for vector control in Uganda and add to the evidence base for evaluation of vector control impact and best practice. These data are collected through different surveillance activities: susceptibility tests of local vector species to different insecticides and determination of the underlying mechanisms, cone bioassays to evaluate the quality and residual efficacy of spraying on different wall types, and monthly longitudinal monitoring of the vector density, behavior, and composition in districts that received indoor residual spraying and those that did not.

Longitudinal entomological monitoring was conducted in three indoor residual spray (IRS) intervention districts: Bugiri, Lira, and Tororo; and in three non-IRS districts: Apac, Otuke, and Soroti. Apac received PBO nets (Permanet 3.0) and pyriproxyfen nets (Royal Guard) in the 2020 mass campaign, while all other districts listed received pyrethroid-only ITNs. In all districts, entomological monitoring data were collected using pyrethrum spray catches (PSCs) and human landing catches (HLCs) indoors and outdoors. Larval collections of *An. gambiae* s.l. provided adults of known age for insecticide susceptibility tests in eight districts: three IRS districts (Bugiri, Lira, and Tororo) and five non-IRS districts (Apac, Arua, Kanungu, Mityana, and Moroto). These districts were representative of the different epidemiological settings in the country. Cone assays of surfaces sprayed with IRS were performed using pyrethroid-susceptible mosquitoes reared at insectaries in Gulu, Tororo, and Kampala.

In Uganda, malaria vectors and malaria transmission increase within 2–4 weeks after the start of the rains and peak transmission follows the peak of the rainy season. Most of Uganda experiences a bi-modal rainfall pattern. March to May constitutes the first (and main) rainfall season; September to November is the second rainfall season and occurs in most parts of the country. June to August is generally dry in the southwestern, Central, and Lake Victoria basin regions and some parts of the Eastern region, but there is continued rainfall for much of northern Uganda during these months. December to February is dry for most parts of the country. Annually, the east and north receive 1200–1600mm of rain; typically higher than the northeast and southwest (Figure 2). For the Eastern region's IRS districts, the first rains are usually experienced between late February and mid-March and peak around late April, with cessation of rains expected around late May/mid-June except during El Niño years. For the IRS districts in the Northern region, the first rains are usually experienced in mid-March and end around late June/early July. However, over recent years, the rainfall patterns have become unpredictable, and there have been unusually heavy and extended rains in the country, which has made it more difficult to plan when it is most appropriate to conduct IRS campaigns and to find enough larvae for susceptibility studies.

FIGURE 1: PMI VECTORLINK PROJECT DISTRICTS FOR ENTOMOLOGICAL MONITORING IN 2021

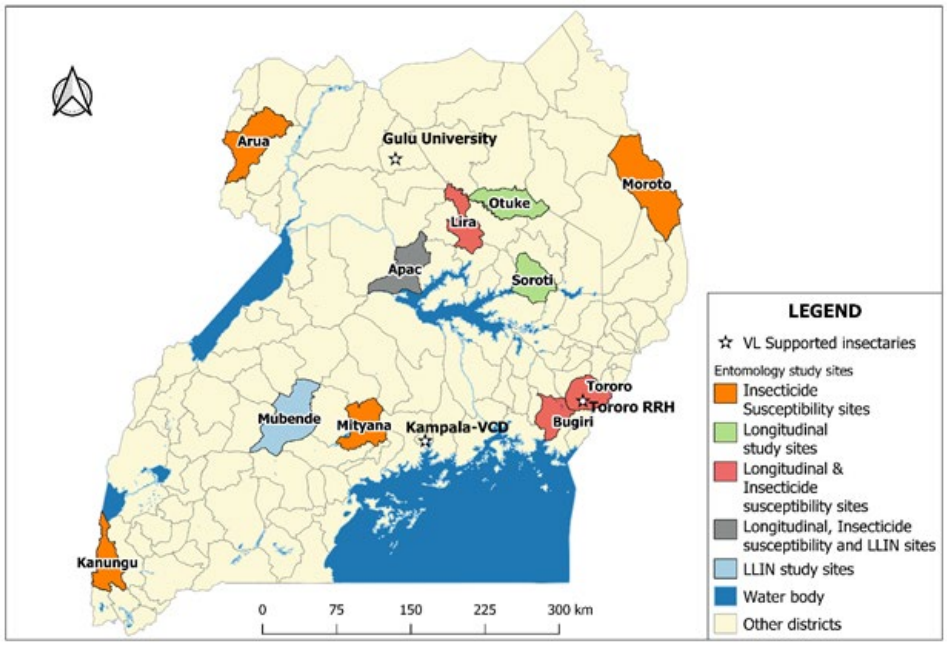
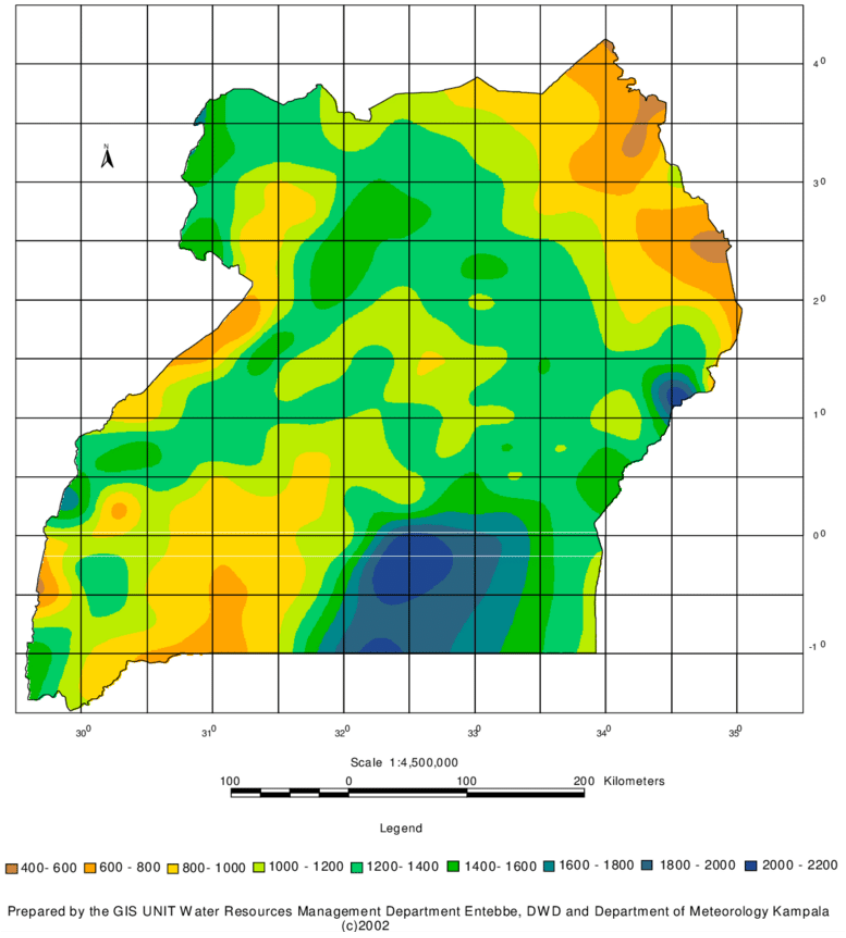


FIGURE 2: MAP OF UGANDA SHOWING MEAN ANNUAL RAINFALL (MM)



2. METHODOLOGY

2.1 LONGITUDINAL MONITORING (BIONOMICS STUDIES)

VectorLink Uganda collected adult mosquitoes on a monthly basis from January through December 2021 using PSCs (in accordance with PMI VectorLink Standard Operating Procedure (SOP) 03/01[1]) and HLCs (PMI VectorLink SOP 02/01) in six sentinel sites: Bugiri, Lira, and Tororo (current IRS districts), and Apac, Otuke, and Soroti (non-IRS/control districts). Table 1 summarizes the longitudinal monitoring methods.

TABLE 1. LONGITUDINAL MONITORING ADULT MOSQUITO COLLECTION METHODS

Collection Methods	Time	Frequency	Sample
PSCs	6:00 a.m. to 10:00 a.m.	Two days every month	Twenty houses in three villages per sentinel site (10 houses each day for two days) – same houses used every month
HLCs	6:00 p.m. to 7:00 a.m.	Two consecutive nights every month	Two houses per sentinel site – same houses used every month

2.2 BEHAVIOR AND DENSITY

2.2.1 PYRETHRUM SPRAY CATCH

In each district where PSCs were conducted (Apac, Bugiri, Lira, Otuke, Soroti, and Tororo), 20 single-room, grass-thatched houses in each of three villages were selected. PSCs were conducted from 6a.m. to 10 a.m., once per month over two days in each district from January through December 2020, except during July and August, when the country experienced lockdown down due to the COVID-19 pandemic. The same houses were visited each month. A pyrethroid-based aerosol (KillIt commercial spray, containing d-Tetramethrin 0.135% w/w, d-Allethrin 0.06% w/w, and cypermethrin 0.46% w/w) was used to knock down indoor resting mosquitoes. The room was closed for 10 minutes after spraying the aerosol, and then the knocked-down mosquitoes, which had fallen on the white sheets laid on the floor, were collected using forceps and placed in a labeled petri dish. The samples were identified morphologically (Coetzee 2020) and preserved in 1.5 mL microfuge tubes. A hole was pierced in the lid of all tubes, and these were kept in plastic containers containing silica gel. A subset of samples collected by this method was sent for further identification and tests using the Polymerase Chain Reaction (PCR) technique at the Infectious Diseases Research Collaboration (IDRC) molecular laboratories.

2.2.2 HUMAN LANDING CATCH

HLCs were conducted indoors in two houses in each district on two consecutive nights. Outdoor HLCs were conducted concurrently, at least 5 meters away from the same houses. This resulted in eight person-nights of collection per district per month (two houses x two collection nights = four person-nights indoors and four person-nights outdoors). Human volunteers (trained adult mosquito collectors), one inside the house and the other outside, collected mosquitoes in four-hour shifts. Collections were conducted from 6:00 p.m. to 7:00 a.m. using 12 volunteers in total. For each hour of collection, collectors collected mosquitoes for 55 minutes and rested for 5 minutes, during which they exchanged positions. During the time of collection, the collectors exposed their legs up to the knees and arms up to the elbow; when they detected mosquitoes landing on their limbs, they turned on a torch and collected the mosquitoes using a mouth aspirator. The mosquitoes were transferred into paper cups labeled for each hourly collection. They were subsequently killed by exposure to

[1] Complete SOPs can be found here: https://pmivectorlink.org/resources/tools-and-innovations/

cotton wool soaked in diethyl ether, identified to species, counted, and preserved in 1.5 mL microfuge tubes. A subset of samples from these collections were sent to IDRC molecular laboratories for PCR analyses. Data obtained from HLCs were used to directly determine human biting rate (HBR). HBR was calculated by dividing the number of mosquitoes of a single species or species complex (*An. gambiae* s.l. and/or *An. funestus* s.l.) by the number of human-nights.

2.3 STRAIN CHARACTERIZATION OF INSECTARY-REARED *AN. GAMBIAE* S.S. KISUMU STRAIN

During the reporting period, the project conducted susceptibility testing for strain characterization of 3–5-day old *An. gambiae* s.s. Kisumu reared at the Vector Control Division in Kampala, Tororo Hospital, and Gulu University insectaries. The Kisumu colonies were exposed to alpha-cypermethrin 0.05%, deltamethrin 0.05%, and permethrin 0.75% using the World Health Organization (WHO) tube assay (WHO 2016).

2.4 VECTOR SUSCEPTIBILITY TESTING OF WILD POPULATIONS

Insecticide susceptibility studies were conducted using the WHO tube bioassays and Centers for Disease Control and Prevention (CDC) bottle bioassays to determine *An. gambiae* s.l susceptibility or resistance to insecticides recommended by the WHO for use in public health. The five classes of insecticides tested included: neonicotinoids (clothianidin), pyrroles (chlorfenapyr), organophosphates (pirimiphos-methyl 0.25%), pyrethroids (deltamethrin 0.05%, permethrin 0.75%, and alpha-cypermethrin 0.05%,) and carbamates (bendiocarb 0.1%). The pyrethroids, bendiocarb, and pirimiphos-methyl were tested using the WHO tube bioassays. Diagnostic doses of the insecticides were used following the PMI VectorLink SOP 06/01 based on the standard WHO bioassay method (WHO 2016). When pyrethroid resistance was detected, resistance intensity was determined using 5x and 10x diagnostic doses. Synergist assays with a pre-exposure to 4% piperonyl butoxide (PBO) were conducted in tube bioassays to assess the contribution of mixed function oxidases to phenotypic resistance. Synergist assays using PBO were run in parallel to controls using the insecticide only, PBO only, and acetone only. All exposures using the WHO tube tests 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.

Clothianidin and chlorfenapyr were tested in CDC bottle bioassays. For both insecticides, 20 to 25 mosquitoes were aspirated into each of four 250mL glass Wheaton bottle bottles coated with solutions prepared from technical grade insecticide. After the exposure time of 1 hour, the mosquitoes were released in a clean cage and aspirated back to paper cups and fed with 10% sugar solution on wet cotton balls to assess delayed mortality. For clothianidin, mortality was scored 24 hours after exposure. For chlorfenapyr, mortality was scored every 24 hours up to 72 hours after exposure.

Technical grade clothianidin was dissolved in a mixture of Mero (81% Rapeseed oil methyl ester) and acetone. Clothianidin tends to crystallize if used with acetone alone and the uptake of active ingredient by the mosquito becomes low. Therefore, the Mer adjuvant manufactured by Bayer Crop Science was used to prevent crystallization. Bottles were coated with 4µg technical grade clothianidin dissolved in a mixture of 800ppm Mero® (81% Rapeseed oil methyl ester) and acetone. Two control bottles were coated using a solution of the acetone/Mero mixture. Clothianidin stock and working solutions were made fresh or stored at 4°C in the fridge in amber bottles to protect against UV light and used within 24 hours. The solution was left for 1h at room temperature and vigorously shaken for 10 seconds before pipetting into bottles. Testing of the susceptibility of *An. gambiae* s.l. to chlorfenapyr followed the PMI VectorLink SOP 04/01. The bottles were coated with chlorfenapyr provided by CDC Atlanta at a concentration of 100µg/mL. Two bottles impregnated with acetone only were set up similarly and served as negative controls. All bottles were treated a day before conducting the tests.

All WHO tube and CDC bottle susceptibility tests were performed using adults reared from field-collected larvae of *An. gambiae* s.l. at field insectaries established in the selected study sites in the districts, following the PMI VectorLink SOP 14/01 protocol. All susceptibility tests were conducted to the greatest extent possible

under the recommended optimal conditions, at temperatures of 27±2°C and 75%±10% relative humidity during exposure and post-exposure holding periods. Maximum and minimum temperature and humidity were recorded at the end of the testing and holding periods and recorded on the results form. When control mortality was higher than 5% but equal to or less than 10%, Abbott's correction was applied to test mortalities (Abbott 1925). Control-adjusted mortality rates equal to or higher than 98% were classified as susceptible, between 90% and 97% as suggestive of resistance and requiring further investigation, and below 90% as resistant. A subset of *An. gambiae* s.l. samples from the susceptibility tests were sent to the IDRC molecular laboratories for PCR assays to identify sibling species and detect the presence of knockdown (*kdr*) and acetylcolinesterase-1 (*Ace-1*) genes and to determine sporozoite rates.

2.5 IRS QUALITY ASSURANCE, RESIDUAL EFFICACY, AND FUMIGANT EFFECT

WHO cone bioassay tests were performed according to the standard PMI VectorLink SOP 09/01 in each of seven spray districts (Bugiri, Butaleja, Dokolo, Kibuku, Lira, Serere, and Tororo) within one week of the start of spraying to assess the quality of spraying. Thereafter, cone bioassays were conducted in the same houses each month in four districts – Bugiri, Lira, Serere, and Tororo – to determine IRS residual efficacy. Bioassays were run until control-adjusted mortality dropped below 80% for two consecutive months. Six houses of different wall types (two cement plastered and painted, two plain brick, and two mud-walled) were randomly selected in each study village in each study district. Cones were fixed with self-adhesive tape onto sprayed walls at heights of 0.5, 1.0, and 1.5m from the floor. Control cones were fixed to a wall lined with paperboard in an unsprayed house. Two- to five-day-old female susceptible An. gambiae s.s. Kisumu strain mosquitoes were used for the tests. Mosquitoes were introduced into the plastic cones in batches of 10 and exposed for 30 minutes. The number of mosquitoes knocked down at 30 minutes was recorded. At the end of the exposure period, the mosquitoes were carefully transferred to paper cups and provided with 10% sugar solution soaked into cotton wool pads. Mortality was recorded every 24 hours up to 120 hours.

Tests for the fumigant effect of Fludora Fusion were conducted using mosquitoes inside a cage suspended 10 cm away from the sprayed wall surface at a height of 1.0 m above the floor. The exposure time was 30 minutes; knock-down was recorded at 30 mins and 60 mins after exposure. After exposure mosquitoes were transferred into clean paper cups and mortality was monitored every 24 hours up to 120 hours.

2.6 MOLECULAR ASSAYS

The molecular assays were performed by the IDRC molecular laboratory and included the vector species identification, determination of sporozoite rate (detection of *Plasmodium falciparum*), and detection of insecticide resistance genetic markers *kdr* and *Ace-1* using established protocols as stipulated in the Methods of *Anopheles* Research (MR4 2014).

2.6.1 VECTOR SPECIES IDENTIFICATION

Following morphological identification of individual mosquitoes in the field, a cohort of these was subjected to PCR assays to identify sibling species. The MR4 *An. gambiae* s.l. assay for species diagnosis (Scott et al. 1993) was used to separate *An. gambiae* s.s. and *An. arabiensis*. *An. gambiae* s.s. will be further analyzed and separated into *An. gambiae* s.s. and *An. coluzzii*. *An. funestus* s.l. species identification was conducted according to Koekemoer et al. (2002).

2.6.2 DETECTION OF MALARIA PARASITES

Detection and identification of malaria parasites in *An. gambiae* s.l. and *An. funestus* s.l., by district and by month of collection, were performed using *Plasmodium falciparum* ELISA assays following the method of Wirtz et al. (1989) and expressed as sporozoite rates.

2.6.3 DETECTION OF INSECTICIDE RESISTANCE MARKERS

To determine the prevailing resistance mechanisms, molecular assays were used to detect presence of *kdr* and *Ace-1* genes. This was done in mosquito samples whose insecticide resistance phenotype had been determined using standard WHO susceptibility assays. DNA extraction provided a template used for determining the underlying genotype for the *kdr* mutation. PCR was used as a diagnostic method for detection of *kdr* mutations following protocols described by Martinez-Torres et al. (1998) (*kdr* L1014F) and Ranson et al. (2000) (*kdr* L1014S). Mosquitoes were also screened for insensitive *Ace-1*R by the PCR method of Weill et al. (2004).

3. RESULTS

3.1 LONGITUDINAL MONITORING

3.1.1 ALL COLLECTION METHODS COMBINED

During the reporting period, 30,949 female *Anopheles* mosquito species were collected using the two trapping methods (PSCs and HLCs) from the six districts, and morphologically identified (Table 2). Overall, *An. funestus* s.l. was the most abundant species complex followed by *An. gambiae* s.l.: a total of 23,721 (76.7%) *An. funestus* s.l. were collected compared with 7,197 (23.3%) *An. gambiae* s.l. The relative proportion of the two species complexes was dependent on location – *An. funestus* s.l. was more common than *An. gambiae* s.l. in the four northern districts of Apac, Otuke, Soroti, and Lira, but the reverse was found in the eastern districts of Bugiri and Tororo. Other *Anopheles* species comprised 0.1% (n=31) of the total number caught and included only *An. coustani* and *An. ziemanni.*

TABLE 2: NUMBER OF FEMALE *ANOPHELES* MOSQUITOES COLLECTED IN EACH DISTRICT BY PSC AND HLC COMBINED, JANUARY THROUGH DECEMBER 2021

Mosquito Species	Non-IRS Districts			IRS Districts			All Districts	
	Apac	Otuke	Soroti	Bugiri	Lira	Tororo	Total	%
An. funestus s.l.	2,439	2,100	17,503	54	1,625	0	23,721	76.6
An. gambiae s.l.	259	2,001	2,543	604	1,196	594	7,197	23.3
Other Anopheles species	2	0	9	7	13	0	31	0.1
Total per district	**2,700**	**4,101**	**20,055**	**665**	**2,834**	**594**	**30,949**	**100**

3.1.2 PYRETHRUM SPRAY CATCH

PSC collections yielded 19,883 *Anopheles* mosquitoes: 14,345 (72.1%) *An. funestus* s.l., 5,526 (27.8%) *An. gambiae* s.l., and 12 (0.1%) other *Anopheles* species (Table 3). Most *Anopheles* vectors caught resting indoors were from the unsprayed districts of Apac, Otuke, and Soroti. More *An. funestus* s.l. were caught in these districts than in any of the IRS districts, and also more *An. gambiae* s.l. except for in Apac. *An. funestus* s.l. was the predominant species complex resting indoors in all districts apart from Bugiri and Tororo (Figure 3). The relative proportions of the two dominant species complexes in each district resemble the 2020 data, with the notable exception of Otuke, where the percentage of *An. funestus* s.l. has decreased sharply from 91% to 54%. This reflects an increase in absolute numbers of *An. gambiae* s.l. caught by PSC in Otuke rather than a decrease in *An. funestus* s.l. numbers. *An. gambiae* s.l. numbers increased from 234 in 2020 to 1,288 in 2021. This is likely to be the result of IRS withdrawal in Otuke in 2021 – there are two peaks in *An. gambiae* s.l. densities in Otuke in 2021 that were suppressed by IRS in 2020 (Figure 4).

TABLE 3: NUMBER OF MOSQUITOES BY SPECIES COLLECTED USING PSC IN THE STUDY DISTRICTS, JANUARY THROUGH DECEMBER 2021

Mosquito Species	Non-IRS Districts			IRS Districts			All Districts	
	Apac	Otuke	Soroti	Bugiri	Lira	Tororo	Total	%
An. funestus s.l.	2,284	1,538	9,075	47	1,401	0	14,345	72.1
An. gambiae s.l.	168	1,288	2,316	502	921	331	5,526	27.8
Other Anopheles species	0	0	3	2	7	0	12	0.1
Total per district	**2,452**	**2,826**	**11,394**	**551**	**2,329**	**331**	**19,883**	**100**

In the IRS districts, there was evidence that the IRS suppressed indoor resting densities of *An. gambiae* s.l. in Bugiri; densities did increase from June to November but did not reach pre-IRS levels (Figure 5). In Tororo, *An. gambiae* s.l. densities decreased post-IRS from March to May, but by November densities exceeded pre-IRS levels. Nevertheless, it is important to note that, in both districts, absolute numbers of *An. gambiae* s.l. were low throughout the reporting period and lower than in the non-IRS districts of Otuke and Soroti. The indoor resting densities of *An. funestus* s.l. in Bugiri and Tororo were extremely low both before and after IRS. In Lira, the IRS did not have an immediate impact on *An. gambiae* s.l. densities, which were highest during April (the month of spraying) and May, but thereafter declined to the extent that *An. funestus* s.l. densities were higher after June. An increase in *An. funestus* s.l. from September to November was observed in Lira – this trend was also observed in the non-IRS districts of Otuke and Soroti, where higher densities were reached than in Lira (Figure 5). The distribution of PBO (Permanet 3.0) and pyriproxyfen (Royal Guard) long-lasting insecticide-treated nets (LLINs) in Apac in the fourth quarter of 2020 likely reduced the usefulness of Apac as a control district for the IRS districts as it was expected that these nets would be effective against *An. gambiae* s.l. and *An. funestus* s.l. populations in that district.

Figures 6 and 7 show the impact that IRS had on the abdominal stages *of An. gambiae* s.l. and *An. funestus* s.l. caught by PSC in IRS districts compared to those from non-IRS districts. The proportion of half-gravid and fully gravid *An. gambiae* s.l. mosquitoes caught in IRS districts was considerably less than in non-IRS districts indicating that the IRS was killing mosquitoes that were resting on the walls before bloodmeals were digested and eggs were produced. This was much less apparent for *An. funestus* s.l. – the proportion of fully gravid mosquitoes caught in IRS districts was only marginally smaller than for non-IRS districts.

FIGURE 3: *ANOPHELES* SPECIES COMPOSITION BY STUDY SITE USING PSCS, JANUARY THROUGH DECEMBER 2021

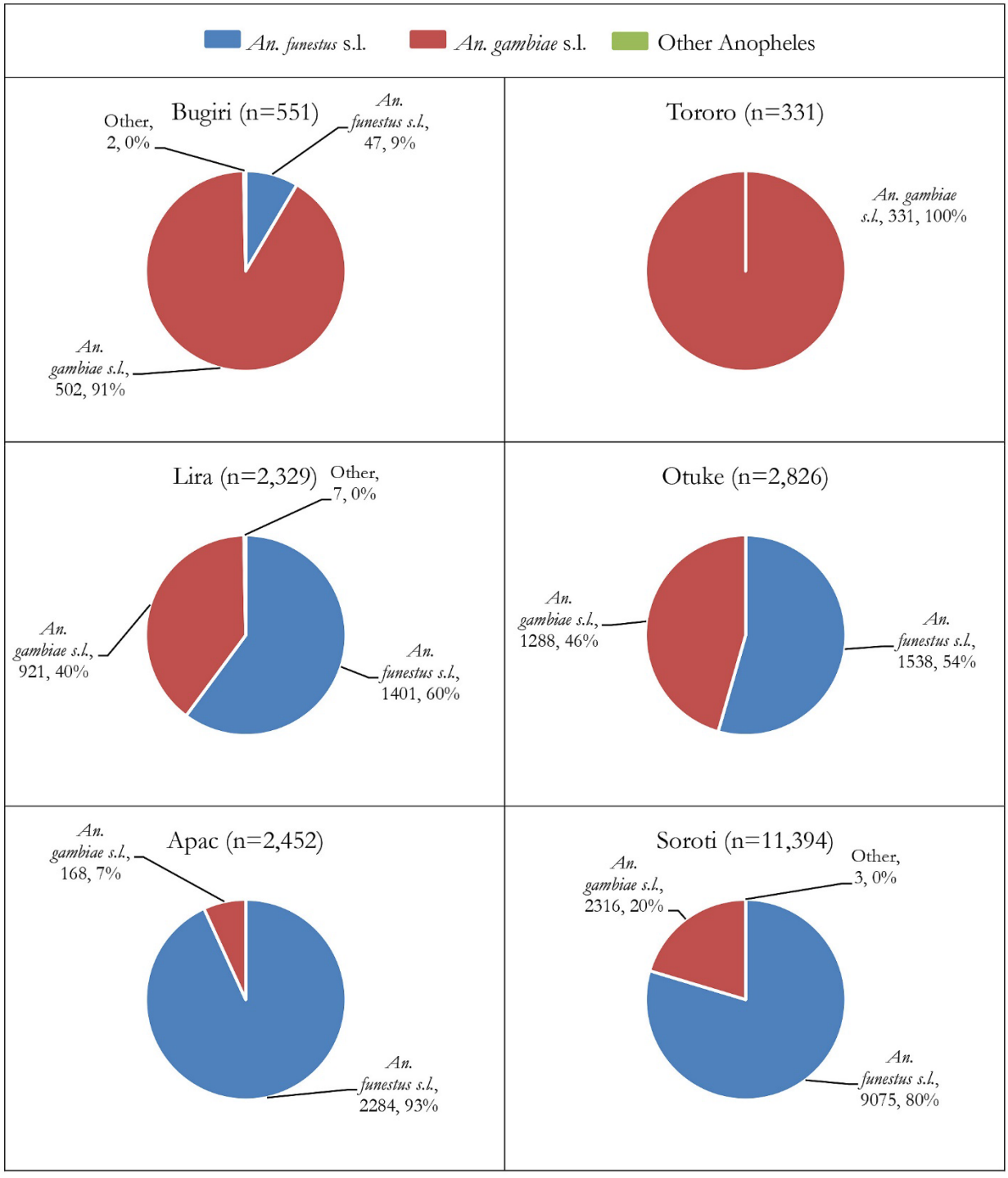


FIGURE 4: INDOOR RESTING DENSITY OF MALARIA VECTORS FROM PSCS IN OTUKE, JANUARY 2020 THROUGH DECEMBER 2021

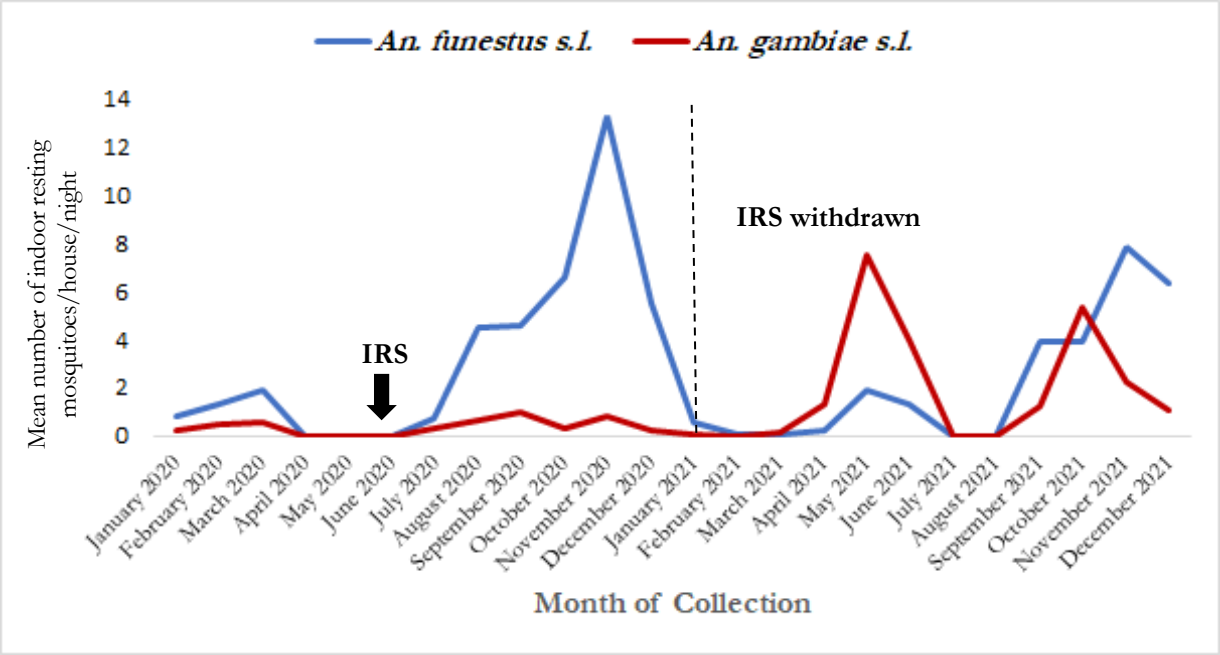
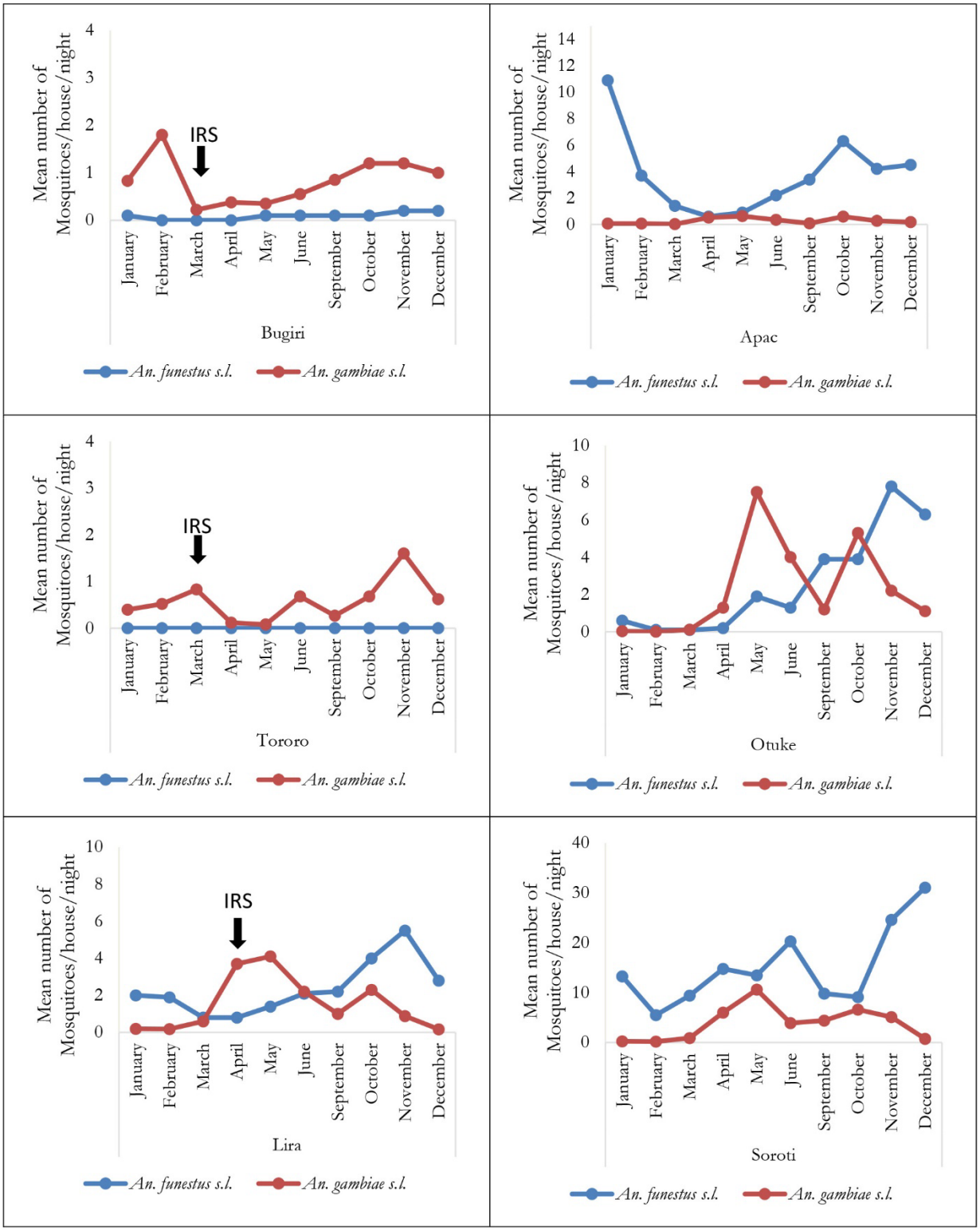


FIGURE 5: INDOOR RESTING DENSITIES OF *AN. FUNESTUS* S.L. AND *AN. GAMBIAE* S.L. FROM PSCS, BY MONTH, JANUARY THROUGH DECEMBER 2021



Note: No PSCs were conducted in July and August 2021 due to the COVID-19 pandemic lockdown.

FIGURE 6: ABDOMINAL STAGE OF INDOOR RESTING *AN. GAMBIAE* S.L. MOSQUITOES COLLECTED BY PSC FROM A: IRS DISTRICTS; B: NON-IRS DISTRICTS

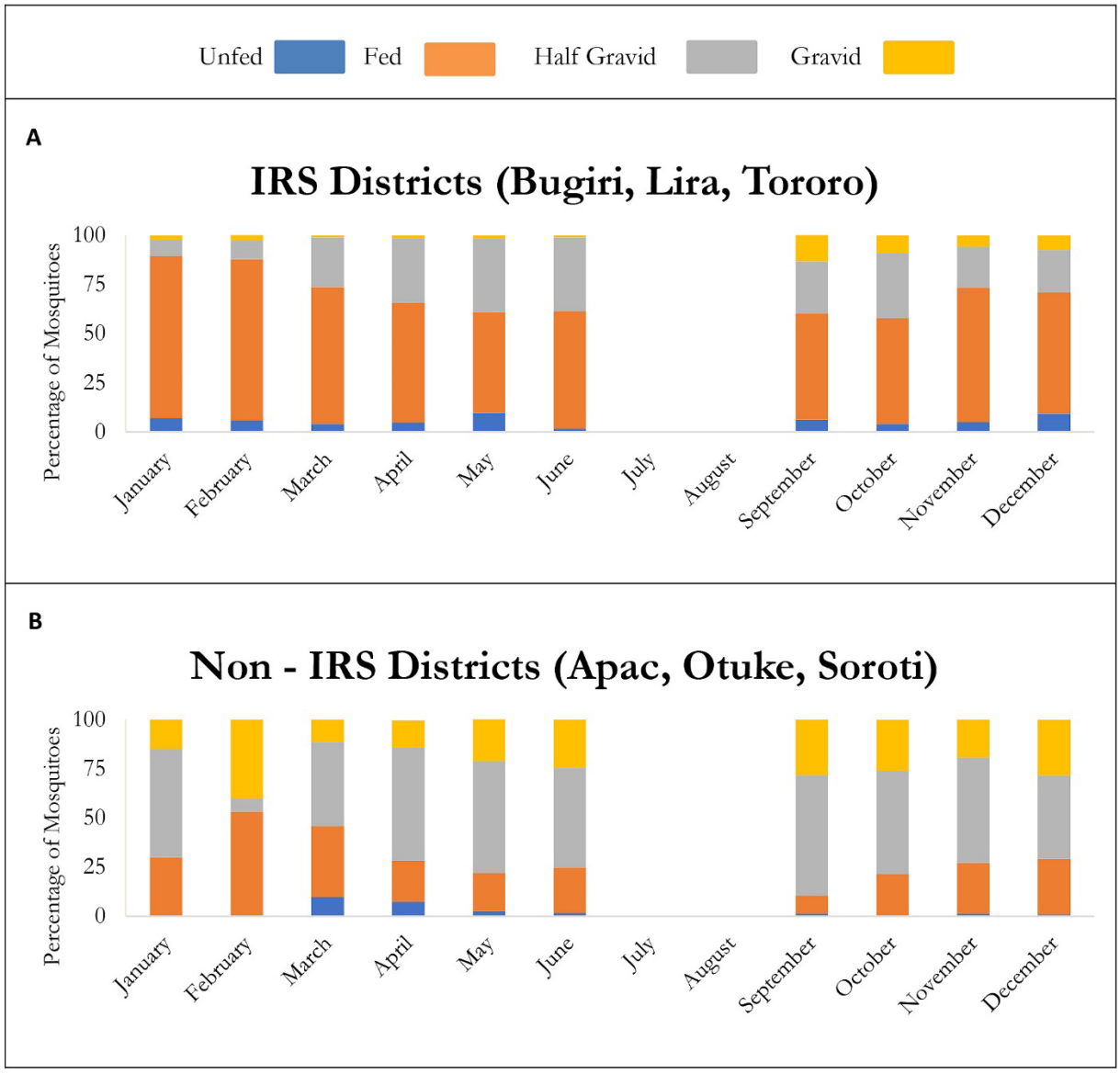
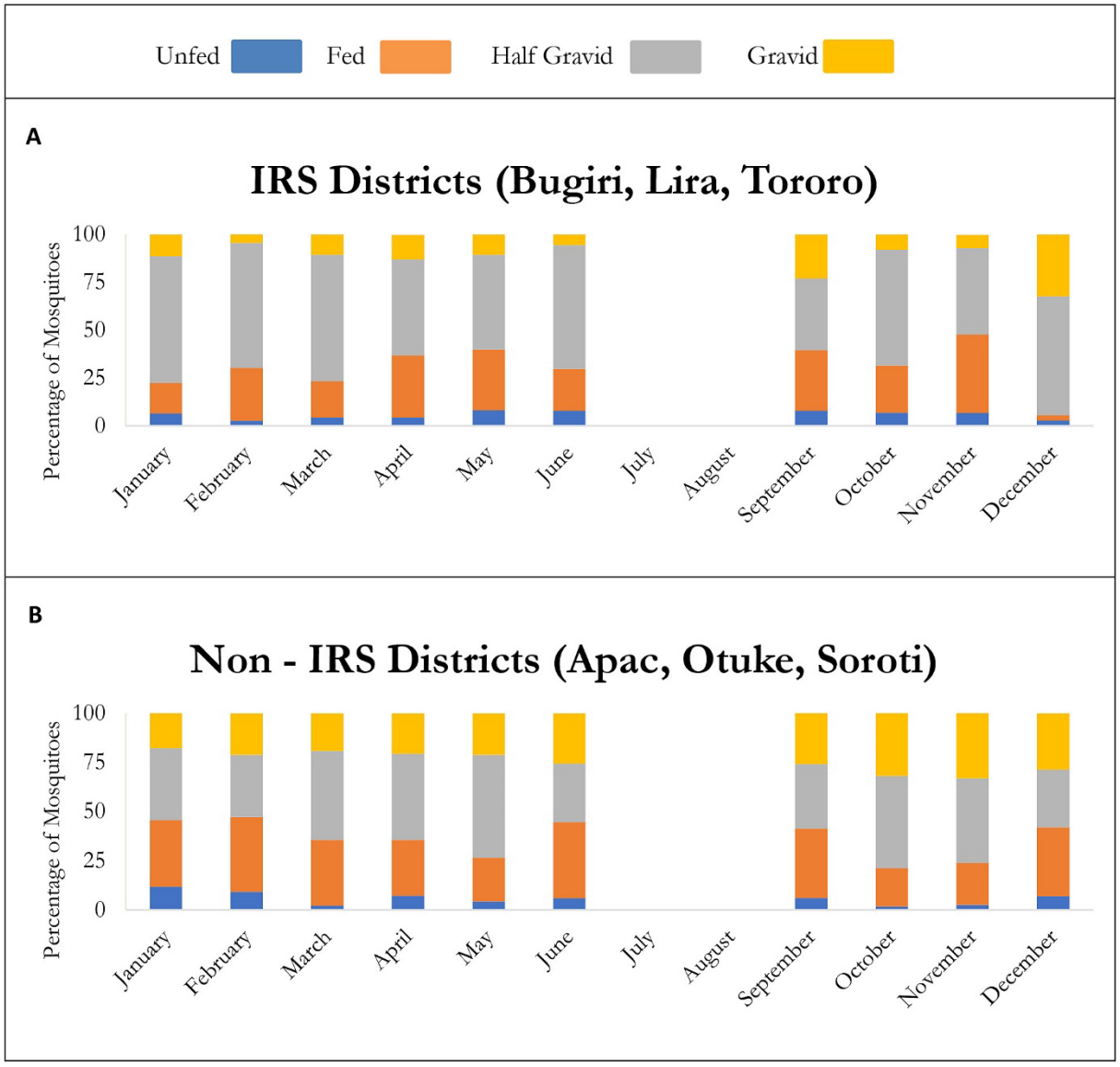


FIGURE 7: ABDOMINAL STAGE OF INDOOR RESTING *AN. FUNESTUS* S.L. MOSQUITOES COLLECTED BY PSC FROM A: IRS DISTRICTS; B: NON-IRS DISTRICTS



3.1.3 HUMAN LANDING CATCHES

HLCs collected 11,066 *Anopheles* mosquitoes from January through December 2021 including 9,376 (84.7%) *An. funestus* s.l., 1,671 (15.1%) *An. gambiae* s.l., and 19 (0.2%) other *Anopheles* species (Table 4). *An. gambiae* s.l. was the most abundant mosquito species collected both indoors and outdoors in Bugiri and Tororo, while *An. funestus* s.l. was the most abundant mosquito species collected both indoors and outdoors in the districts of Apac and Soroti. In Lira and Otuke, there were marginally more *An. funestus* s.l. relative to *An. gambiae* s.l. indoors but the reverse was observed for outdoor collections (figures 8 and 9). In four of the six districts, a higher percentage of *An. gambiae* s.l. were caught seeking a bloodmeal outdoors than indoors, including two IRS districts, Bugiri and Tororo (52.0% and 54.4% respectively). However, in the third IRS district, Lira, *An. gambiae* s.l. was strongly endophagic and this skews the endophagic index for the IRS districts combined (Table 5). *An. funestus* s.l. was much more endophagic that *An. gambiae* s.l. – only in Bugiri was a higher percentage of *An. funestus* s.l. caught outdoors than indoors and numbers were extremely low. For both species complexes, the endophagic index was higher in the IRS districts combined compared to the non-IRS districts combined (Table 5).

In Lira, Otuke, and Soroti, where numbers of *An. funestus* s.l. were moderate or high, the indoor HBRs reflected seasonal changes in densities (Figure 10A-C). The numbers caught in Soroti were far higher than in other districts (10A). The pattern of biting rate changes in Lira and Otuke were similar – there was a small peak in May and a larger peak in October to November. Despite IRS in April in Lira, the peak late in the year was still apparent (10B), though absolute numbers of *An. funestus* s.l. were lower than in the unsprayed district of Otuke, and much lower than Soroti (10C). In Soroti, one peak in HBR occurred in June, and another from October through December, when the *An. funestus* s.l. HBR increased markedly. Too few *An. funestus* s.l. were caught in Tororo or Bugiri to merit an analysis of HBR in those districts. The seasonal pattern in outdoor HBR for *An. funestus* s.l. was similar to that observed for indoor HBR, but generally the rates were lower (Figure 11A-C). In Soroti, the outdoor HBR peaked in June and November (11C).

The seasonal pattern in indoor and outdoor HBR for *An. gambiae* s.l. was similar to that of *An. funestus* s.l. insofar as there were two peaks: one in May-June and a second higher peak in October-November. This was true for all districts regardless of whether they received IRS or not (figures 12A and 13A). The impact of the IRS on indoor HBR in Lira was apparent – biting rates declined sharply between May and June – but not in Tororo where the indoor HBR continued to increase after spraying (12B). In Lira, the IRS may have been sprayed too late as indoor and outdoor HBR were already increasing in April. In the non-IRS districts, the highest HBRs for *An. gambiae* s.l. were recorded in Otuke in October, reaching 52.5 bites per person per night indoors (12C) and 44.3 outdoors (13C).

TABLE 4: HLCS INDOORS AND OUTDOORS IN SIX STUDY DISTRICTS, JANUARY THROUGH DECEMBER 2021

Anopheles species	IRS Districts									Non-IRS districts									Total		Grand Total
	Bugiri			Lira			Tororo			Apac			Otuke			Soroti					
	Indoors	Outdoors	Total	Indoors	Outdoors	Total	Indoors	Outdoors	Total	Indoors	Outdoors	Total	Indoors	Outdoors	Total	Indoors	Outdoors	Total	Indoors	Outdoors	
An. gambiae s.l.	49	53	102	178	97	275	120	143	263	24	67	91	397	316	713	76	151	227	844	827	**1671**
An. funestus s.l.	2	5	7	192	32	224	0	0	0	82	73	155	426	136	562	5273	3155	8428	5975	3401	**9376**
Other Anopheles	1	4	5	2	4	6	0	0	0	0	2	2	0	0	0	1	5	6	4	15	**19**
Total per district	**52**	**62**	**114**	**372**	**133**	**505**	**120**	**143**	**263**	**106**	**142**	**248**	**823**	**452**	**1275**	**5350**	**3311**	**8661**	**6823**	**4243**	**11066**

TABLE 5: INDOOR AND OUTDOOR HBRS AND ENDOPHAGIC INDEX IN IRS AND NON-IRS DISTRICTS

Species Collected and location	IRS Districts			Non-IRS Districts		
	Total Numbers Collected	Total Person Nights	HBR	Total Numbers Collected	Total Person Nights	HBR
An. gambiae s.l. indoors	347	60	5.8	497	60	8.3
An. gambiae s.l. outdoors	293	60	4.9	534	60	8.9
An. gambiae s.l. endophagic index	-	-	0.54	-	-	0.48
An. funestus s.l. indoors	194	60	3.2	5781	60	96.4
An. funestus s.l. outdoors	37	60	0.6	3364	60	56.1
An. funestus s.l. endophagic index	-	-	0.84	-	-	0.63
Other *Anopheles* indoors	3	60	0.05	1	60	0.01
Other *Anopheles* outdoors	8	60	0.13	7	60	0.12
Other *Anopheles* endophagic index	-	-	0.28	-	-	0.08

FIGURE 8: *ANOPHELES* SPECIES COMPOSITION BY DISTRICT USING HLCS INDOORS, JANUARY THROUGH DECEMBER 2021

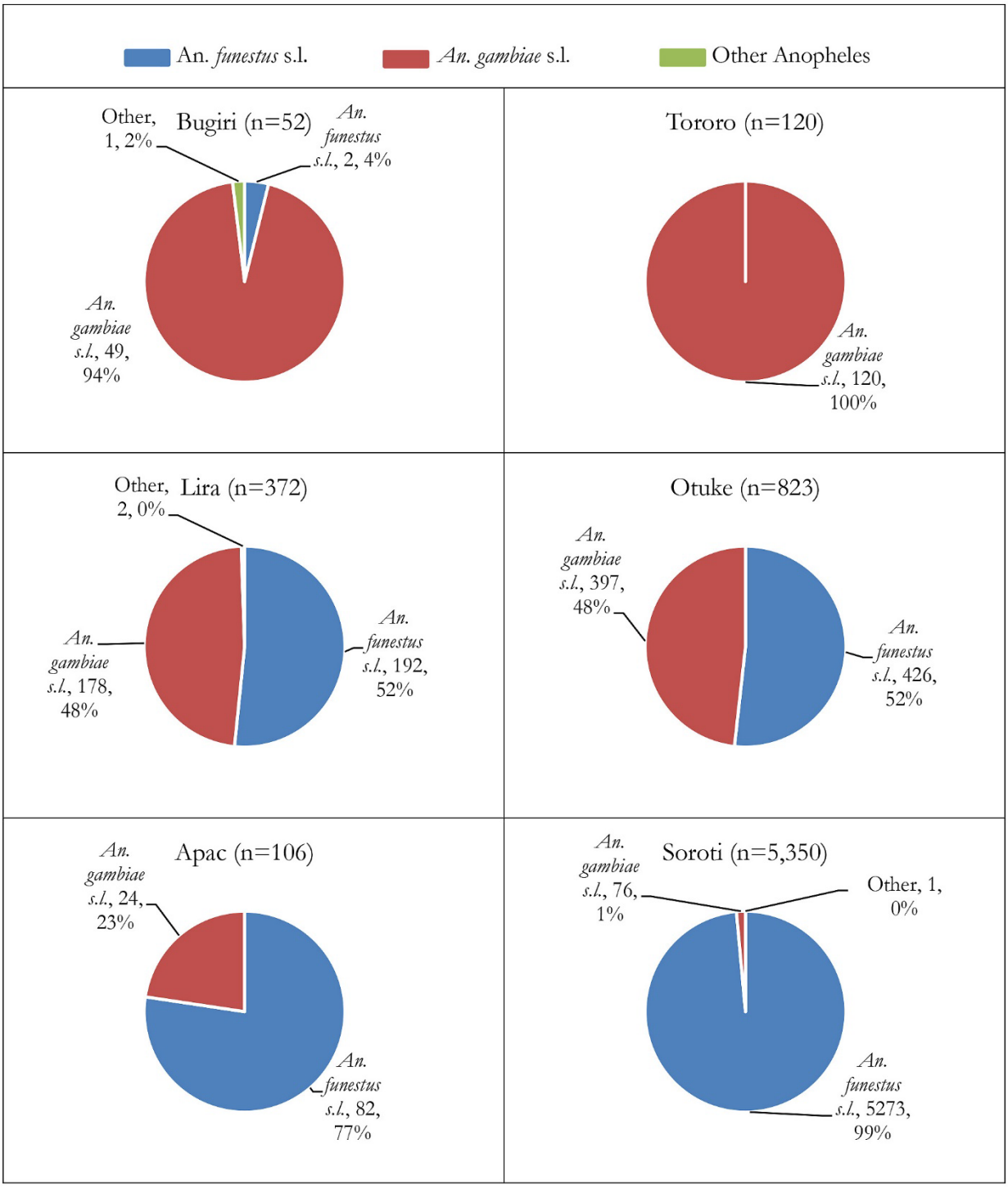


FIGURE 9: *ANOPHELES* SPECIES COMPOSITION BY DISTRICT USING HLCS OUTDOORS, JANUARY THROUGH DECEMBER 2021

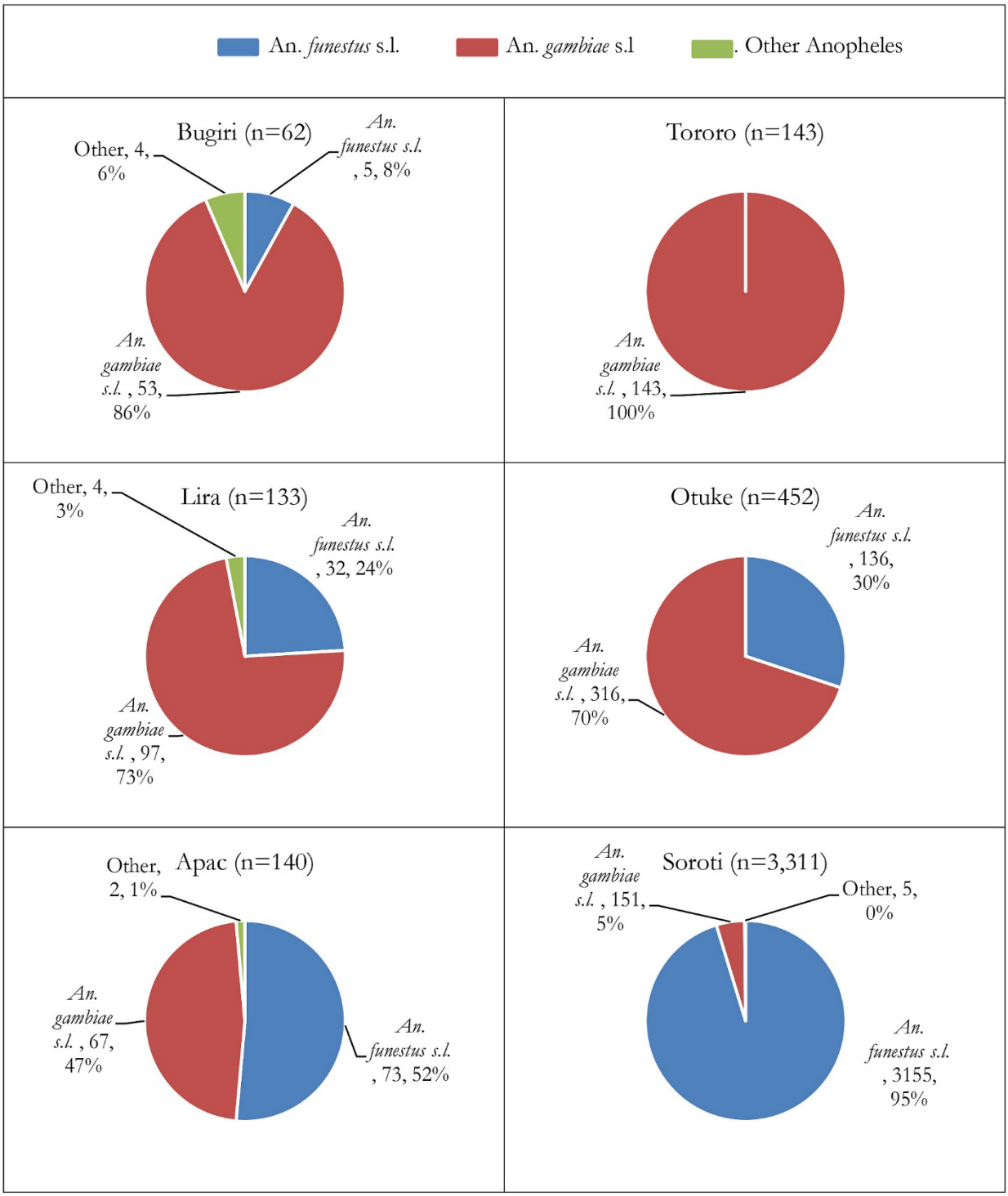
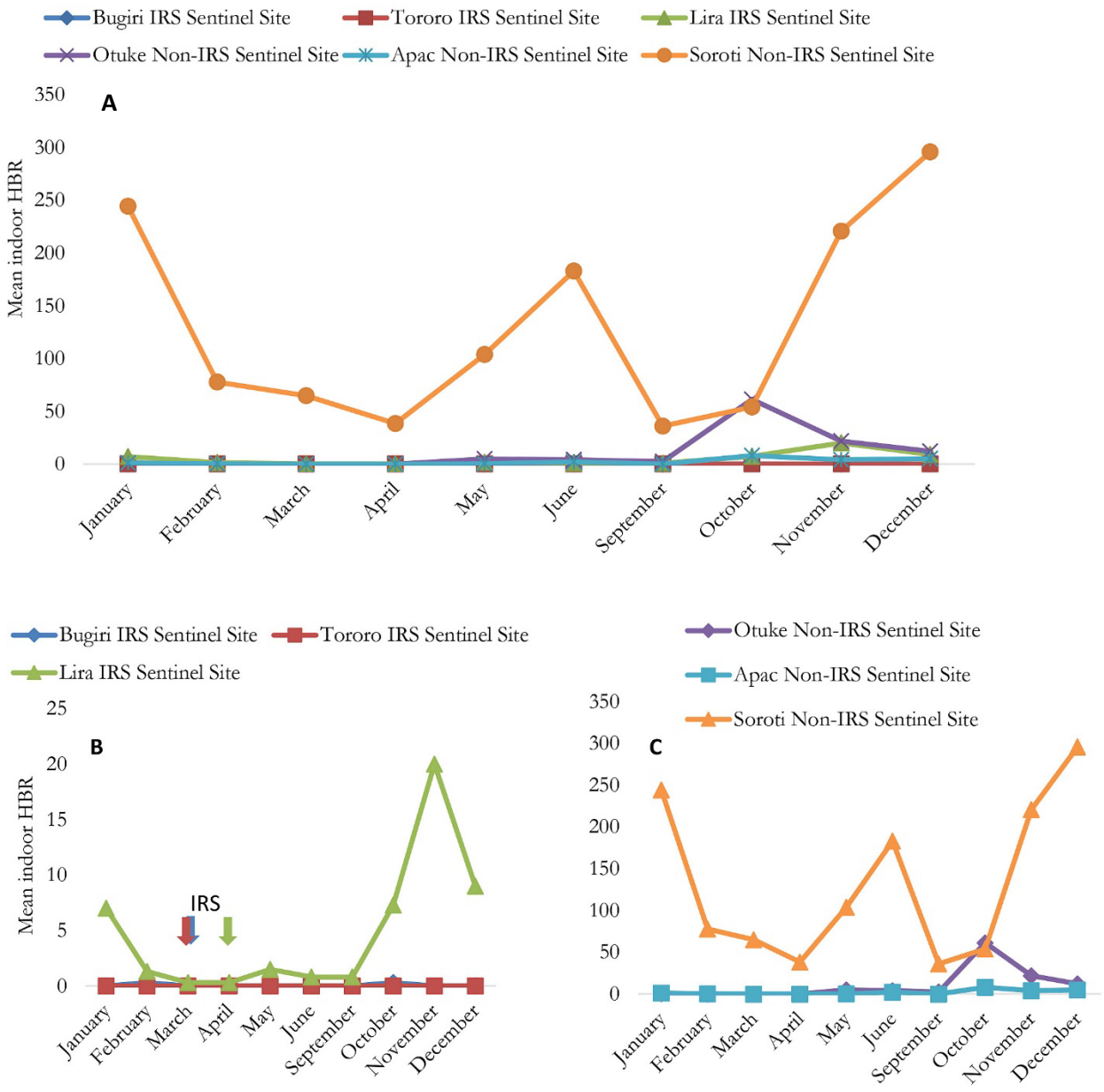
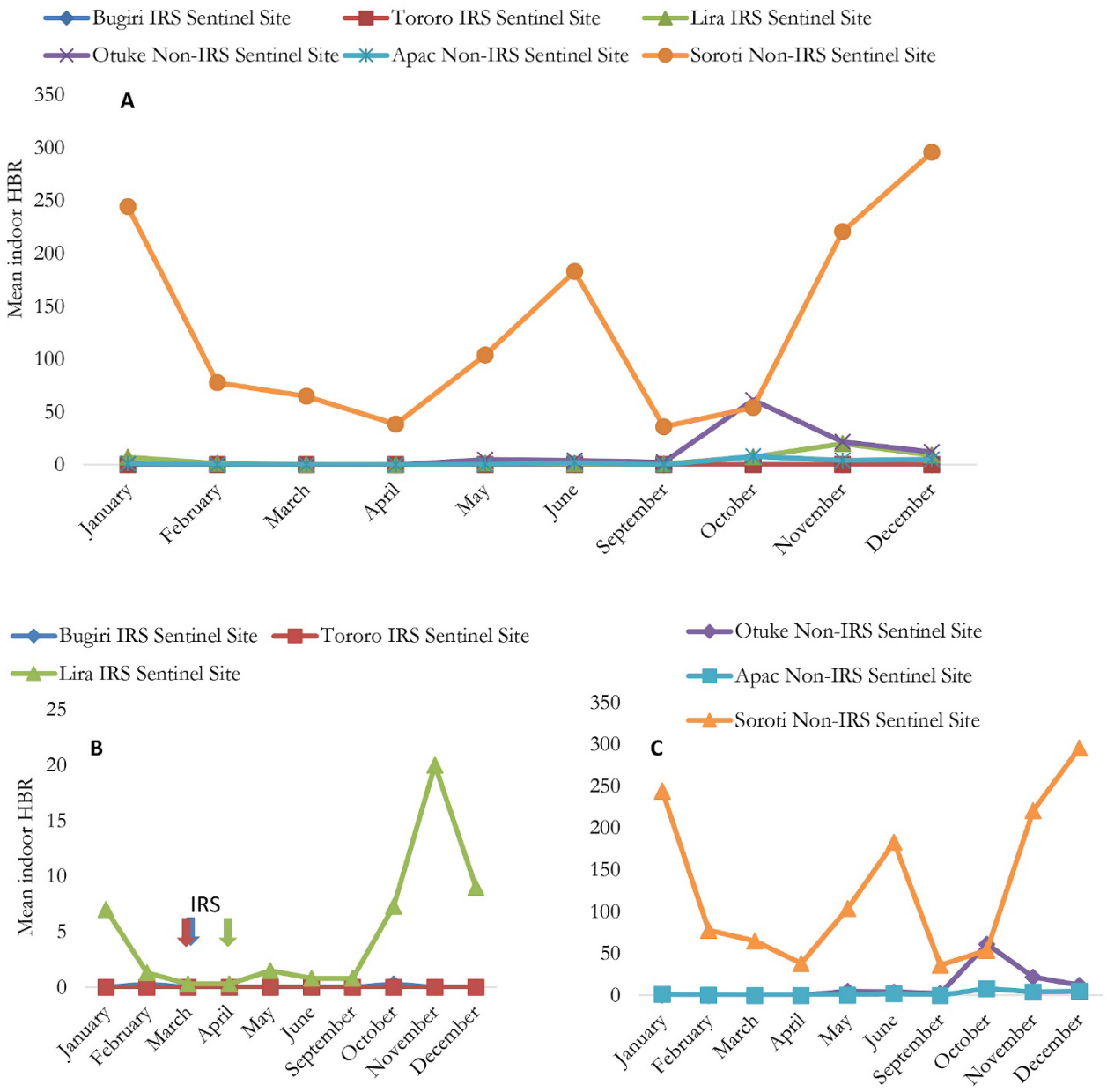


FIGURE 10: *AN. FUNESTUS* S.L. MEAN MONTHLY INDOOR HBRS: A: ALL DISTRICTS; B: IRS DISTRICTS; C: NON-IRS DISTRICTS



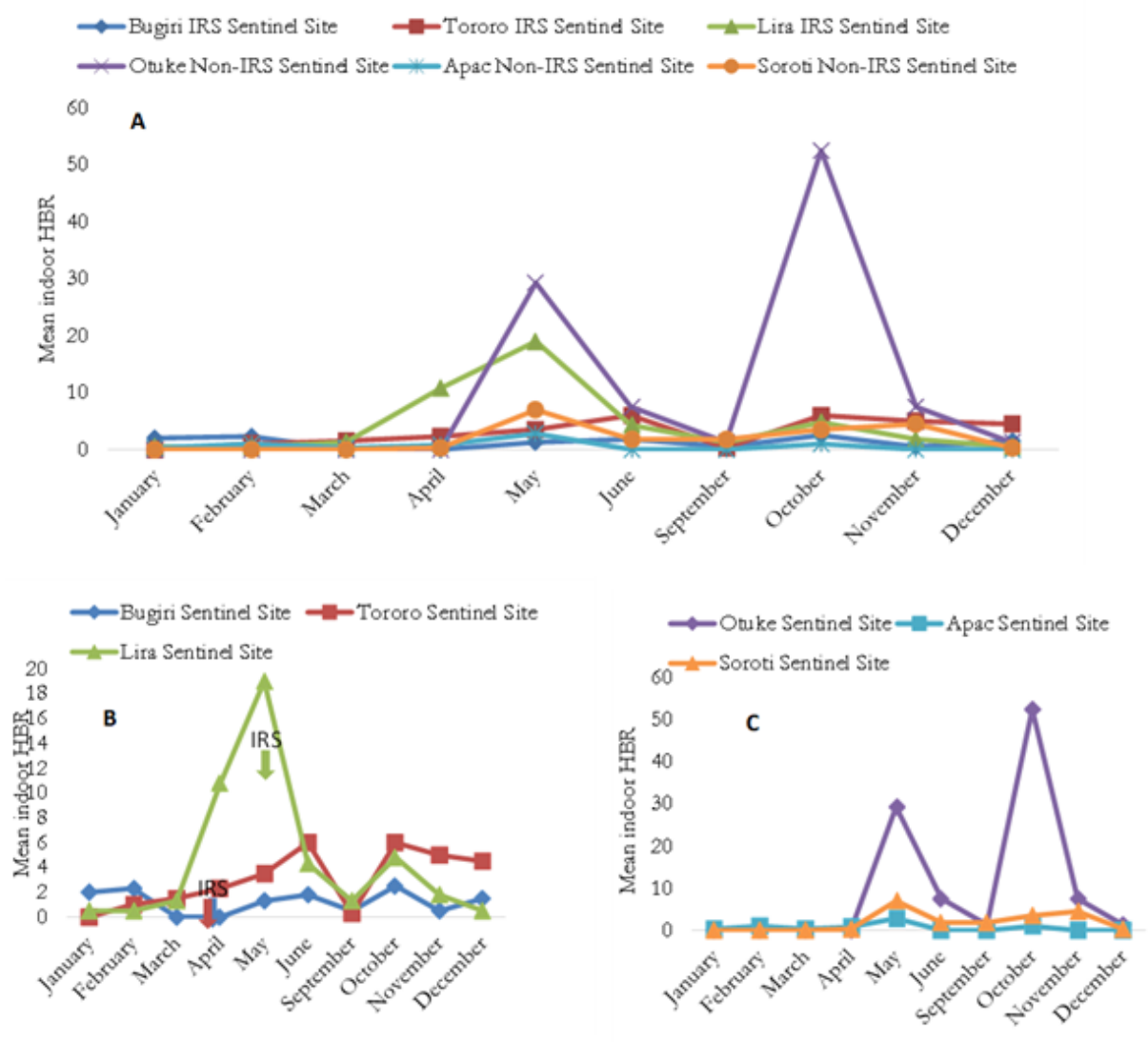
Note: No HLCs were conducted in July and August 2021 due to the COVID-19 pandemic lockdown.

FIGURE 11: *AN. FUNESTUS* S.L. MEAN MONTHLY OUTDOOR HBRS: A: ALL DISTRICTS; B: IRS DISTRICTS; C: NON-IRS DISTRICTS



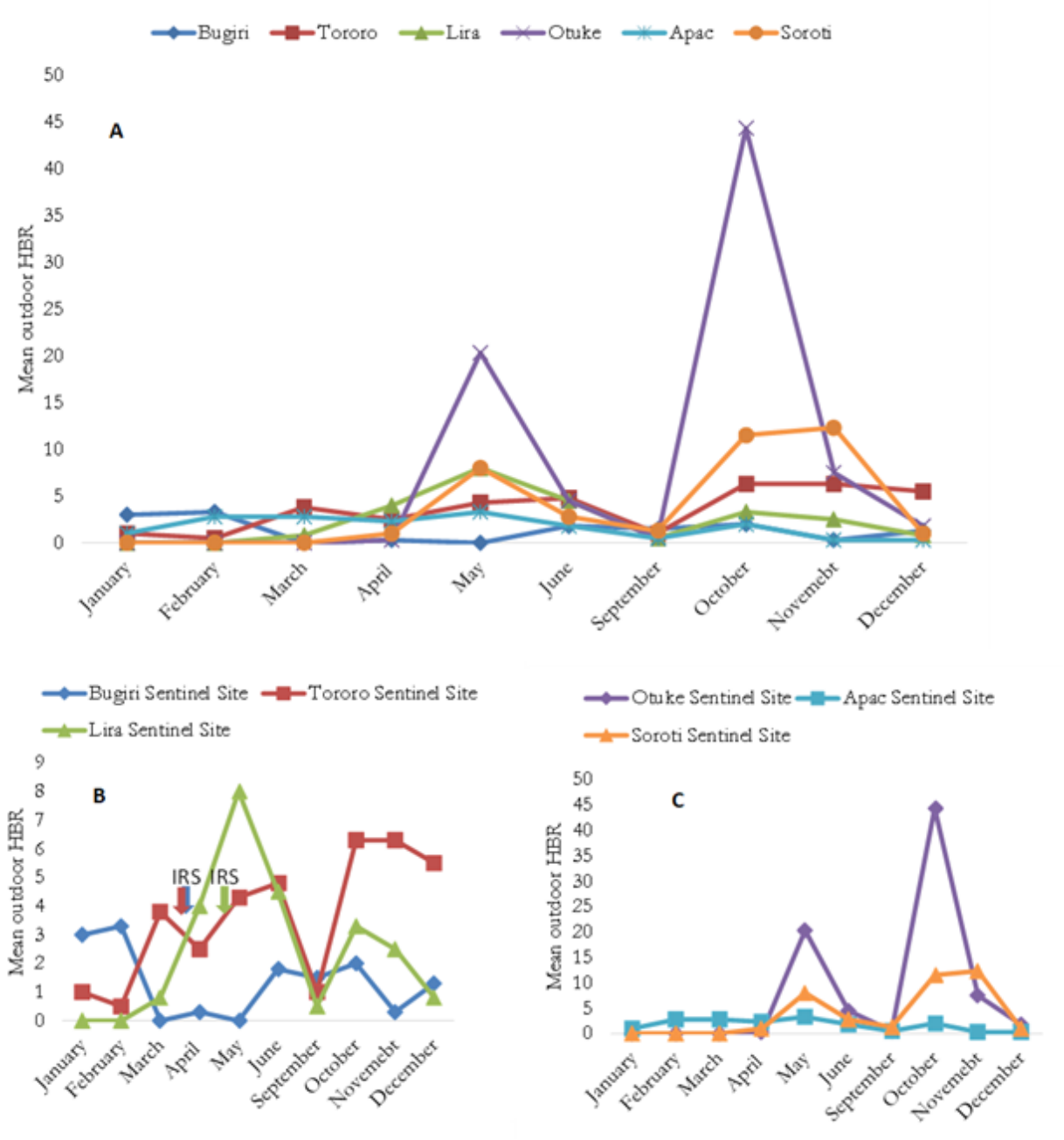
Note: No HLCs were conducted in July and August 2021 due to the COVID-19 pandemic lockdown.

FIGURE 12: *AN. GAMBIAE* S.L. MEAN MONTHLY INDOOR HBRS: A: ALL DISTRICTS; B: IRS DISTRICTS; C: NON-IRS DISTRICTS



Note: No HLCs were conducted in July and August 2021 due to the COVID-19 pandemic lockdown.

FIGURE 13: *AN. GAMBIAE* S.L. MEAN MONTHLY OUTDOOR HBRS: A: ALL DISTRICTS; B: IRS DISTRICTS; C: NON-IRS DISTRICTS



Note: No HLCs were conducted in July and August 2021 due to the COVID-19 pandemic lockdown.

HLCs were also used to estimate the hourly biting rates in all six sentinel sites. *An. funestus* s.l. and *An. gambiae* s.l. contributed 99.9% and 98.8% of the bites in the IRS and non-IRS areas, respectively. Tables 6 and 7 show the indoor and outdoor mean hourly biting rates of *An. funestus* s.l. and *An. gambiae* s.l. in the IRS and non-IRS districts.

For *An. gambiae* s.l., in Tororo, the indoor biting peaked at midnight–1a.m. (Figure 14B), while outdoor biting was very low (14E). In Lira, the highest indoor hourly biting rate occurred later, at 3–4 a.m. (14B). By contrast, in Bugiri, indoor biting was very low but there was a peak in outdoor biting at 1–2 a.m. (14E). In the non-IRS districts there no clear patterns – in Soroti and Apac the biting rates were extremely low, while in Otuke, both indoors and outdoors, the hourly biting rates increased steadily from 9 p.m. reaching a peak at 4–5 a.m. (14C and 14F). For *An. funestus* s.l., hourly biting rates were higher indoors than outdoors (Figure 15) but rates were generally very low aside from in Soroti, where rates increased steadily from 9 p.m. reaching a peak at 5-6 both indoors (15C) and outdoors (15F).

TABLE 6: MEAN HOURLY BITING RATES OF *AN. FUNESTUS* S.L. IN ALL SENTINEL SITES, JANUARY THROUGH DECEMBER 2021

District	Collection location	Time of Collection												
		6–7 p.m.	7-8 p.m.	8-9 p.m.	9-10 p.m.	10-11 p.m.	11-12 p.m.	12-1 a.m.	1-2 a.m.	2-3 a.m.	3-4 a.m.	4-5 a.m.	5-6 a.m.	6-7 a.m.
Apac (non-IRS)	Indoor	0	0	0	0.13	0.15	0.05	0.2	0.23	0.23	0.43	0.18	0.33	0.15
	Outdoor	0	0	0.1	0	0.03	0.23	0.15	0.08	0.25	0.2	0.28	0.38	0.15
Otuke (non-IRS)	Indoor	0.05	0.03	0.08	0.08	0.4	0.5	0.75	0.75	1.9	1.4	2.6	1.5	0.63
	Outdoor	0.03	0	0	0.08	0.1	0.15	0.1	0.63	0.45	0.53	0.7	0.6	0.05
Soroti (non-IRS)	Indoor	1.2	1.7	2.5	3.5	4.5	8.7	12.1	10.4	11.9	21.9	17.9	21.7	13.9
	Outdoor	0.3	0.55	1.1	2.2	3.9	5.6	5.7	7.5	7.1	10.3	10.9	12.7	11.2
Bugiri` (IRS)	Indoor	0	0	0	0	0.03	0	0	0	0	0	0.03	0	0
	Outdoor	0	0	0	0	0	0	0.03	0.03	0	0	0	0.05	0.03
Lira (IRS)	Indoor	0	0.03	0.05	0.05	0.35	0.45	0.43	0.83	0.38	0.85	0.6	0.45	0.35
	Outdoor	0	0	0.03	0	0.03	0.15	0.08	0.1	0.1	0.1	0.1	0.05	0.08
Tororo (IRS)	Indoor	0	0	0	0	0	0	0	0	0	0	0	0	0
	Outdoor	0	0	0	0	0	0	0	0	0	0	0	0	0

TABLE 7: MEAN HOURLY BITING RATES OF *AN. GAMBIAE* S.L. IN ALL SENTINEL SITES, JANUARY THROUGH DECEMBER 2021

District	Collection location	Time of Collection												
		6–7 p.m.	7-8 p.m.	8-9 p.m.	9-10 p.m.	10-11 p.m.	11-12 p.m.	12-1 a.m.	1-2 a.m.	2-3 a.m.	3-4 a.m.	4-5 a.m.	5-6 a.m.	6-7 a.m.
Apac (non-IRS)	Indoor	0	0	0.03	0.03	0.03	0.1	0.05	0.05	0.1	0.1	0.05	0.05	0.03
	Outdoor	0	0.08	0.05	0.08	0.08	0.3	0.2	0.13	0.28	0.08	0.1	0.25	0.08
Otuke (non-IRS)	Indoor	0.05	0.05	0.05	0.2	0.43	0.65	1.4	0.88	1.3	1.3	1.8	1.5	0.38
	Outdoor	0.05	0.25	0.13	0.2	0.43	0.88	0.7	0.63	1.2	1.2	1.3	0.73	0.35
Soroti (non-IRS)	Indoor	0.03	0.03	0.18	0.05	0.15	0.18	0.3	0.23	0.18	0.23	0.23	0.15	0
	Outdoor	0	0.08	0.1	0.15	0.1	0.48	0.23	0.25	0.53	0.4	0.73	0.55	0.2
Bugiri (IRS)	Indoor	0	0.05	0.05	0.05	0.15	0.15	0.15	0.05	0.1	0.1	0.1	0.18	0.1
	Outdoor	0.03	0.1	0.08	0.08	0.2	0.05	0.13	0.05	0.1	0.05	0.25	0.15	0.08
Lira (IRS)	Indoor	0	0.08	0.1	0.23	0.3	0.38	0.55	0.33	0.58	1	0.43	0.4	0.1
	Outdoor	0	0.15	0.13	0.2	0.3	0.18	0.25	0.2	0.38	0.25	0.15	0.15	0.1
Tororo (IRS)	Indoor	0.03	0.23	0.23	0.18	0.23	0.3	0.58	0.23	0.25	0.28	0.13	0.2	0.18
	Outdoor	0	0.15	0.28	0.23	0.23	0.28	0.28	0.85	0.4	0.1	0.15	0.5	0.15

FIGURE 14: MEAN NUMBER OF *AN. GAMBIAE* S.L. COLLECTED PER PERSON PER HOUR BY HLC: A: ALL DISTRICTS INDOORS; B: IRS DISTRICTS INDOORS; C: NON-IRS DISTRICTS INDOORS; D: ALL DISTRICTS OUTDOORS; E: IRS DISTRICTS OUTDOORS; F: NON-IRS DISTRICTS OUTDOORS

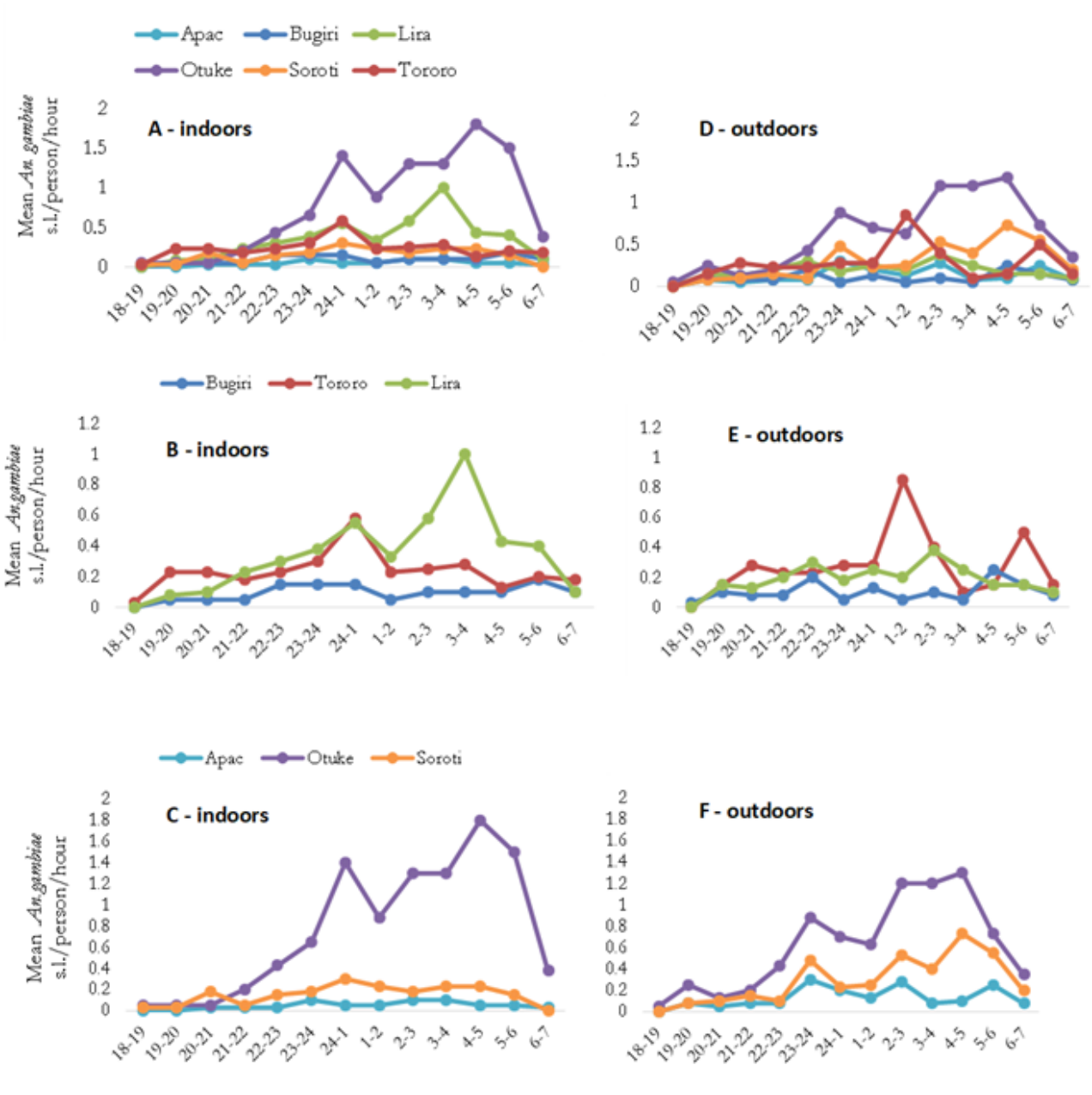
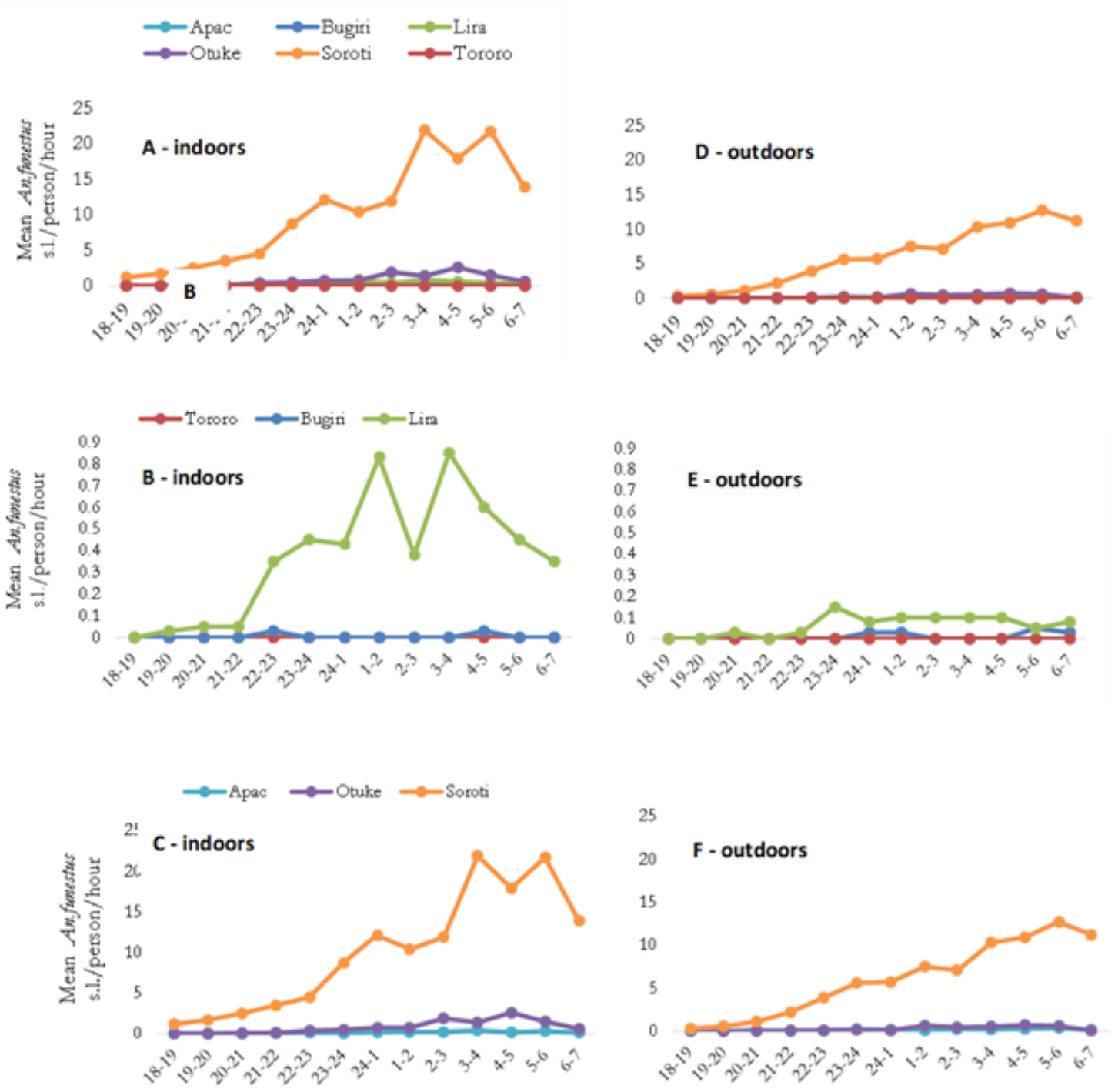


FIGURE 15: MEAN NUMBER OF *AN. FUNESTUS* S.L. COLLECTED PER PERSON PER HOUR BY HLC. A: ALL DISTRICTS INDOORS; B: IRS DISTRICTS INDOORS; C: NON-IRS DISTRICTS INDOORS; D: ALL DISTRICTS OUTDOORS; E: IRS DISTRICTS OUTDOORS; F: NON-IRS DISTRICTS OUTDOORS



3.2 CONE WALL BIOASSAY TESTS

WHO cone bioassays were conducted in one site in each of the seven sentinel spray districts (Bugiri, Butaleja, Dokolo, Kibuku, Lira, Serere, and Tororo) within one week of the start of spraying to assess the quality of the IRS. Thereafter, the residual efficacy and fumigant effect of Fludora Fusion were monitored in four districts (Bugiri, Lira, Serere, and Tororo) on a monthly basis for three types of wall surfaces (cement painted, plain brick, and mud), which represent the common wall surfaces in the IRS districts. The susceptible *An. gambiae* s.s. Kisumu strain was used for both the quality assessment and residual efficacy bioassays.

3.2.1 QUALITY ASSURANCE OF IRS

100% mosquito mortality was recorded from the cone assays conducted within one week after spraying in all seven sentinel spray districts.

3.2.2 INSECTICIDE RESIDUAL EFFICACY

The Fludora Fusion residual efficacy was at least eight months in both Bugiri and Tororo districts on all surfaces, and extended to nine months on cement painted wall surfaces in Tororo (Table 8 and Figure 16A). In Lira and Serere districts, which were sprayed with Fludora Fusion in May, residual efficacy on all surfaces was at least six months in Serere and seven months in Lira. In both Lira and Serere, this residual efficacy was extended by at least one month for painted cement wall surfaces (Table 8 and Figure 16B). Cone bioassays continue for painted surfaces in Lira and Tororo, because mortality did not drop below the 80% threshold during the reporting period.

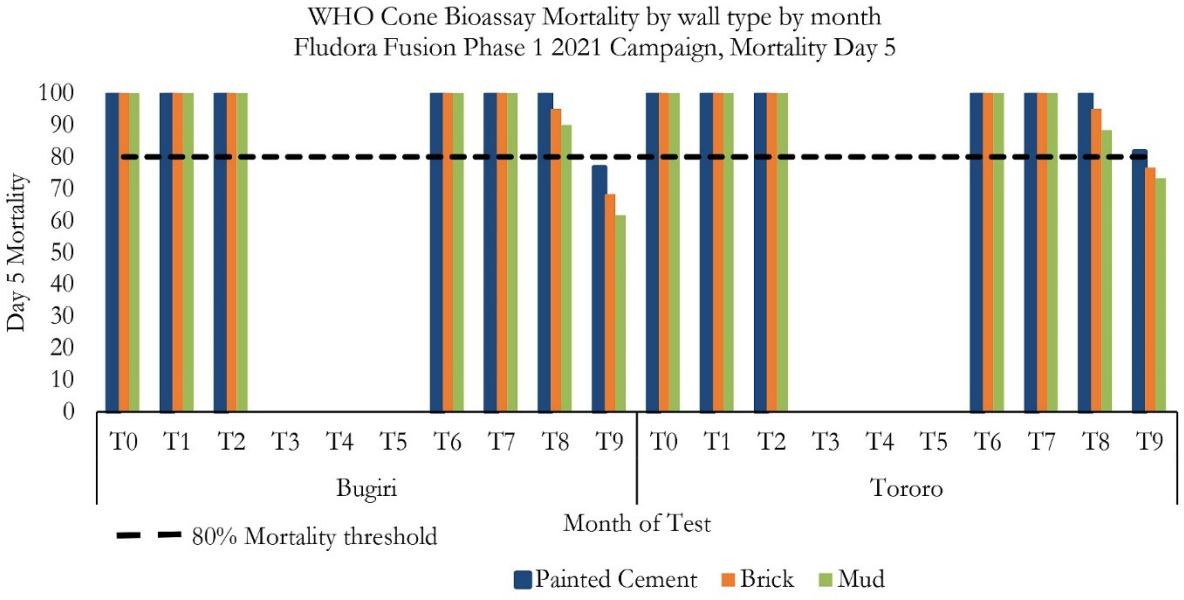
TABLE 8: WALL BIOASSAY RESULTS IN BUGIRI, LIRA, SERERE, AND TORORO INSECTICIDE DECAY RATE MONITORING SITES, DECEMBER 2021

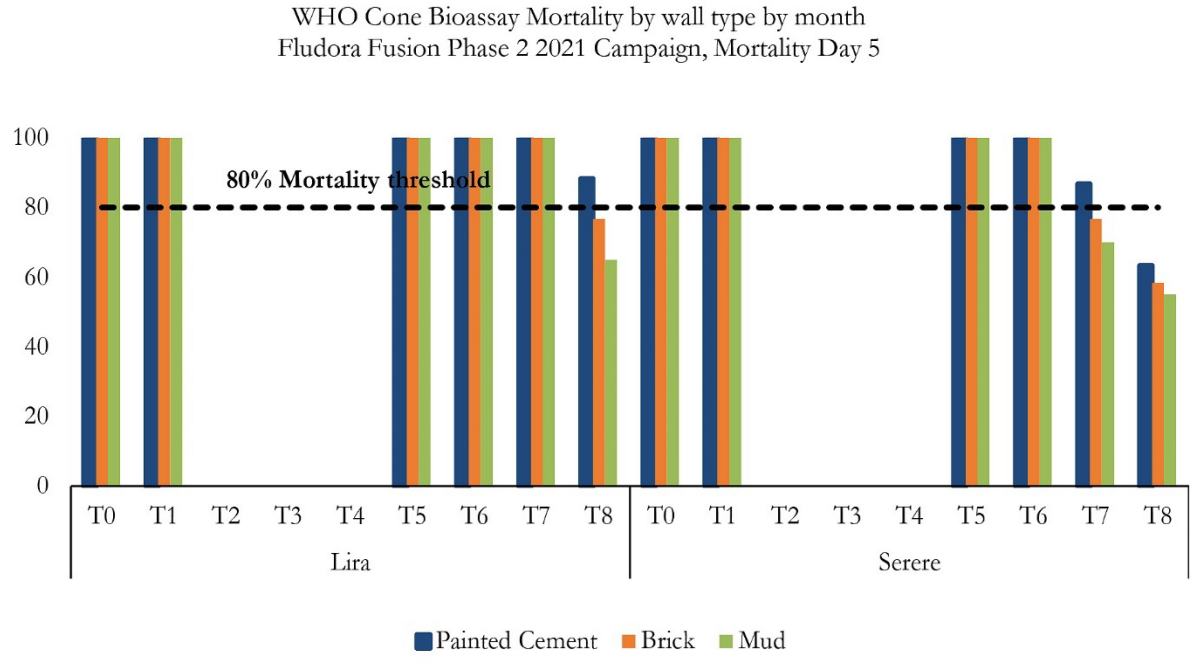
Time	% Mortality of *An. gambiae* s.s. (Kisumu strain)																Overall Mean
	Fludora Fusion Bugiri (T0 = March 2021)				Fludora Fusion Tororo (T0 = March 2021)				Fludora Fusion Lira (T0 = April 2021)				Fludora Fusion Serere (T0 = April 2021)				
	Painted	Plain Brick	Mud	Mean	Painted	Plain Brick	Mud	Mean	Painted	Plain Brick	Mud	Mean	Painted	Plain Brick	Mud	Mean	
T0	100 (60/60)	100 (60/60)	100 (60/60)	100	100 (60/60)	100 (60/60)	100 (60/60)	100	100 (60/60)	100 (60/60)	100 (60/60)	100	100 (60/60)	100 (60/60)	100 (60/60)	100	100
T1	100 (60/60)	100 (60/60)	100 (60/60)	100	100 (60/60)	100 (60/60)	100 (60/60)	100	100 (60/60)	100 (60/60)	100 (60/60)	100	100 (60/60)	100 (60/60)	100 (60/60)	ND	100
T2	100 (60/60)	100 (60/60)	100 (60/60)	100	100 (60/60)	100 (60/60)	100 (60/60)	100	ND	ND	ND	ND	ND	ND	ND	ND	ND
T3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
T4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	100	ND	ND	ND	100	ND
T5	ND	ND	ND	ND	ND	ND	ND	ND	100 (60/60)	100 (60/60)	100 (60/60)	100	100 (60/60)	100 (60/60)	100 (60/60)	100	100
T6	100 (60/60)	100 (60/60)	100 (60/60)	100	100 (60/60)	100 (60/60)	100 (60/60)	100	100 (60/60)	100 (60/60)	100 (60/60)	100	100 (60/60)	100 (60/60)	100 (60/60)	100	100 4
T7	100 (60/60)	100 (60/60)	100 (60/60)	100	100 (60/60)	100 (60/60)	100 (60/60)	100	100 (60/60)	100 (60/60)	100 (60/60)	100	86.7 (52/60)	76.7 (46/60)	70 (42/60)	77.8	94.4
T8	100 (60/60)	95 (57/60)	90 (54/60)	95.0	100 (60/60)	95 (57/60)	88.3 (53/60)	94.4	88.3 (53/60)	76.7 (46/60)	65 (39/60)	76.7	63.3 (38/60)	58.3 (35/60)	55 (33/60)	58.9	81.3
T9	76.7 (46/60)	68.3 (41/60)	61.7 (37/60)	68.9	81.7 (49/60)	76.7 (46/60)	73.3 (44/60)	77.2	ND	ND	ND	ND	ND	ND	ND	ND	72.8

For Bugiri and Tororo T0 = March 2021; for Lira and Serere, T0 = April 2021

T0 is the test done within 1 week after spraying; T1, T2, T3 etc. represent the test results of studies conducted monthly after spraying i.e., 1, 2, 3 months post-spraying.

FIGURE 16: RESIDUAL EFFICACY OF FLUDORA FUSION: A: SPRAYED IN BUGIRI AND TORORO (T0 = MARCH); B: SPRAYED IN LIRA AND SERERE (T0 = APRIL). NOTE: NO WALL BIOASSAY STUDIES WERE CONDUCTED IN JUNE, JULY, AND AUGUST 2021 DUE TO THE COVID-19 PANDEMIC LOCKDOWN





3.3 WHO INSECTICIDE SUSCEPTIBILITY TESTING

3.3.1 DETERMINATION OF THE INSECTICIDE SUSCEPTIBILITY STATUS USING WHO TUBE TESTS

Susceptibility testing was conducted in eight districts: Apac, Arua, Bugiri, Kanungu, Lira, Mityana, Moroto, and Tororo in September and October 2021. The PMI VectorLink Protocol SOP 06/01 standard susceptibility test method was used in all districts to test the main malaria vector *An. gambiae* s.l. *An. funestus* s.l. was not tested because insufficient larvae were found in any district to rear into adults for testing, and a limited number of tests were performed for *An. gambiae* s.l. in Mityana District for the same reason.

An. gambiae s.l. was susceptible to pirimiphos-methyl in all seven districts (Apac, Arua, Bugiri, Kanungu, Lira, Moroto, and Tororo) where the test was completed and to bendiocarb in five of the seven districts (Apac, Arua, Bugiri, Lira, and Tororo) (Annex Table A-1 and Figure 17). Low intensity resistance was observed in Moroto (90% mortality) and Kanungu districts (86% mortality) – in both districts exposure to a 5x dose of bendiocarb resulted in 100% mortality.

An. gambiae s.l. was resistant to all three pyrethroids tested in the study districts (Annex Table A-1 and Figure 18). For alpha-cypermethrin, mortality ranged between 1% in Arua and 35% in Mityana; deltamethrin mortality ranged between 5.0% in Moroto and 82% in Apac; and permethrin mortality ranged between 0% in Arua and 86% in Bugiri.

FIGURE 17: MORTALITY OF FEMALE *AN. GAMBIAE* S.L. 24 HOURS AFTER EXPOSURE TO BENDIOCARB AND PIRIMIPHOS-METHYL IN SEVEN DISTRICTS, SEPTEMBER–OCTOBER 2021

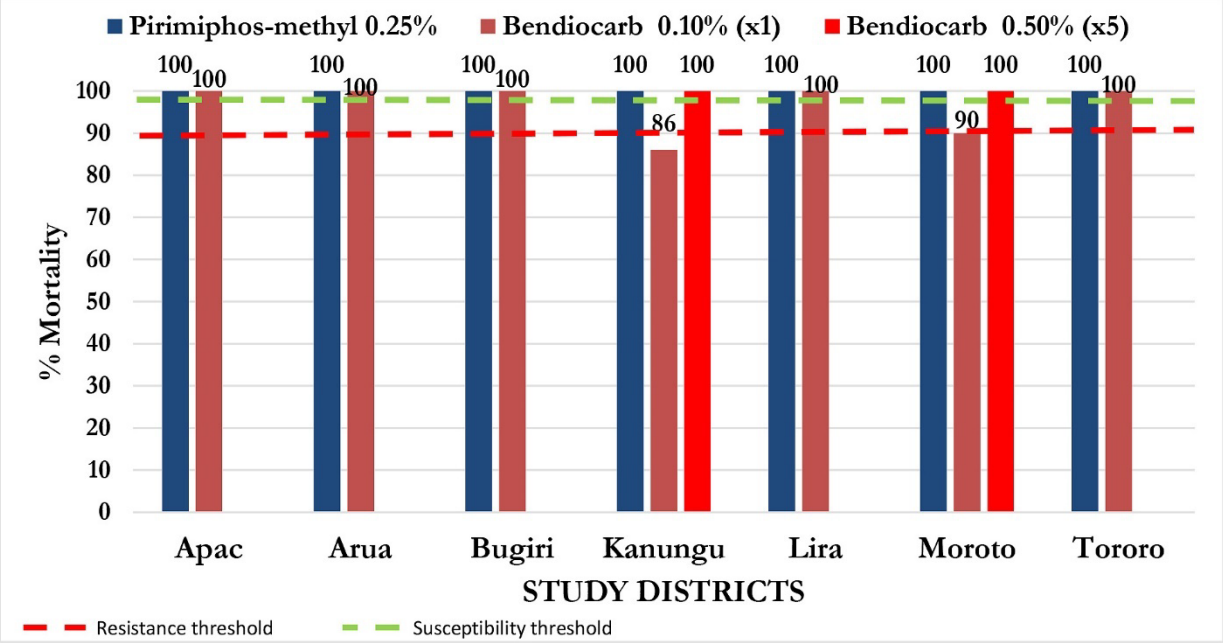
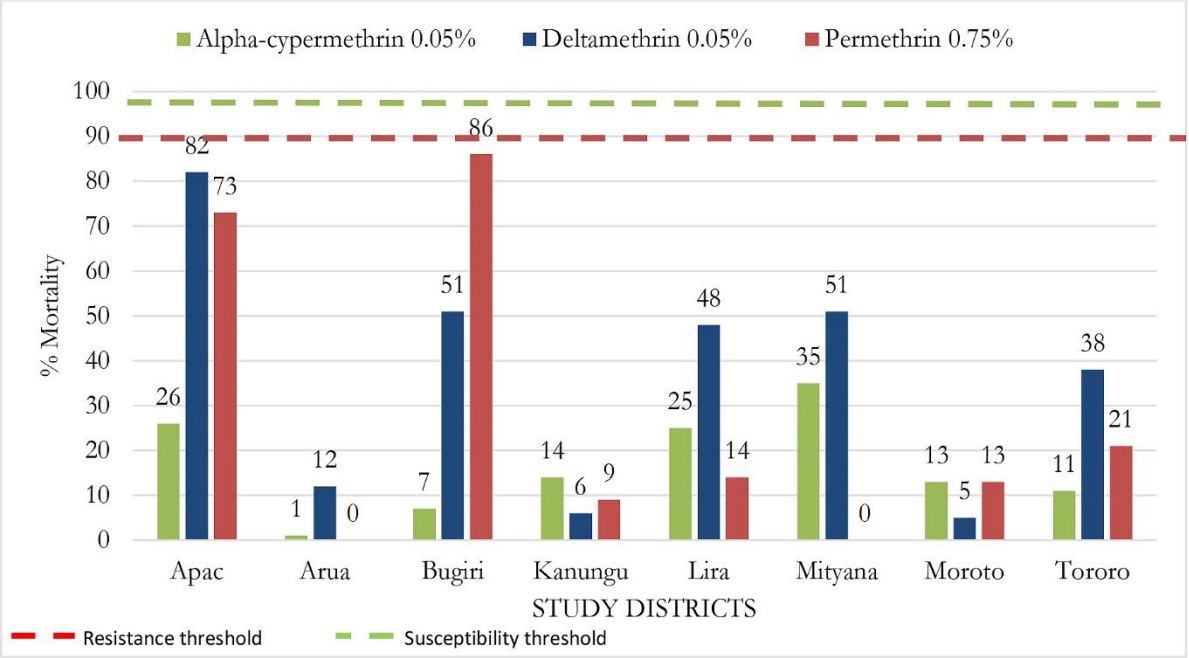


FIGURE 18: PERCENT 24-HOUR HOLDING MORTALITY OF FEMALE *AN. GAMBIAE* S.L. AFTER EXPOSURE TO THREE PYRETHROID INSECTICIDES IN EIGHT DISTRICTS, SEPTEMBER–OCTOBER 2021



3.3.2 DETERMINATION OF THE INTENSITY OF PYRETHROID RESISTANCE USING WHO TUBE TESTS

Bioassays for intensity of resistance were conducted where *An. gambiae* s.l. resistance was detected with the discriminating concentrations (24-hour mortality <98%). These were not performed in all districts for all insecticides, or for all concentrations, due to difficulties in finding and rearing enough larvae to supply the tests. High alpha-cypermethrin resistance intensity was detected in Apac (96% mortality after 24h at 10× concentration) and Tororo (75%) and moderate resistance intensity in Moroto (100% mortality at 10x but 90% at 5x dose) (Annex Table A-1 and Figure 19). The 10x dose was not tested at Kanungu or Arua but because of results using the 5x dose it is possible to conclude that resistance to alpha-cypermethrin in those districts is *at least* of moderate intensity. Moderate resistance intensity of *An. gambiae* s.l. to deltamethrin was observed in Apac, Lira, and Tororo. The 10x dose was not tested at Bugiri, Kanungu, or Moroto but, because of results using the 5x dose, it is possible to conclude that resistance to deltamethrin in those districts is *at least* of moderate intensity (Annex Table A-1 and Figure 20). Low resistance intensity of *An. gambiae* s.l. to permethrin was observed in Apac, Bugiri, Moroto, and Lira. Moderate resistance intensity to permethrin was observed in Tororo (100% mortality at 10× concentration) (Annex Table A-1 and Figure 21).

FIGURE 19: PERCENT MORTALITY *OF AN. GAMBIAE* S.L. AFTER EXPOSURE TO DIFFERENT CONCENTRATIONS OF ALPHA-CYPERMETHRIN IN FIVE DISTRICTS, SEPTEMBER–OCTOBER 2021

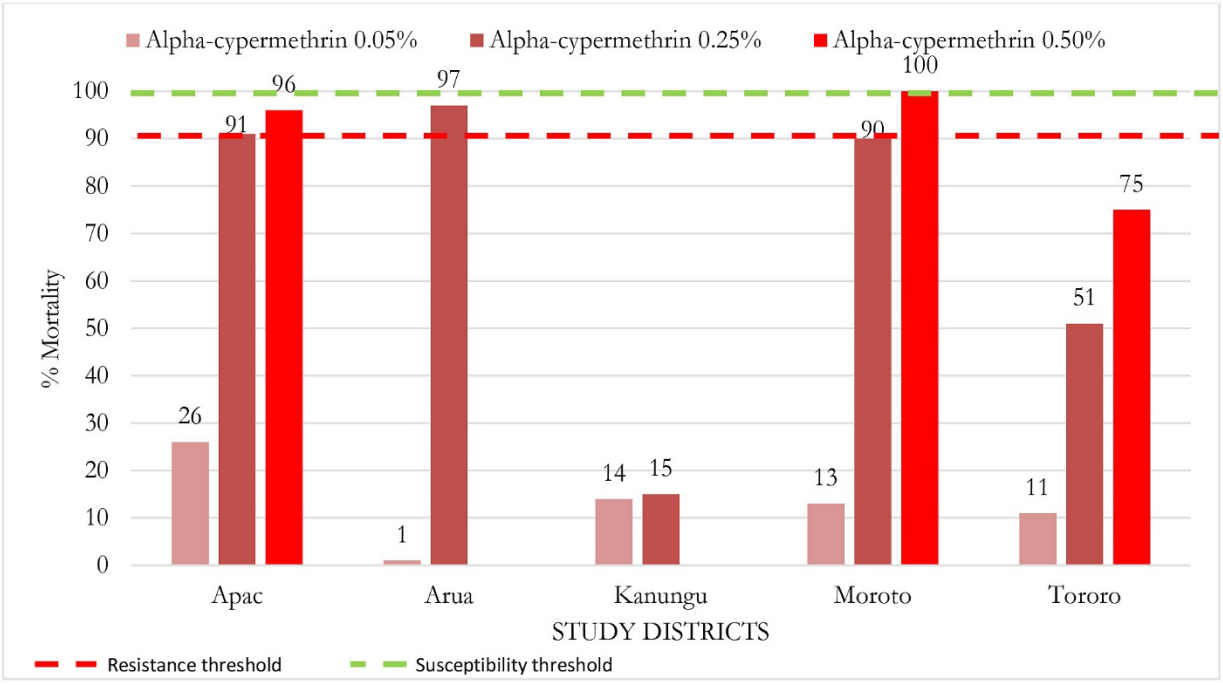


FIGURE 20: PERCENTAGE MORTALITY OF *AN. GAMBIAE* S.L. AFTER EXPOSURE TO DIFFERENT CONCENTRATIONS OF DELTAMETHRIN IN SIX DISTRICTS, SEPTEMBER–OCTOBER 2021

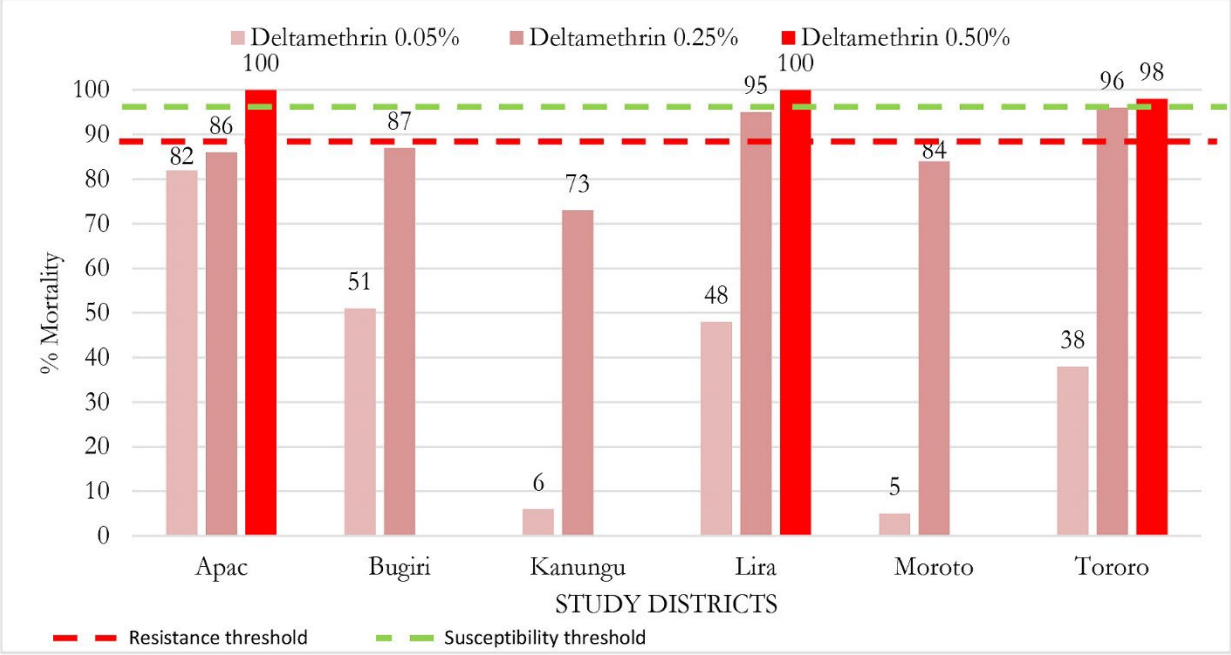
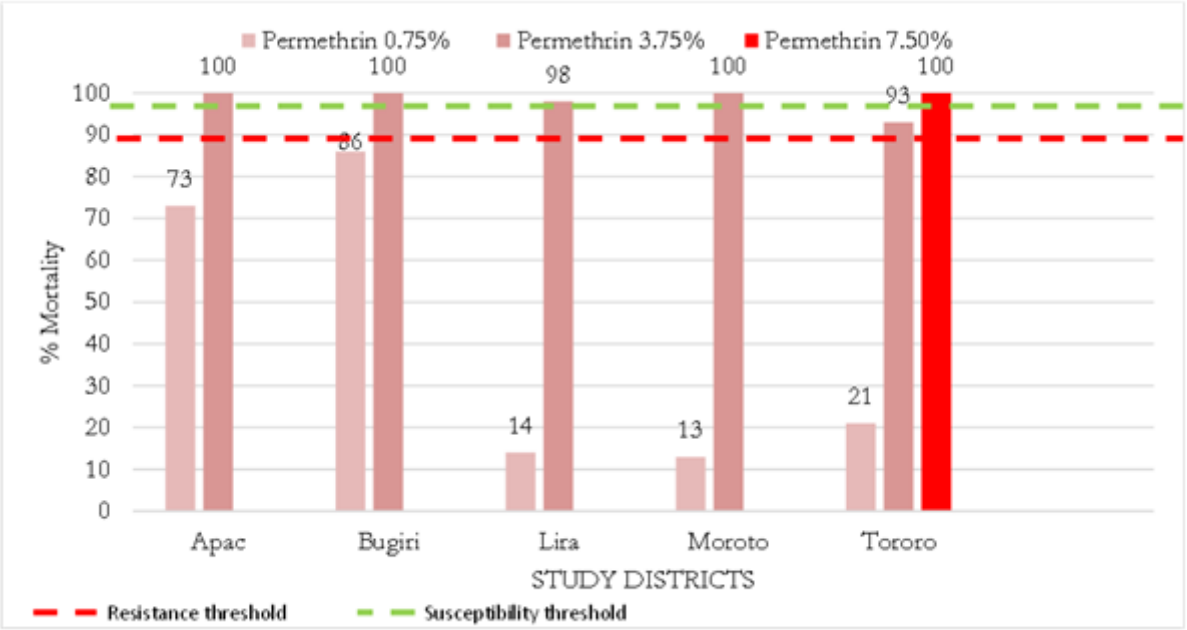
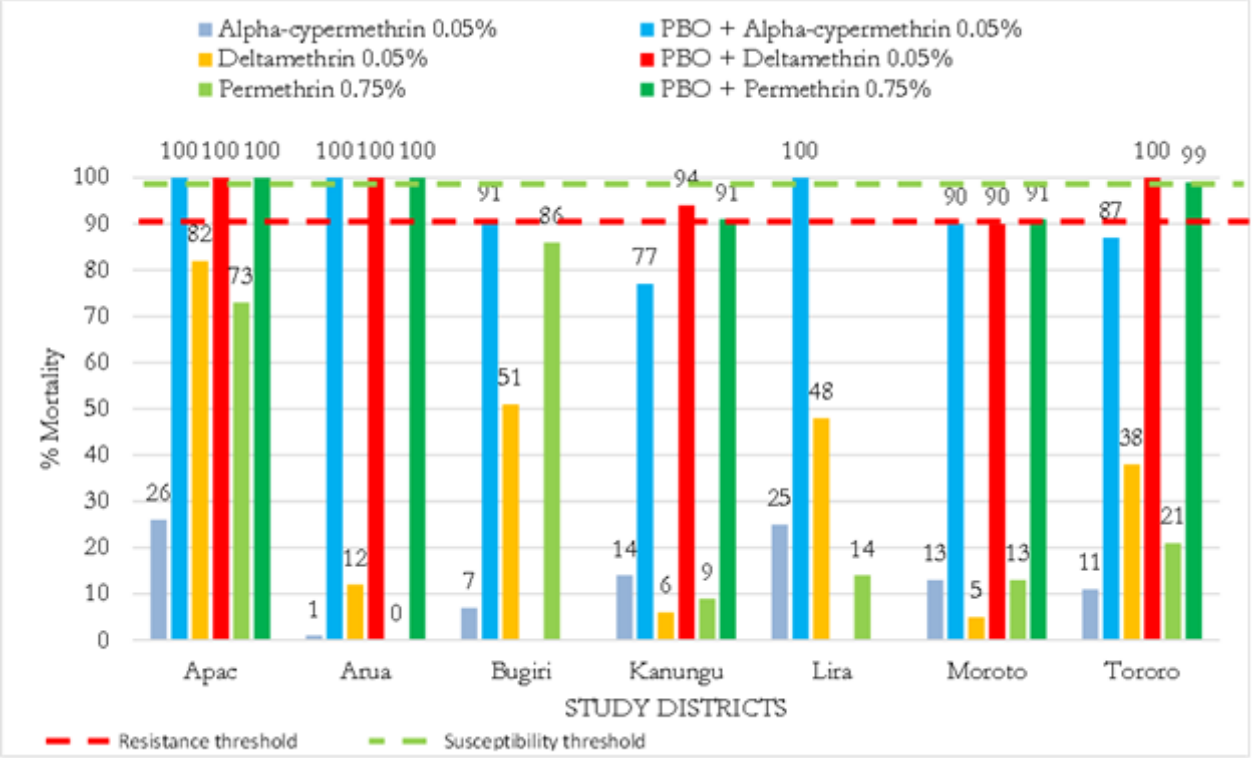


FIGURE 21: PERCENTAGE MORTALITY OF *AN. GAMBIAE* S.L. AFTER EXPOSURE TO DIFFERENT CONCENTRATIONS OF PERMETHRIN IN FIVE DISTRICTS, SEPTEMBER–OCTOBER 2021DETERMINATION OF THE INSECTICIDE RESISTANCE MECHANISMS (SYNERGIST ASSAYS) USING WHO TUBE TESTS



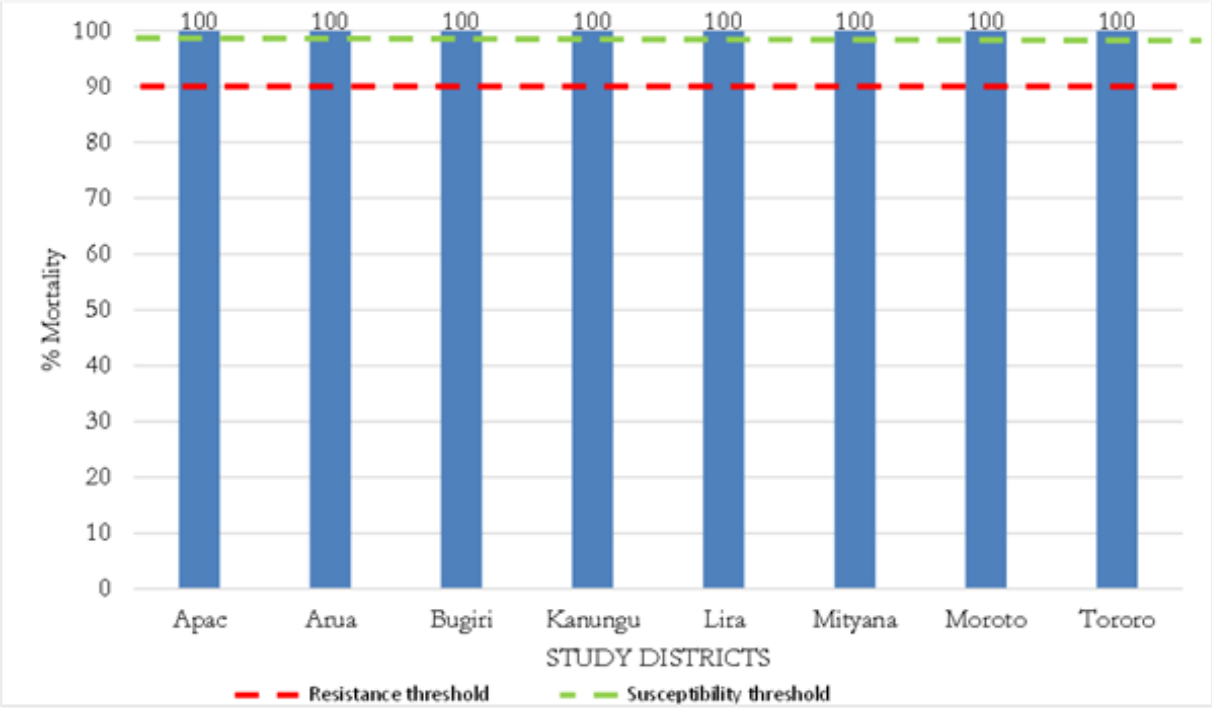
Synergist bioassays were conducted to assess the involvement of oxidase enzymes in *An. gambiae* s.l. resistance to pyrethroid insecticides using PBO. Pre-exposure to PBO fully restored *An. gambiae* s.l. susceptibility (98–100%) to alpha-cypermethrin in three of the seven districts (Apac, Arua, and Lira) and partially restored susceptibility in another four districts (Bugiri, Kanungu, Moroto, and Tororo) with mortality varying between 77% in Kanungu and 91% in Bugiri. PBO fully restored *An. gambiae* s.l. susceptibility to deltamethrin in three out of five districts (Apac, Arua, and Tororo) and partially restored susceptibility in Kanungu (94% mortality) and Moroto (90% mortality). Lastly, PBO fully restored *An. gambiae* s.l. susceptibility to permethrin in three of five districts (Apac, Arua, and Tororo) and partially restored susceptibility in Kanungu (91% mortality) and Moroto (91% mortality) (Annex Table A-1 and Figure 22). Full restoration of the efficacy of pyrethroid insecticides in some districts suggest that monoxygenases are the only form of metabolic resistance prevailing in those sites. Partial restoration of the efficacy of the insecticides suggests that, though monoxygenases are the main form of metabolic resistance prevailing in the area, other forms of resistance mechanisms are also present and need to be investigated.

FIGURE 22: SYNERGIST ASSAY MORTALITY RESULTS IN *AN. GAMBIAE* S.L. FROM SEVEN OUT OF EIGHT STUDY DISTRICTS UPON EXPOSURE TO ALPHA-CYPERMETHRIN, DELTAMETHRIN, AND PERMETHRIN ONLY OR WITH 4% PBO PRE-EXPOSURE, SEPTEMBER–OCTOBER 2021CLOTHIANIDIN SUSCEPTIBILITY TEST RESULTS



The clothianidin tests were performed on An. gambiae s.l. collected in all eight districts surveyed. One hundred percent (100%) mortality was recorded within 24 hours after exposure in all districts (Annex Table A-1 and Figure 23).

FIGURE 23: PERCENT 24-HOUR HOLDING MORTALITY OF *AN. GAMBIAE* S.L. REARED FROM LARVAE AFTER EXPOSURE TO CLOTHIANIDIN AT A CONCENTRATION OF 4MG/BOTTLE IN EIGHT DISTRICTS, SEPTEMBER–OCTOBER 2021

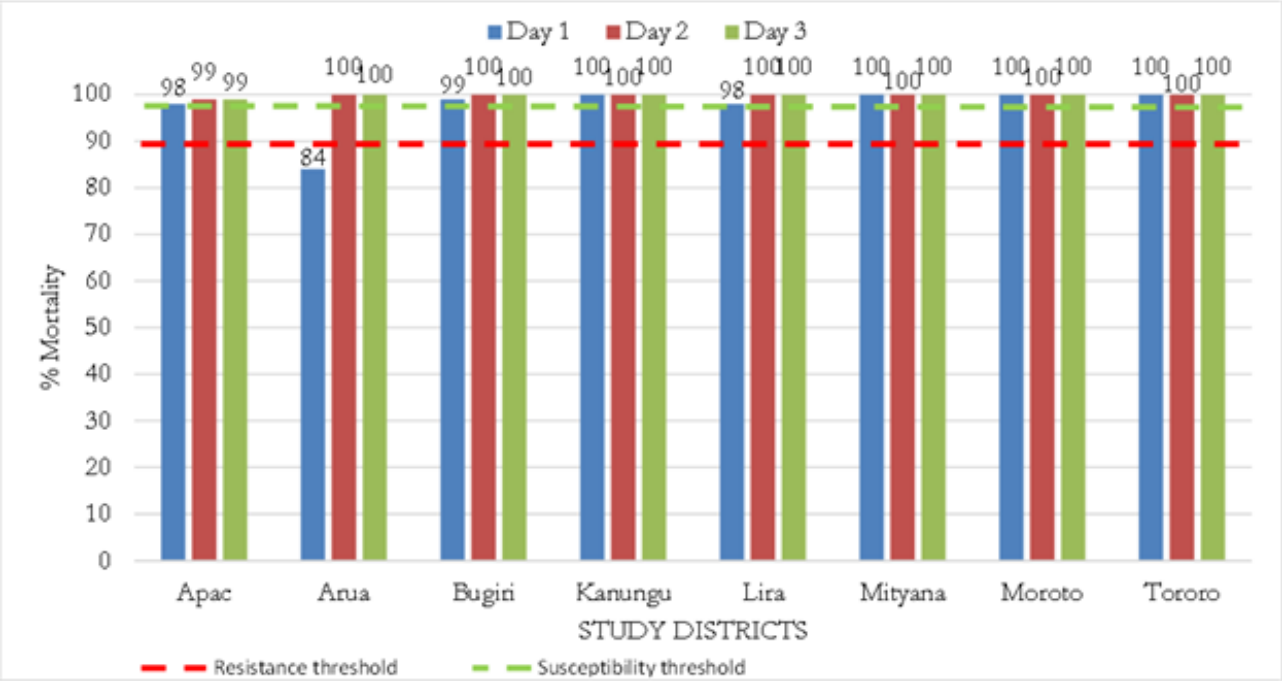


3.3.3 CHLORFENAPYR SUSCEPTIBILITY TEST RESULTS

Chlorfenapyr tests were conducted on samples collected in all eight districts surveyed with 99–100% mortality within three days after exposure was observed in all districts (Annex Table A-1 and Figure 24).

Both clothianidin and chlorfenapyr tests were conducted on the wild *An. gambiae* s.l. in parallel with the laboratory susceptible *An. gambiae* s.s. Kisumu strain in the districts of Bugiri, Lira, and Tororo, where access to susceptible *An. gambiae* s.s. strains was possible, and all were killed in all the tests. Parallel testing with the laboratory susceptible *An. gambiae* s.s. Kisumu strain was not possible in the districts of Arua, Kanungu, Mityana, and Moroto, where there was no nearby colony of susceptible *An. gambiae* s.s. to be used as reference.

FIGURE 24: PERCENTAGE MORTALITY OF *AN. GAMBIAE* S.L. AFTER EXPOSURE TO CHLORFENAPYR AT A CONCENTRATION OF 100MG/BOTTLE IN EIGHT DISTRICTS, SEPTEMBER–OCTOBER 2021 SUSCEPTIBILITY STATUS FOR INSECTARY-REARED *AN. GAMBIAE* S.S. (KISUMU STRAIN)



Susceptibility studies conducted on 2–5-day old *An. gambiae* s.s. (Kisumu strain) reared at the Vector Control Division in Kampala, Tororo Hospital, and Gulu University insectaries showed that the strain was fully pyrethroid-susceptible and therefore suitable for use in wall bioassays and as controls in susceptibility studies (Table 9).

TABLE 9: RESULTS OF INSECTICIDE SUSCEPTIBILITY STATUS OF INSECTARY-*REARED AN. GAMBIAE* S.S. (KISUMU STRAIN), MAY 2021

Insecticide	Vector Control Division Insectary		
	Kampala	Gulu University	Tororo Hospital
	# Tested (Mortality)	# Tested (Mortality)	# Tested (Mortality)
Alpha-cypermethrin 0.05%	80 (100.0%)	100 (100.0%)	100 (100.0%)
Deltamethrin 0.05%	80 (100.0%)	100 (100.0%)	100 (100.0%)
Permethrin 0.75%	60 (100.0%)	100 (100.0%)	100 (100.0%)

3.3.4 MOLECULAR ASSAY RESULTS

A report on molecular assay analysis results for the speciation of the *An. gambiae* and *An. funestus* complexes, infection rates, and characterization of insecticide resistance markers *(kdr* and *Ace-1R*) will be provided once it becomes available from IDRC.

4. DISCUSSION, LESSONS LEARNED, AND CHALLENGES

4.1 DISCUSSION

Results of PSCs conducted as part of longitudinal studies found that *An. funestus* s.l. was the most abundant species collected resting indoors in Apac, Otuke, and Soroti (control districts) and in Lira (current IRS district) whereas much lower densities were collected in Bugiri and none in Tororo (IRS districts). *An. gambiae* s.l. was also predominant in the HLCs in the IRS intervention districts of Bugiri and Tororo. It might be expected that the more endophillic and endophagic *An. funestus* s.l., as demonstrated by the PSC data and the high endophagic index for this species complex, is impacted more by IRS than is *An. gambiae* s.l., but there is no evidence of this: *An. funestus* s.l. densities have been historically low in Tororo (since 2013) and the relative proportion of *An. funestus* s.l. to *An. gambiae* s.l. in Lira is actually higher in 2021 (58%) compared to 2019 (28%), when entomological surveillance of IRS impact started in that district. The impact of IRS on *An. gambiae* s.l. is complicated by the changing prevalence of the exophilic and exophagic *An. arabiensis* species, but conclusions cannot be drawn in relation to that species until the molecular laboratory analyses are complete. Molecular assays are ongoing at IDRC molecular laboratories in Kampala involving vector mosquito species identification, infection rate determination, detection and identification of genetic resistance mutations, *kdr* and *Ace-1* genes in *An. gambiae* s.l. The relative distribution of both species complexes is also related to the sampling locations with the districts – in Apac and Soroti, they are near permanent swamps, an ideal breeding habitat for *An. funestus* s.l. *An. funestus* s.l. and *An. gambiae* s.l. dominated all mosquito collections – *An. ziemanni* and *An. coustani* were caught in very low numbers and other species like *An. ardensis, An. pretoriensis, and An. squamosus* that were caught in 2020 were not caught in 2021.

From HLCs it was seen that Soroti had the highest mean *An. funestus* s.l. biting rate both indoors and outdoors. In Otuke, where *An. gambiae* s.l. were low in previous years when the district was sprayed, this vector had a higher HBR than *An. funestus* s.l. outdoors, and there were more *An. gambiae* s.l. caught overall. This resurgence may be due to the withdrawal of IRS this year. In Apac, the indoor and outdoor HBR for both species complexes were lower than in the other non-IRS districts, which suggests the PBO LLINs distributed there may be effective. Apac was a district with historically high HBRs, but after several rounds of IRS and now PBO LLINs, mosquito densities are kept under control. Therefore in Otuke, consideration should be given to the distribution of PBO LLINs where IRS is withdrawn, in order to maintain reductions in density that were achieved with IRS.

The HLCs also revealed within-species differences between outdoor and indoor biting densities in several districts. Observed biting activity was marginally higher outdoors than indoors for *An. gambiae* s.l. in the IRS districts of Bugiri and Tororo. However, *An. gambiae* s.l. biting was much higher indoors than outdoors in Lira, as a result of which it is hard to attribute any obvious effect of IRS. The majority of *An. funestus* biting occurred indoors.

An. gambiae s.l. biting rates were highest immediately preceding the start of spraying and during part of the spraying period in Lira. Biting rates after IRS in Tororo also increased, though this is probably due to the prolonged heavy rains – the heaviest in the district in the last five years – which could have significantly increased the number of anopheline mosquito breeding sites in the months after IRS. These increases after IRS was sprayed were not as marked as the increases in densities in non-IRS districts over the same timeframe, and densities were much lower. That mean indoor and outdoor HBRs of *An. gambiae* s.l. and *An. funestus* s.l. were generally higher in the non-IRS districts than in the IRS districts give some indication of the effectiveness of IRS in controlling mosquito vectors, but it will be important to reconsider the timing of the 2022 IRS campaign

in light of these data, and to collect data on local rainfall patterns to help better interpret the entomological surveillance data.

The hourly biting rate of *An. funestus* s.l. in Soroti increased steadily throughout the night both indoors and outdoors, reaching a peak in the early morning hours. In other districts biting rates were too low to discern obvious biting patterns for this species complex. For *An. gambiae* s.l. there seem to be two peaks in biting activity, between midnight and 1am, and later between 3 and 5am. In light of these data it seems reasonable to conclude that most exposure occurs during hours when people will be in bed protected by nets, but this would need to be confirmed with human behavior observation studies, planned for 2022. As there is some biting activity starting as early as 7pm nets and IRS will not be wholly protective.

The WHO cone wall bioassay quality assurance results from seven sprayed districts showed that the spray quality of the 2021 spray campaign was satisfactory at all monitored sites. The monitoring for insecticide decay rate showed that Fludora Fusion on average stayed effective on sprayed surfaces between seven and nine months in Tororo and Bugiri, and between six and eight months in Serere and Lira, depending on the wall surface type sprayed. It is not clear why residual efficacy was superior in Tororo when compared to Serere. Plastered and painted cement surfaces were consistently associated with longer residual efficacy than mud and brick surfaces. Encouraging householders to plaster and/or paint walls before IRS is sprayed may help retain insecticide on the wall surface for longer periods and thus allow for longer residual efficacy of the insecticide because of greater bioavailability.

An. gambiae s.l. was found to be susceptible to pirimiphos-methyl, clothianidin and chlorfenapyr in all districts where the susceptibility tests were performed. These data raise no alarms for continuation of use of Actellic, SumiShield and Fludora Fusion insecticides for IRS. Full susceptibility to chlorfenapyr also supports the use of IG2 LLINs or chlorfenapyr-based IRS products in future if WHO PQ listed. In contrast, *An. gambiae* s.l. was susceptible to bendiocarb in two of seven districts, and resistant to all pyrethroids in all districts. Though the intensity of this pyrethroid resistance was not universally high, it is sufficiently concerning that the deployment of next generation LLINs like the PBO-synergized LLINs where resistance intensity is low or moderate, and new WHO-prequalified dual active ingredient LLINs where resistance is high should be encouraged. The synergist assays using PBO fully or partially restored *An. gambiae* s.l. susceptibility to pyrethroids in all districts, lending support to the use of these nets in Uganda. Results suggest the presence of metabolic resistance mainly due to monoxygenases, although other resistance mechanisms appear to also play a minor role in some study districts. We anticipate improved malaria vector control with PBO-synergized LLINs and Royal Guard® (alpha-cypermethrin and pyriproxyfen incorporated into polyethylene, all panels), distributed in Apac and Mubende districts, than with the pyrethroid-only LLINs that were deployed in most districts of Uganda during the last universal LLIN distribution in July 2020–March 2021.

In conclusion, the vector bionomics data indicates that IRS continues to be an appropriate intervention for control of malaria vectors in all these districts, though its impact may be lessened after multiple years of application where *An. funestus* s.l. populations are fully controlled and *An. gambiae* s.l. is comprised of more *An. arabiensis* than *An. gambiae s.s.*, or where *An. gambiae* s.s. is found more often outdoors. The full susceptibility of *An. gambiae* s.l. to pirimiphos-methyl, clothianidin, and chlorfenapyr (98–100% mortality) indicates that both pirimiphos-methyl and clothianidin can be used in rotation to assist in the management of insecticide resistance in IRS programs. The majority of malaria vectors are biting after 10:00 p.m. and the majority of mosquitoes, especially *An. funestus* s.l., are resting indoors. Increased rainfall and unpredictable rainfall was experienced during the review period, probably resulting in the creation of numerous vector breeding sites and increased vector populations before and after IRS when compared to previous years. Additionally it is concerning that the protection that IRS provides does not seem to last long enough to prevent a resurgence in mosquito densities in October–November. Attention should be given to the timing of IRS campaigns, especially for the northern districts, so that spraying is conducted prior to any increase in mosquito densities. Finally, where IRS is withdrawn, consideration should be given to replacing it with PBO-nets or dual-active nets.

4.2 CHALLENGES

The lockdown occasioned by COVID-19 pandemic affected the implementation of some of the planned entomological monitoring activities. Secondly, the unpredictable rainfall pattern and unusually heavy and extended rains over recent years has made it very difficult to plan when it is most appropriate to conduct susceptibility studies in Uganda. In most districts, collecting enough mosquito vectors for all tests was a challenge. The heavy rains also affected the road network in most districts, and this interfered with field collections. Lastly, there was delayed molecular analysis of mosquito samples and reporting by IDRC molecular laboratories in Kampala, which prevented the inclusion of molecular assay results in this report.

4.3 LESSONS LEARNED

The involvement of district-based vector control officers (VCOs) in entomological monitoring activities has strengthened their capacity to conduct similar studies in their respective districts in future. Similarly, involving VCOs not currently deployed to support district activities has improved the human-resource technical capacity available to the Ministry of Health for deployment in future entomological surveillance activities in various areas of the country. By involving and facilitating district-based VCOs, VectorLink Uganda can collect and rear mosquitoes in advance of the project study team arriving at those districts to conduct insecticide resistance tests. The unpredictable rainfall pattern in the country over recent years has made it very difficult to plan when it is most appropriate to conduct susceptibility studies, and it is necessary to stagger the implementation of susceptibility studies to accommodate this. Involving the VCOs will help streamline the process.

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ANNEX A

TABLE A-1: PERCENT 24-HOUR HOLDING MORTALITY OF *AN. GAMBIAE* S.L. AFTER EXPOSURE TO 6 INSECTICIDES, SEPTEMBER–OCTOBER 2021 (RESULTS FROM ADULTS REARED FROM LARVAE)

Insecticide	Apac	Arua	Bugiri	Kanungu	Lira	Mityana	Moroto	Tororo
Pirimiphos-methyl 0.25%	100 (100.0%)	100 (100.0%)	100 (100.0%)	100 (100.0%)	100 (100.0%)	-	100 (100.0%)	100 (100.0%)
Bendiocarb 0.10% (x1)	100 (100.0%)	100 (100.0%)	100 (100.0%)	100 (86.0%)	100 (100.0%)	-	100 (90.0%)	100 (100.0%)
Bendiocarb 0.50 % (x5)	-	-	-	100 (100.0%)	-	-	100 (100.0%)	-
Deltamethrin 0.05% (x1)	100 (82.0%)	100 (12.0%)	100 (51.0%)	100 (6.0%)	100 (48.0%)	100 (51.0%)	100 (5.0%)	100 (38.0%)
Deltamethrin 0.25% (x5)	100 (86.0%)	-	100 (87.0%)	100 (73.0%)	100 (95.0%)	-	100 (84.0%)	100 (96.0%)
Deltamethrin 0.5% (x10)	100 (100.0%)	-	-	-	100 (100.0%)	-	-	100 (98.0%)
PBO + Deltamethrin 0.05%	100 (100.0%)	100 (100.0%)	-	100 (94.0%)	-	-	100 (90.0%)	100 (100.0%)
Permethrin 0.75% (x1)	100 (73.0%)	100 (0.0%)	100 (86.0%)	100 (9.0%)	100 (14.0%)	-	100 (13.0%)	100 (21.0%)
Permethrin 375% (x5)	100 (100.0%)	-	100 (100.0%)	-	100 (98.0%)	-	100 (100.0%)	100 (93.0%)
Permethrin 7.5% (x10)	-	-	-	-	-	-	-	100 (100.0%)
PBO + Permethrin 0.75%	100 (100.0%)	100 (100.0%)	-	100 (91.0%)	-	-	100 (91.0%)	100 (99.0%)

Insecticide	Apac	Arua	Bugiri	Kanungu	Lira	Mityana	Moroto	Tororo
Alpha-cypermethrin 0.05%	100 (26.0%)	100 (01.0%)	100 (07.0%)	100 (14.0%)	100 (25.01%)	100 (35.0%)	100 (13.0%)	100 (11.0%)
Alpha-cypermethrin 0.25% (x5)	100 (91.0%)	100 (97.0%)	-	100 (15.0%)	100 (83.0%)	-	100 (90.0%)	100 (51.0%)
Alpha-cypermethrin 0.5% (x10)	100 (96.0%)	-	-	-	-	-	100 (100.0%)	100 (75.0%)
PBO + Alpha-cypermethrin 0.05%	100 (100.0%)	100 (100.0%)	100 (91.0%)	100 (77.0%)	100 (100.0%)	-	100 (90.0%)	100 (87.0%)
Clothianidin 4 µg/bottle + 800ppm MERO	100 (100.0%)	100 (100.0%)	100 (100.0%)	100 (100.0%)	100 (100.0%)	100 (100.0%)	152 (100.0%)	100 (100.0%)
KEY	**Confirmed resistance**			**Probable resistance**			**Susceptible**	

Key: - = Tests not conducted due to inadequate vector specimens and limited number of *An. gambiae* s.l.