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PMI VECTORLINK ETHIOPIA PROJECT
FINAL ENTOMOLOGY REPORT
MAY 2018-APRIL 2019

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Acronyms

AIRS	Africa Indoor Residual Spraying (project)
CDC	Centers for Disease Control and Prevention
CSP	Circumsporozoite Proteins
ELISA	Enzyme-linked Immunosorbent Assay
HLC	Human Landing Catch
IRS	Indoor Residual Spray
LLIN	Long-lasting Insecticidal Net
ND	Not Done
NMCEP	National Malaria Control and Elimination Program
PBO	Piperonyl Butoxide
PCR	Polymerase Chain Reaction
PMI	U.S. President's Malaria Initiative
PSC	Pyrethrum Spray Catch
WHO	World Health Organization

EXECUTIVE SUMMARY

BACKGROUND

The U.S. President's Malaria Initiative (PMI) VectorLink Ethiopia project conducted entomological monitoring from May 2018 through April 2019. The activity included monthly collection of data on species composition and diversity, resting density, human biting rate and night biting patterns, feeding location, and *Plasmodium* sporozoite infection rates to assess the impact of indoor residual spraying (IRS) on entomological indicators. Human landing catches (HLCs), pyrethrum spray catches (PSCs), and Centers for Disease Control and Prevention (CDC) light trap collections were carried out in three PMI VectorLink Ethiopia monitoring sites: Lare in Gambela, Abaya in Oromia, and Bambasi in Benishangul-Gumuz. All three sites were sprayed with the organophosphate pirimiphos-methyl (Actellic 300CS).

The susceptibility of *Anopheles gambiae* s.l. to various insecticides used in public health was investigated in 13 sentinel sites (Abaya, Abobo, Amibara, Babile, Bahirdar, Bamabasi, Benatsemay, Dangur, Halaba, Humera, Metema, Omonada, and Ziway-Dugda) situated in seven regions of the country. In addition, synergist assays with piperonyl butoxide (PBO) were conducted and resistance intensity was evaluated in six of the 13 sites (Abaya, Amibara, Bambasi, Halaba, Metema, and Ziway-Dugda). The *An. gambiae* s.l. response to chlothianidin was examined in five sites (Abaya, Amibara, Halaba, Omonada, and Ziway-Dugda) and to chlorfenapyr in four sites (Halaba, Abaya, Omonada, and Zeway-Dugda) sites. The quality of spraying and decay rate of Actellic 300 CS was assessed using cone bioassays in 12 houses in each of four sites: Lare, Abaya, Bambasi, and Goro. Molecular species identification of the mosquitoes and *Plasmodium* circumsporozoite protein detection were conducted by Jimma University.

In addition, the project surveyed for the presence of *An. stephensi* in 10 selected urban sites in the eastern and north-eastern parts of the country; in two of the sites, Dire Dawa and Kebridehar, it also assessed *An. stephensi* susceptibility to various public health insecticides.

RESULTS

Mosquito collections using HLC, PSC, and CDC light trap methods demonstrated the presence of the principal malaria vector, *An. gambiae* s.l., secondary vectors *An. funestus* s.l. and *An. pharoensis,* and other potential or non-vectors *An. coustani* and *An. squamosus*. The proportion of *An. gambiae* s.l. in Lare, Bambasi, and Abaya was 12%, 10%, and 58%, respectively. In Bambasi, *An. funestus* s.l. was the dominant species representing 41% of all collections, whereas it was the second-most abundant vector in Lare (28%) after *An. pharoensis* (40%).

Host-seeking malaria vectors *An. gambiae* s.l., *An. funestus* s.l., and *An. pharoensis* were collected both indoors and outdoors. In almost all collection sites, their biting densities were higher outdoors than indoors. Interestingly different seasonal abundances were observed among the three vectors where they co-existed in Lare. *An. gambiae* s.l. biting density peaked in February, *An. funestus* s.l. in November-December, and *An. pharoensis* in August. This might be the reason why malaria transmission in Gambela is relatively stable. Peak biting densities of *An. gambiae* s.l. were recorded from July to September in Bambasi. In Abaya, *An. gambiae* s.l. biting occurred throughout the year (except October), with multiple peak biting densities in May, November, December, March, and April.

As determined from CDC light traps, the mean density of *An. funestus* group peaks in Lare in November and December, and yet, in Bamabsi from August to November although data was lacking for October.

An. gambiae s.l. in Lare was active throughout the night (6:00 pm to 6:00 am) both indoors and outdoors with one peak outdoors, between 7:00 pm and 8:00 pm, and two peaks indoors, between 7:00 pm and 8:00 pm and 10:00 pm and 11:00 pm. *An. funestus* s.l. in Lare was active outdoors all night long, but its indoor activity was limited from 6:00 pm to 2:00 am. *An. pharoensis* was caught outdoors all night long but absent indoors from 3:00 am to 6:00 am. The biting activities of *An. gambiae* s.l. in Abaya extended from dusk to dawn.

Populations of *An. gambiae* s.l. were susceptible (98-100% mortality) to bendiocarb and pirimiphos-methyl in all 13 sentinel sites, and to propoxur in 11 of the 13 sites. Possible resistance to propoxur was recorded in two of the 13 sites (Abaya and Abobo), with 97% mortality. Resistance to the pyrethroid insecticides (deltamethrin, permethrin, and alpha-cypermethrin) was widely distributed. Low and high intensity of resistance to deltamethrin and permethrin was observed. Pre-exposure to Piperonyl butoxide (PBO) either partially or fully restored susceptibility of *An. gambiae* s.l. to deltamethrin in 2 out of 6 and 4 out of 6, respectively and permethrin in 3 out of 6 sites for each. Clothianidin susceptibility tests resulted in lower mortalities of *An. gambiae* s.l. at low temperatures (16-22°C) than at temperature of 27-29°C.

At T0 (bioassays conducted within a week of spraying), mortality of *An. arabiensis* from the cone bioassay test was 100% in all the sites except in Bambasi, where mortality was 95%. The duration of the effect of Actellic 300CS was about two months in Lare and Abaya, 3-5 months in Goro, and more than 5 months in Bambasi.

Adult *An. stephensi* surveys in 10 urban sites revealed the presence of this species in all survey sites in eastern Ethiopia. Two populations of this species, from Dire Dawa and Kebridehar, were highly resistant to bendiocarb, propoxur, deltamethrin, permethrin, and alpha-cypermethrin but susceptible to pirimiphos-methyl. PBO synergist tests restored susceptibility to deltamethrin and permethrin, showing a role for the oxidase enzymes family in the resistance of the two insecticides.

CONCLUSIONS

The entomological monitoring data obtained from Lare indicated variation in the seasonal abundances among the three malaria vectors located in the area namely *An. arabiensis*, *An. funestus* s.l., and *An. pharoensis*, which might partially explain why transmission of malaria in the area is relatively stable as compared to other parts of the country. .

The invasion of *An. stephensi* in urban sites in eastern Ethiopia and perhaps in the rest of the country, together with its high insecticide resistance profile, might be a concern in the epidemiology of malaria, particularly in urban settings. At this stage, however, information is lacking on its infection and infectivity, longevity, and other important indices that incriminate a vector.

1. INTRODUCTION

In September 2017, the U.S. President's Malaria Initiative (PMI) launched the five-year PMI VectorLink Project. The project works in 24 countries in sub-Saharan Africa as well as Cambodia, equipping the countries to plan and implement safe, cost-effective, and sustainable vector control programs with the overall goal of reducing the malaria burden.

Ethiopia has received PMI support to do indoor residual spraying (IRS) since 2008. From 2012 to 2017, that support flowed through the PMI Africa Indoor Residual Spraying (AIRS) project. Now, PMI VectorLink Ethiopia is implementing IRS operations in 44 districts in three regional states: Gambela, Benishangul-Gumuz, and Oromia. The project supports the National Malaria Control and Elimination Program (NMCEP) in implementing IRS, capacity building, monitoring and evaluation, and producing entomological surveillance data. Such entomological information is the cornerstone of evidence-based, effective decision making for the implementation of vector control interventions including IRS and long-lasting insecticidal mosquito nets (LLINs). For example, the data can be used to evaluate the impact of vector control interventions on entomological outcomes such as vector density and infection, assess vector susceptibility to insecticides, and change in vector behavior in response to scale-up of vector control interventions. PMI VectorLink Ethiopia and the NMCEP use the data to decide which insecticide will be most effective for IRS. (Since 2015, the project has used the organophosphate pirimiphos-methyl, (Actellic 300 CS) in Ethiopia.)

In 2018-2019, PMI VectorLink Ethiopia collected mosquitoes for entomological monitoring using three sampling methods: human landing catches (HLCs), pyrethrum spray catches (PSCs), and Centers for Disease Control and Prevention (CDC) light traps. For susceptibility tests, larval collections were carried out to rear larvae to the adult stage of *An. gambiae* s.l. needed for the tests.

The project also did a cross–sectional survey to assess the spread of *An. stephensi* in eastern Ethiopia. Introduction of *An. stephensi* vectors from other countries or continents might jeopardize Ethiopia's malaria control effort, although the role of this species in Ethiopia is not known yet.

This report covers entomological monitoring activities conducted in Ethiopia from May 2018 to April 2019. The entomological monitoring activities were aimed at:

- Assessing malaria vector density and species composition pre-/ post-IRS in three sites (Lare, Bambasi, and Abaya);
- Understanding vector feeding times and locations;
- Monitoring the quality of insecticide application and insecticide decay rates in four sites (Lare, Bambasi, Goro, and Abaya);
- Determining vector infection rates;
- Monitoring the level of insecticide resistance of the populations of *An. gambiae* s.l. to various insecticides, measuring resistance intensity, and conducting synergist assays; and
- Investigating the distribution of *An. stephensi* in 10 sites and conducting insecticide susceptibility tests in two of those sites.

2. METHODOLOGY

This section discusses the various methodologies used to conduct entomological monitoring and to evaluate insecticide resistance and decay rates. It also presents methods of the *An. stephensi* survey.

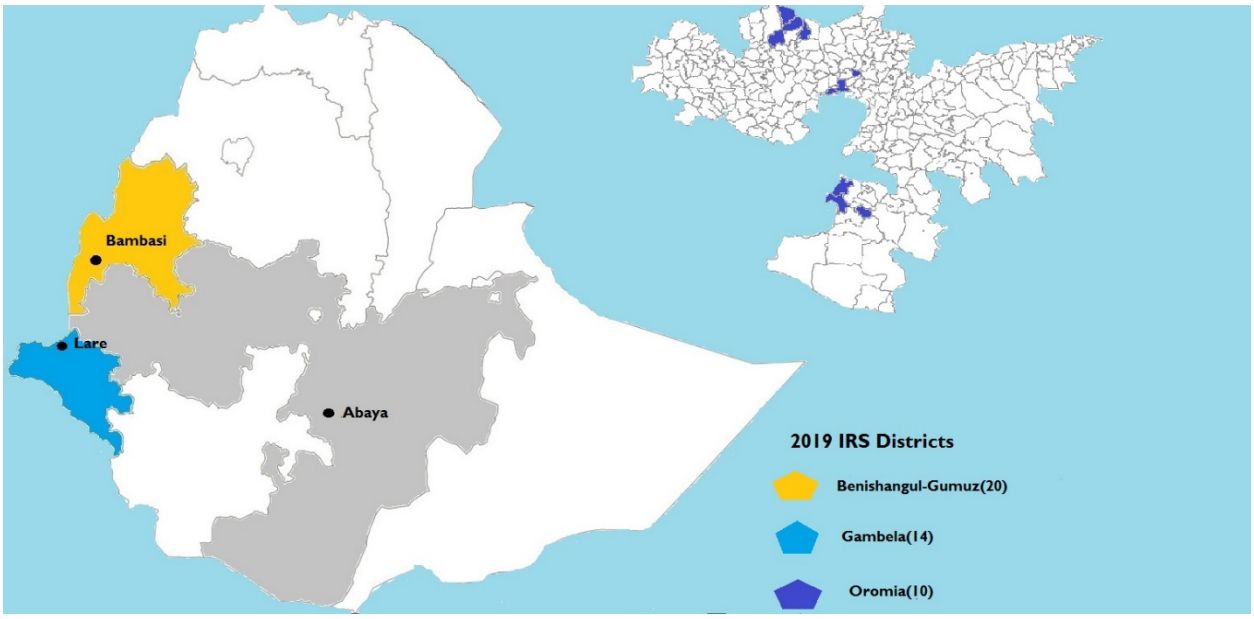
2.1 LONGITUDINAL ENTOMOLOGICAL MONITORING

Monthly entomological monitoring was conducted from May 2018 to April 2019 in three sentinel sites: Lare, Bambasi, and Abaya. Entomological data were collected on species composition and diversity, indoor resting density, biting time and location, and *Plasmodium* sporozoite infection rates of malaria vectors. The 2018 spray campaign started in May and ended in July. Spraying was conducted in Lare in May, in Bambasi and in June, and in Abaya in July. Actellic 300CS was sprayed in all PMI-supported areas. The May data collection took place before spraying and served as baseline while collections from June through November in Lare, July through November in Bambasi, and August through December in Abaya produced post-spray data. The remaining collection period until April in Lare and Abaya represents the dry season.

2.1.1 STUDY SITES

The three sentinel sites of Lare, Bambasi, and Abaya (Figure 1) were selected for routine entomological monitoring based on the recommendation of the regional and district health bureaus. PMI VectorLink Ethiopia conducted monitoring for a full year (12 months) in Lare, 11 months in Abaya, and six months in Bambasi. Security problems interrupted monitoring in June 2018 in Abaya and in October 2018 in Bambasi. Continued unrest in Bambasi forced monitoring to be discontinued as of December 2018.

FIGURE 1: ENTOMOLOGICAL MONITORING SENTINEL SITES IN PMI SUPPORTED IRS REGIONS, 2018



2.1.2 COLLECTION METHODS

Mosquitoes were sampled using HLCs, PSCs, and human-baited CDC light traps (Table 1).

2.1.2.1 HUMAN LANDING CATCH

HLCs yield information on the anthropophagic profile of *Anopheles*, night biting patterns, human biting rate, and biting location (indoors and outdoors). To do the HLCs, three houses were randomly selected from each longitudinal monitoring site and same houses were consistently used for monthly mosquito collections for six nights per month from 6:00 pm to 6:00 am, indoors and outdoors. Two teams comprising four volunteers were put on Mefloquine prophylaxis (which was administered orally) engaged in the mosquito collections at each house. Two volunteers collected mosquitoes from 6:00 pm to midnight, the other two from midnight to 6:00 am. One volunteer sat indoors while the other sat outdoors, and they shifted sites every hour. They used an aspirator, torch, and paper cups to do the collections. Hourly collections were labelled and paper cups containing mosquitoes were kept in boxes. The following day, the caught mosquitoes were identified as *Anopheles* and culicines and their number was recorded. *Anopheles* were further identified to species using the Gillies and Coetzee morphological key (1987) and preserved individually in Eppendorf tubes over silica gel for further molecular and immunological analysis.

The monthly mean biting density of a species of *Anopheles* was determined from the total number of collections over the six days. The mean hourly human biting rate was calculated as the total number of female mosquitoes collected in the hour divided by the number of human baits.

The rate of exophagy in comparison to endophagy of the main malaria vectors was scored as a ratio of the number collected outdoors to the number collected indoors.

2.1.2.2 PYRETHRUM SPRAY CATCH

The monthly species composition and indoor resting density of *Anopheles* was determined from PSCs from 20 houses in each of the three sites. The project secured consent to do the collection from each head of household or another family member. The PSC was done between 6:30 am and 8:00 am in at least five houses each day for a total of four days per month. Animals, food and drink items, and big household utensils were removed; collectors also ensured that children left the house. They then used a white cloth to cover the floor, underneath and the tops of beds and other unmovable items. To prevent mosquitoes from escaping, they sealed eaves and other openings. Two entomology assistant technicians wearing protective nose masks were assigned to spray an aerosol insecticide, one inside to knock down indoor resting mosquitoes, and the other outside of the house. The technician inside started from the door spraying the walls and ceiling, after which s/he left the room carefully; the one outside sprayed around the house. After 10 minutes, knocked-down mosquitoes were collected from the cloth, identified to species, categorized based on their abdominal status (unfed, fresh fed, half gravid, or gravid), and preserved in Eppendorf tubes.

2.1.2.3 CDC LIGHT TRAP COLLECTION

Mosquitoes attracted to bite humans indoors were sampled using human-baited CDC light traps. In houses where the light traps were installed, occupants slept under LLINs. In each site, four traps were set in four houses that neighbored an HLC house, which was assigned as an index. Thus, monthly collections were done in 12 houses in each sentinel site for two nights (24 trap-nights) between 6:00 pm and 6:00 am. Traps were collected the next morning; the mosquitoes were collected with aspirators and identified as *Anopheles* and culicines. After recording numbers, culicines were discarded and *Anopheles* were categorized based on their abdominal status. After species identification, mosquitoes were individually preserved in Eppendorf tubes.

TABLE 1: METHOD, TIME, AND FREQUENCY OF SAMPLING

Type of Collection	Time	Frequency	Sample
HLC	6:00 pm to 6:00 am	Six nights/month in the same houses throughout the collection period	Three houses per site, indoors and outdoors (18 indoor HLC and 18 outdoor HLC per site per month)
PSC	6:30 am to 8:00 am	Once a month	Twenty houses per site per month
CDC light traps	6:00 pm to 6:00 am	Two nights/month	Twelve houses per site, all indoors (24 trap nights per site per month)

2.1.3 IDENTIFICATION OF *ANOPHELES* AND DETECTION OF INFECTIONS

Anopheles collections from HLC, PSC, and CDC light traps were tested for species identification and sporozoite infections.

2.1.3.1 MOLECULAR IDENTIFICATION OF *AN. GAMBIAE* S.L. AND *AN. FUNESTUS* S.L.

Molecular identification of *An. gambiae* s.l. was carried out using species-specific polymerase chain reaction (PCR) techniques by using primers for *An. gambiae* s.s., *An. arabiensis*, and *An. quadriannulatus* as described by Scott et al., 1993. From the collections of *Anopheles* in Bamabsi, 37 specimens morphologically identified as *An. funestus* were investigated by PCR at Walter Reed Biosystematics Unit (WRBU) to identify the specific species prevailing in the area.

2.1.3.2 SPOROZOITE DETECTION

The dried head and thorax of the preserved *Anopheles* mosquitoes were carefully separated from the abdomen and tested simultaneously for *Plasmodium falciparum* and *P. vivax* circumsporozoite proteins (CSP) as described by Wirtz et al. 1992.

2.2 INSECTICIDE RESISTANCE MONITORING

For susceptibility tests, larvae and pupae were collected using dippers and reared to adult either in the field or insectary. The resistance status and resistance intensity of *An. gambiae* s.l. was monitored with diagnostic doses (1x) as well as 5x and 10x concentrations of each insecticide. The involvement of oxidases in conferring resistance to deltamethrin and permethrin was assessed through synergist assay. The allelic frequency of *kdr* mutations was assessed using molecular techniques.

2.2.1 INSECTICIDE SUSCEPTIBILITY WITH DISCRIMINATING CONCENTRATIONS

Insecticide resistance monitoring was conducted in 13 sentinel sites (Figure 2) in seven regions: one site in Afar (Amibara), four in Oromia (Ziway-Dugda, Omonada, Babile, Abaya), one in Gambela (Abobo), two in Benishangul Gumuz (Bambasi, Dangur), two in Amhara (Metema, Bahirdar), two in Southern Nations Nationalities and Peoples Region (Halaba, Benatsemay), and one in Tigray (Humera). World Health Organization (WHO) tube tests (WHO 2016) were conducted to measure *An. gambiae* s.l. susceptibility to the diagnostic dose (1x) of bendiocarb (0.1%), propoxur (0.1%), pirimiphos-methyl (0.25%), deltamethrin (0.05%), permethrin (0.75%), and alpha-cypermethrin (0.05%). These insecticides were selected based on their use for IRS and LLINs in the country.

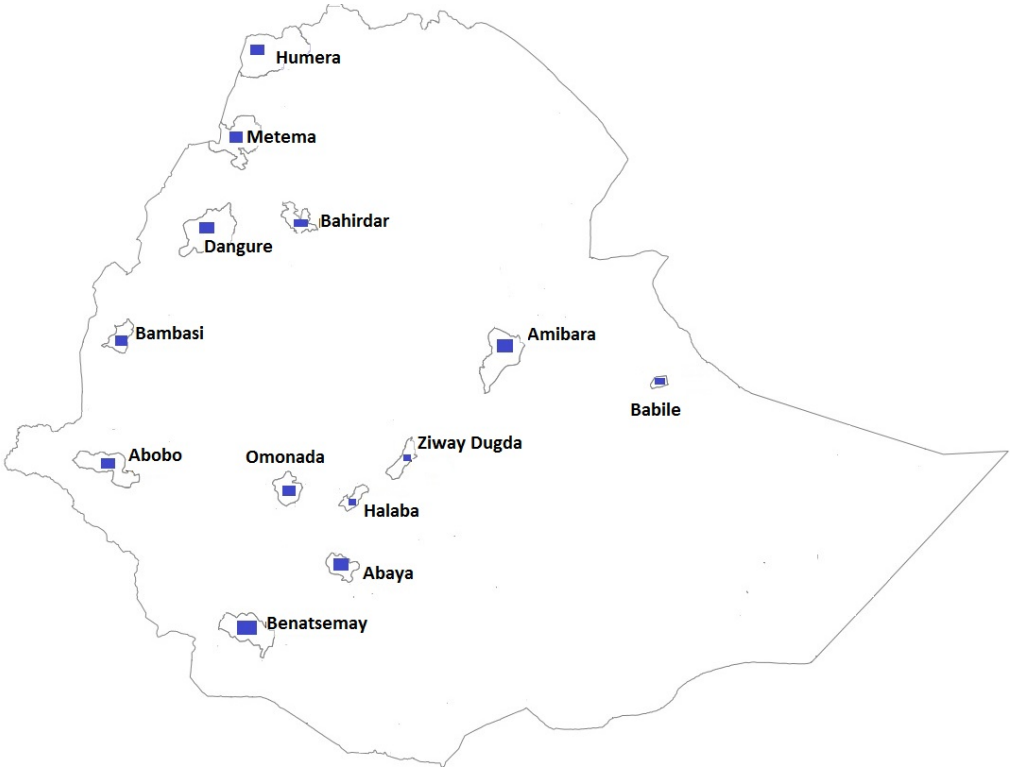
Larvae and pupae were sampled from various breeding sites and raised to adults. After identification to species, 3–5-day-old, non-blood fed *An. gambiae* s.l., females were exposed to each insecticide concentration for one hour, after which the number of knocked-down mosquitoes was recorded; the mosquitoes were kept for a 24-hour holding period by maintaining the microenvironment at a temperature of 25±2°C and relative humidity of 75±20%. After the 24 hours, dead and alive mosquitoes

were counted and the percent mortality was determined. Ten percent of the dead mosquitoes and 40% of the surviving ones were dry-preserved for molecular examination.

Each insecticide was tested against 100 mosquitoes in four replicates except in one sentinel site (Babile in Oromia), where only 75 mosquitoes were tested for each insecticide. The number of mosquitoes for controls (exposed to oil-impregnated papers) was 50 per site. When control mortality was between 5-20%, Abbott's formula was applied.

Vector population susceptibility/resistance status was classified using WHO criteria as: >98% mortality considered as full susceptibility; 97-90% possible resistance, and <90% resistance.

FIGURE 2: INSECTICIDE RESISTANCE MONITORING SITES, 2018



2.2.2 INTENSITY ASSAYS

Populations of *An. gambiae* s.l. that showed resistance to deltamethrin and permethrin were further investigated to elucidate the level of resistance intensity. Intensity assays were conducted by exposing wild mosquitoes (raised from larvae) to insecticide dosages of 5x and 10x, the diagnostic concentrations of permethrin and deltamethrin, according to the standard WHO tube test method. All exposures were for one hour, and final mortality was recorded after a 24-hour holding period during which a 10% sugar solution was made available to surviving mosquitoes. Test sites were Amibara, Ziway-Dugda, Abaya, Bambasi, Halaba, and Metema. Four sites Amibara, Ziway-Dugda, Abaya, and Halaba were planned for this activity. However, the VectorLink entomology team found adequate number of mosquitoes during the field work and included two additional sites Bambasi, and Metema to the test. One hundred 3–5-day-old female mosquitoes (in four replicates) were tested against each concentration of each insecticide. Test conditions were similar to that of diagnostic concentration tests. The results were interpreted according to WHO criteria as follows: mortality >98% with 5x: low resistance; <98% mortality with 5x: moderate or high resistance; >98% mortality with 10x: moderate resistance; and <98% mortality with 10x: high resistance.

2.2.3 SYNERGIST ASSAYS

To assess the involvement of P450s as the resistance mechanism in the observed phenotypic resistance of *An. gambiae* s.l. to deltamethrin and permethrin, synergist assays with piperonyl butoxide (PBO) were conducted on populations from Amibara, Ziway-Dugda, Abaya, Bambasi, Halaba, and Metema. To test a single insecticide from each site, three replicates, each replicate containing 100 mosquitoes were used (3 x100=300 mosquitoes). In each replicate, four tubes each containing 25 mosquitoes were assigned for PBO only, PBO plus insecticide, insecticide only and control (oil). This means a total of 600 mosquitoes to test deltamethrin and permethrin with and without PBO. Knocked-down mosquitoes at the end of a one-hour exposure period and mortality after 24 hours were recorded.

2.2.4 CLOTHIANIDIN SUSCEPTIBILITY TESTS

Clothianidin susceptibility tests were conducted on wild *An. gambiae* s.l. (raised from field-collected larvae and pupae) from Amibara, Ziway-Dugda, Abaya, Omonada, and Halaba. Insectary *An. arabiensis* was used as a positive control. Filter papers freshly treated with Sumishield and water (for controls) according to the AIRS protocol were inserted into plastic cylinders and tested according to standard WHO susceptibility test protocols. For each site, four replicates of 25 test mosquitoes and two control replicates were used. Knocked-down mosquitoes were counted at the end of 60 minute exposure. The number of dead and alive mosquitoes was recorded on days 1, 2, 3, 4, 5, and 6, and final mortality on day 7 after exposure. A negative control was tested at the same time and mortality recorded on days 1 through 7. Temperature and relative humidity were maintained at 25±2°C and 75±20%, respectively, as recommended by WHO (2016).

2.2.5 CHLORFENAPYR TEST

For chlorfenapyr susceptibility tests, freshly treated 250-ml Wheaton bottles using CDC-bottle bioassay according to AIRS protocol were used. The exposure time was 60 minutes. Afterwards, mosquitoes were transferred into holding mosquito cages and provided with lightly moistened cotton wool containing 10% sugar solution that was changed daily. The number of knocked-down mosquitoes was recorded after 60 minutes. Mortality was recorded on days 1, 2, and final mortality on day 3 after exposure. Mosquitoes exposed to filter paper treated with acetone alone was used as negative control.

The test and holding temperature was maintained at 27-29°C and relative humidity at 75%±20%. The tests were done on *An. gambiae* s.l. from Ziway-Dugda, Abaya, Omonada, and Halaba; the insectary colony of *An. arabiensis* was used as a positive control.

2.3 MOLECULAR DETECTION OF *KDR* ALLELES

Following the bioassays, 392 surviving and dead mosquitoes were randomly selected from each test site and insecticide-tested for molecular species identification of the *gambiae* complex and *kdr* allele detection. DNA extraction, molecular identification of mosquito specimens, and detection of *kdr* mutation was done at the Molecular Biology Laboratory at the Tropical and Infectious Diseases Research Center of Jimma University. Genomic DNA from the sub-samples of both surviving and dead mosquitoes was extracted following the procedure described in Collins et al. (1987). DNA was re-suspended in 25 ml sterile TE-buffer (10 mM Tris–HCl pH 8, 1 mM EDTA).The protocol used for the detection of the West African *kdr* (L1014F) and East African *kdr* (L1014S) alleles by allele-specific polymerase chain reaction assay (AS-PCR) was adapted from established protocols (Martinez-Torres et al. 1998; Ranson et al. 2000).

2.4 DECAY RATE EVALUATION

The WHO cone wall bioassays and fumigation bioassays were conducted in four spray sites (Lare, Bambasi, Goro, and Abaya) using a susceptible insectary colony of *An. arabiensis* and also *An. gambiae* s.l. (when available) raised from wild collected larvae and pupae. Twelve houses were randomly selected from each site, representing wall surfaces that were common in the area. In Gambela, all houses had mud wall surfaces. In Bambasi and Abaya, wall surfaces were mud and paint. In Goro, there were three surface types: dung, mud, and paint.

Three cones were fixed at the height of 0.5, 1.0, and 1.5 meters in sprayed houses. Ten mosquitoes were introduced to test cones and exposed for 30 minutes. A similar number of mosquitoes was also exposed to an unsprayed house as a control. Knocked-down mosquitoes were counted at the end of 30 and 60 minutes. At the end of exposure, mosquitoes were transferred to paper cups and kept for 24 hours at 25±2°C temperature and 75±20% relative humidity.

To measure the fumigant effect of Actellic 300 CS, 10 mosquitoes in a cage were placed in a room at a height of 1.0 meter from the floor and at a distance of 10 centimeters from the wall and tested in parallel to the cone bioassays. At the end of the exposure time, mosquitoes were transferred into clean paper cups. Mosquitoes were handled in a similar manner to that of the cone bio-assayed mosquitoes.

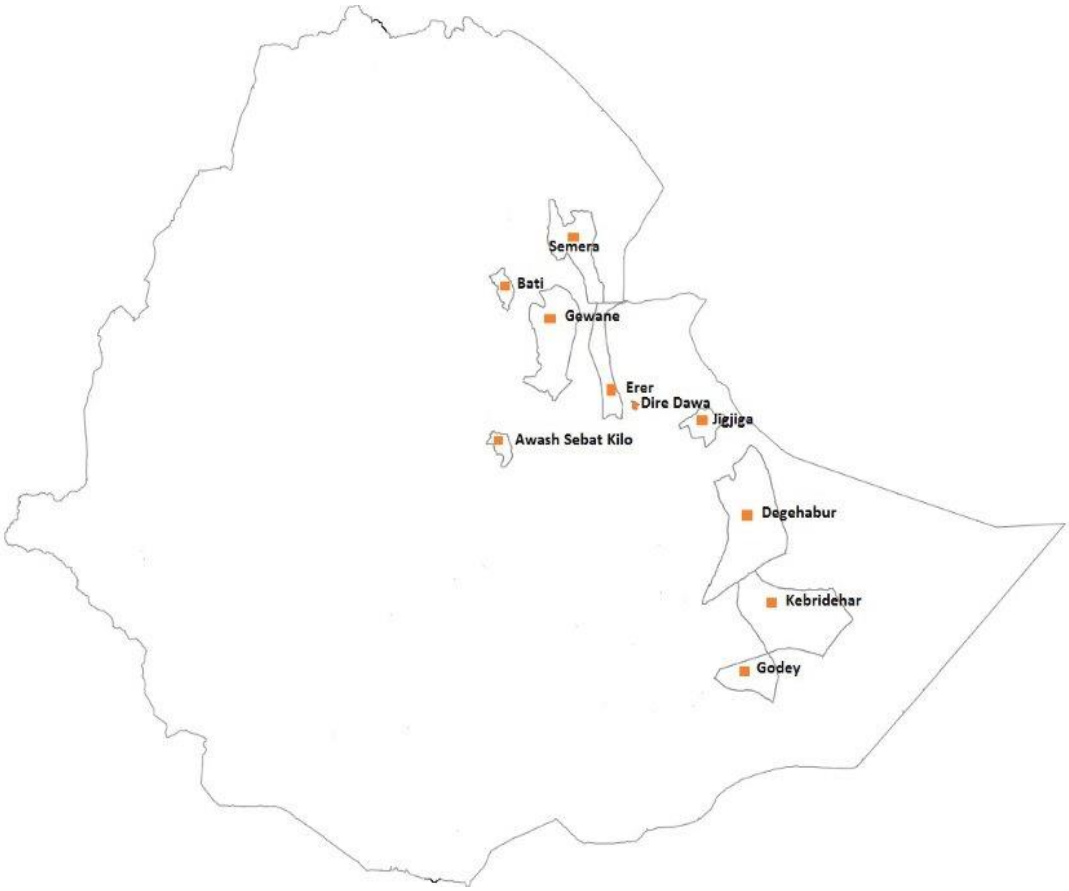
2.5 SURVEY AND INSECTICIDE SUSCEPTIBILITY STATUS OF *AN. STEPHENSI*

In 2016, Carter and colleagues (2018) reported the presence of *An. stephensi* from Kebridehar, a town in Somali Region of eastern Ethiopia. The finding led to further investigation of the geographical distribution of this species in the eastern and northeastern parts of the country. Accordingly, PMI VectorLink Ethiopia in coordination with Dire Dawa and Jigjiga Universities conducted entomological surveys from August to November 2018 in 10 urban localities. In addition, insecticide susceptibility tests were performed in two of the 10 sites to understand the level of resistance of the populations of *An. stephensi* to various insecticides.

2.5.1 *AN. STEPHENSI* SURVEY AREAS

The entomological survey investigated the presence and distribution of *An. stephensi* in 10 urban sites in eastern and northeastern Ethiopia: Jigjiga, Degehabur, Kebridehar, Godey, Erer, Semera, Gewane, Awash Sebat Kilo, Bati and Dire Dawa city (Figure 3). The sites were selected because they are on or near the main roads that connect Ethiopia and Djibouti.

FIGURE 3: *AN. STEPHENSI* SURVEY SITES, 2018



Adult sampling methods in each site included HLCs from five houses for five nights, PSCs from 30 houses, and CDC light trap collections from 20 houses per month in each sites. To identify the preferred breeding habitats of *An. stephensi,* collectors looked for immature mosquitoes in water in manmade containers and natural pools. Larvae and pupae were collected and reared to adults in order to identify *An. stephensi* from *An. gambiae* s.l. and other *Anopheles* species. All specimens identified as *An. stephensi* were preserved on silica gel for molecular species identification and blood meal sources.

2.5.2 INSECTICIDE SUSCEPTIBILITY TESTS OF *AN. STEPHENSI*

Populations of *An. stephensi* from Dire Dawa and Kebridehar were tested against bendiocarb, propoxur, pirimiphos-methyl, deltamethrin, permethrin, and alpha-cypermethrin. Synergist bioassays with PBO were done for deltamethrin and permethrin, as described above.

3. TRAINING ON *AN. STEPHENSI* IDENTIFICATION

As part of local human capacity development, PMI VectorLink Ethiopia held a training workshop on sampling and identification of *An. stephensi* in Adama in July 2018. The eight workshop participants were from Dire Dawa and Jigjiga Universities, Oromia Public Health Research Capacity Building and Quality Assurance Laboratory, and PMI VectorLink Ethiopia itself. After the training, two staff from the two aforementioned universities led *An. stephensi* surveys in Dire Dawa, Erer, Jigjiga, Kebridehar, Degehabur, and Godey. In addition, they conducted investigations on insecticide susceptibility of the *An. stephensi* populations of Dire Dawa and Kebridehar.

4. RESULTS

4.1 LONGITUDINAL MONITORING

4.1.1 *ANOPHELES* SPECIES COMPOSITION AND DIVERSITY

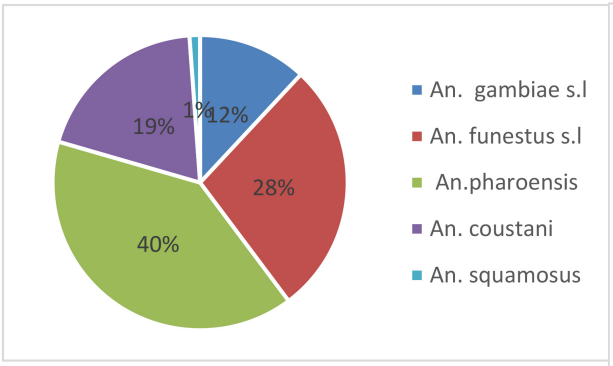
A total of 4,365 *Anopheles* (2,878 from Lare, 1,158 from Bambasi, and 329 from Abaya) were collected using the three collection methods (HLCs, PSCs, and CDC light traps) described in Section 2. The *Anopheles* comprised at least five species: *An. gambiae* s.l., *An. funestus* s.l., *An. pharoensis, An. coustani,* and *An. squamosus*. All five species co-existed in Lare and Bambasi. Three species, *An. gambiae* s.l., *An. pharoensis,* and *An. coustani,* were found in Abaya (Figure 4, Annex A).

In Ethiopia, *An. gambiae* s.l. is represented by *An. arabiensis* and *An. amharicus* (Coetzee et al. 2013) and the former species is the principal malaria vector. The occurrence of this species together with the secondary vectors, *An. funestus* s.l. and *An. pharoensis,* in Lare and Bambasi is indicative of the complex vectorial system in the two regions in terms of seasonality, abundance, infection, and behavior.

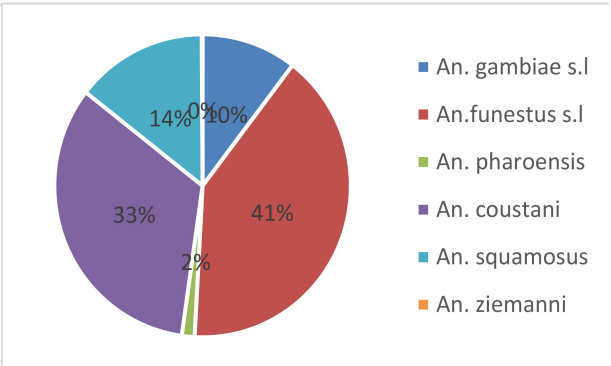
There was variability in the proportions of *Anopheles* mosquitoes found in the three sites. In Lare, the most abundant was *An. pharoensis* (40%) followed by *An. funestus* s.l. (28%) and *An. coustani* (19%). *An. gambiae* s.l. was 12% of all collections in Lare. In Bambasi, the proportion of *An. funestus* s.l., *An. gambiae* s.l., and *An. pharoensis* was 41%, 10%, and 2%, respectively. In Abaya, *An. gambiae* s.l. was the predominant species and comprised 58%, while *An. pharoensis* represented 41% of all collections (Figure 4).

FIGURE 4. *ANOPHELES* SPECIES COMPOSITION IN LARE, BAMBASI, AND ABAYA FROM HLCS, PSCS, AND CDC LIGHT TRAPS, MAY 2018-APRIL 2019

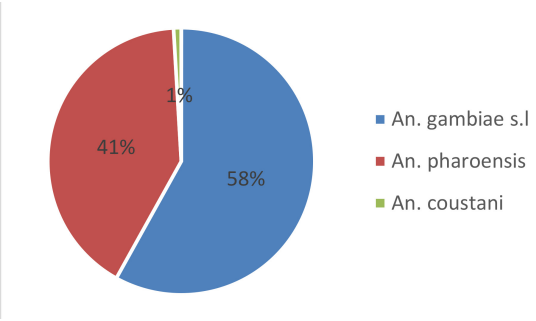
Lare, Gambela



Bambasi, Benishangul-Gumuz



Abaya, Oromia



4.1.2 *ANOPHELES* ABUNDANCE BY METHOD OF COLLECTION

In Lare, the HLC, CDC light trap, and PSC collections yielded 73.8%, 22.5%, and 3.7% of *Anopheles,* respectively (Table 2). In Bambasi, CDC light traps caught the highest proportion (75.7%); catches from HLC and PSC were 15.4% and 8.9%, respectively. In Abaya, 77.3% were from HLCs; CDC light traps sampled only 14.3% and PSCs even less, 8.4%.

In Lare, the majority (66.7-85.8%) of *An. gambiae* s.l., *An. funestus* s.l., and *An. pharoensis* together were caught by HLC (Table 2), whereas in Bambasi, CDC light traps caught more than HLCs and PSCs. Similar to Lare, more than 75% of *An. gambiae* s.l. and *An. pharoensis* collections in Abaya were from HLCs. Only 74 *An. gambiae* s.l. were sampled from the three sites using PSCs.

The small number of mosquitoes collected using PSCs compared with HLCs and CDC light traps might be attributable to: either the insecticide had a lethal effect on the population of mosquitoes entering/resting in houses, or, because of inherent characteristics of the vectors in country, the majority of mosquitoes might rested outdoors. The other possible reason could be related to avoidance of treated surfaces by the vectors because of the development of behavioural resistance.

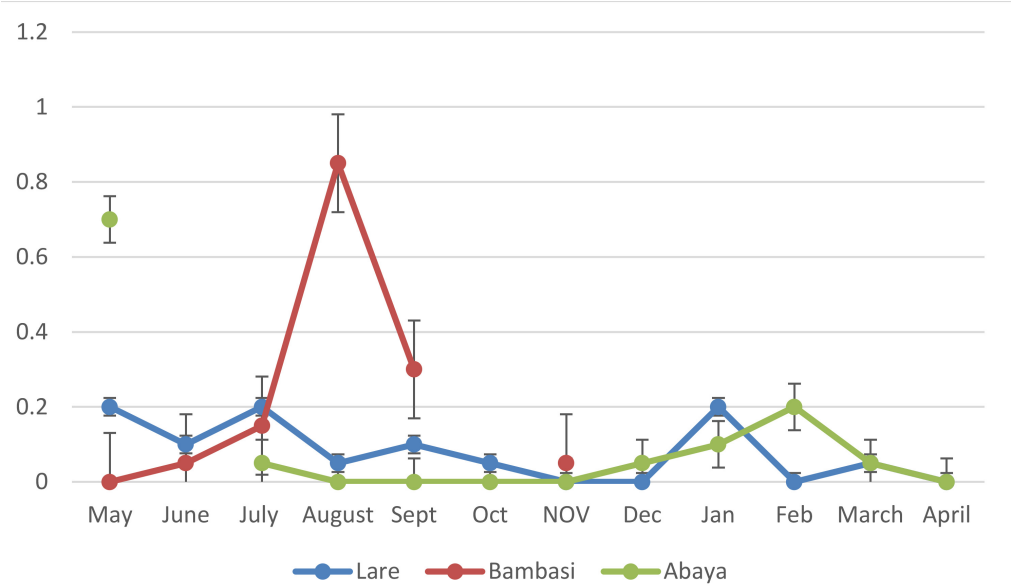
TABLE 2. PROPORTION OF *ANOPHELES* COLLECTED BY HLC, CDC LIGHT TRAPS, AND PSC FROM LARE, BAMBASI, AND ABAYA, MAY 2018-APRIL 2019

Species		Lare				Bambasi				Abaya			
		HLC	CDC	PSC	Total	HLC	CDC	PSC	Total	HLC	CDC	PSC	Total
***An. gambiae* s.l.**	#	241	48	19 (6.2)	308	31	61	28	120	142	18 (9.6)	27	187
	%	(78.2)	(15.6)		(100)	(25.9)	(50.8)	(23.3)	(100)	(75.9)		(14.5)	(100)
***An. funestus* s.l.**	#	748	161	80 (8.1)	989	12 (2.6)	387	70	469	-	-	-	-
	%	(75.6)	(16.3)		(100)		(82.5)	(14.9)	(100)				
An. pharoensis	#	877	142	4 (0.4)	1023	1 (6.3)	14	1 (6.2)	16 (100)	104	28	0 (0)	132
	%	(85.8)	(13.8)		(100)		(87.5)			(78.8)	(21.2)		(100)
An. coustani	#	254	272	2 (0.4)	528	90	293	4 (0.8)	387	10	0	0	10 (100)
	%	(48.1)	(51.5)		(100)	(23.3)	(75.9)		(100)	(100)			
An. squamosus	#	11	19	0 (0)	30 (100)	44	121	1 (0.6)	166	-	-	-	-
	%	(36.7)	(63.3)			(26.5)	(72.9)		(100)				
Total	#	2131	642	105	2878	178	877	104	1158	249	46	27 (8.4)	*329*
	%	(74.0)	(22.3)	(3.7)	(100)	(15.4)	(75.7)	(8.9)	(100)	(77.3)	(14.3)		(100)

4.1.3 INDOOR RESTING DENSITY OF *AN. GAMBIAE* S.L.

In general, the mean indoor resting density of *An. gambiae* s.l. in all areas was low, on average less than one *An. gambiae* s.l./house/day (Figure 5). The indoor resting densities peaked in May (0.7 *An. gambiae* s.l./house/day) in Abaya, in August (0.85 *An. gambiae* s.l./house/day) in Bambasi, and in March (0.5 *An. gambiae* s.l./house/day) in Lare.

FIGURE 5: INDOOR RESTING DENSITY OF *AN. GAMBIAE* S.L. IN LARE, BAMBASI AND ABAYA AS DETERMINED FROM PSC, MAY 2018-APRIL 2019



4.1.4 RATIO OF OUTDOOR TO INDOOR FEEDING OF *AN. GAMBIAE* S.L., *AN. FUNESTUS* S.L., AND *AN. PHAROENSIS*

Based on indoor and outdoor night biting collections, the ratio of exophagy to endophagy of *An. gambiae* s.l., *An. funestus* s.l., and *An. pharoensis* in Lare and *An. gambiae* s.l. and *An. pharoensis* in Abaya was determined. A ratio could not be determined in Bambasi because so few of the three species were collected.

In Lare and Abaya, more of the three species were collected outdoors than indoors (Table 3). In Lare, outdoor collections of *An. gambiae* s.l. and *An. funestus* s.l. were three times that of the indoor collections. *An. pharoensis* also showed the tendency to feed outdoors, albeit at a lesser frequency. In Abaya, the number of *An. gambiae* s.l. and *An. pharoensis* caught outdoors was double that caught indoors.

TABLE 3: RATIO OF EXOPHAGY TO ENDOPHAGY OF *AN. GAMBIAE* S.L., *AN. FUNESTUS* S.L., AND *AN. PHAROENSIS* IN LARE AND ABAYA, MAY 2018-APRIL 2019

Species	Lare			Abaya		
	Indoor	Outdoor	Out/indoor	Indoor	Outdoor	Out/indoor
An. gambiae s.l.	52	189	3.6:1	46	96	2.1:1
An. funestus s.l.	179	569	3.2:1	-	-	-
An. pharoensis	316	561	1.8:1	29	72	2.5:1

4.1.5 MONTHLY NIGHT BITING DENSITIES OF *ANOPHELES*

This section presents data on the monthly mean night biting densities of *An. gambiae* s.l. from the three sites and of *An. funestus* s.l. and *An. pharoensis* from Lare. Night-time biting collections of *An. funestus* s.l. from Bambasi and *An. pharoensis* from Abaya were too small to show on a graph.

4.1.5.1 BITING DENSITIES OF *AN. GAMBIAE* S.L., *AN. FUNESTUS* S.L., AND *AN. PHAROENSIS* IN LARE

An. gambiae s.l. in Lare prevailed throughout the year, with mean seasonal biting density fluctuating from low to high in some months, due to vector control and climatological/environmental factors.

In Lare, the peak mean biting densities of *An. gambiae* s.l. were in May, January, and February (Figure 6) both indoor and outdoor. In addition, the biting density of *An. gambiae* s.l. outdoors was higher than indoors in most months ($P<0.001$). *An. gambiae* s.l. was present in outdoor collections only in October, November, March, and April. The reduced density from October through December could be due to the small number of temporary breeding habitats, which corresponds to the reduced density observed in adult *An. gambiae* s.l. in those months. The greatest outdoor density was in February when it was almost 9.0 *An. gambiae* s.l. bites /person/night followed by nearly 5.0 *An. gambiae* s.l./person/night in January. In general, the mean biting density indoors was very low; it peaked between 1.5 and 3.0 *An. gambiae* s.l. bites/person/night in August, January, and February.

An. funestus s.l. in Lare predominantly occurred from November to January (Figure 7). This may be due to rains expanding swamplands that supported the breeding of *An. funestus* s.l. from November to January, allowing for more mean adult densities during those months. *An. funestus* s.l. and *An. gambiae* s.l. were both present in October but a clear shift to *An. funestus* s.l. was observed in November and December. Although both again were present in January, the biting density of *An. funestus* s.l. indoors (2.7 *An. funestus* s.l./person/night) and outdoors (8.8 *An. funestus* s.l./person/night) was more than *An. gambiae* s.l. (2.0 and 4.8 *An. gambiae* s.l. bites/person/night, respectively). Similar to *An. gambiae* s.l., the outdoor biting density was more than the indoor density, and it was significantly higher in November and December ($P<0.001$).

The seasonal prevalence of *An. funestus* s.l. between November and January on top of its greater outdoor biting and probably also resting activities in Lare implies that the IRS operation in Lare in May had little if any impact on the population of this vector after November, warranting additional vector control interventions like distribution of LLINs to keep the transmission low during the dry season.

An. pharoensis was seasonally present in Lare from June to December, with main mean human biting density peaks in August and September when breeding habitats were available (Figure 8). The population disappeared completely from February to April. This species shared similar outdoor human biting tendencies with *An. gambiae* s.l. and *An. funestus* s.l. Its biting densities in June, August, and September were significantly greater outdoors than indoors ($P<0.001$). In August, the respective outdoor and indoor densities were 44.5 and 25.3 *An. pharoensis* bites /person/night and in September they were 26.2 and 14.7 *An. pharoensis* bites/person/night.

FIGURE 6: MONTHLY BITING DENSITY OF *AN. GAMBIAE* S.L.IN LARE, MAY 2018-APRIL 2019

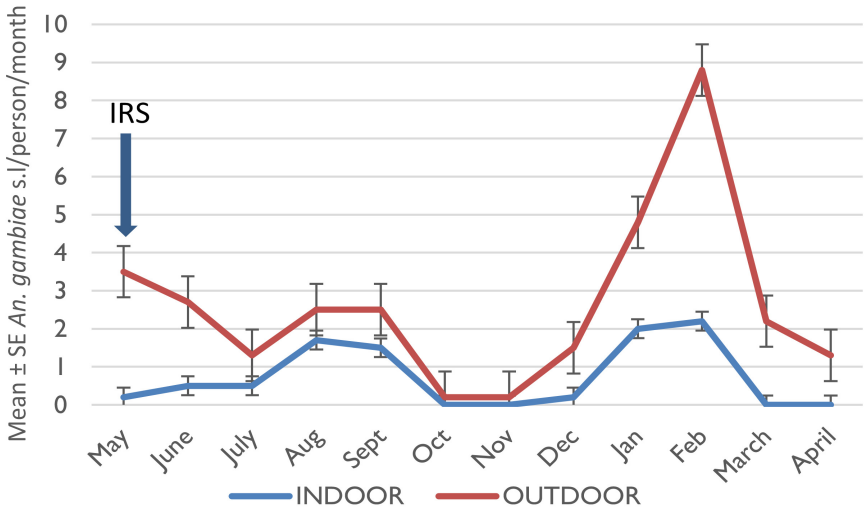


FIGURE 7: MONTHLY BITING DENSITY OF *AN. FUNESTUS* S.L. IN LARE, MAY 2018-APRIL 2019

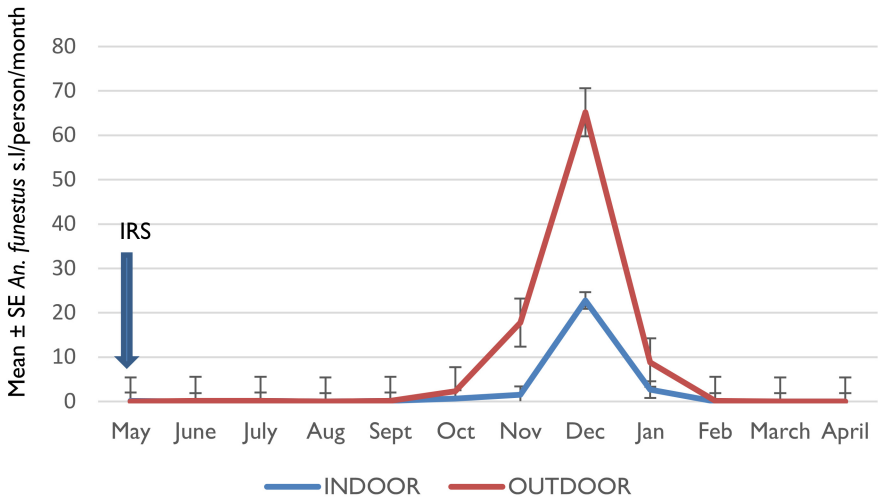
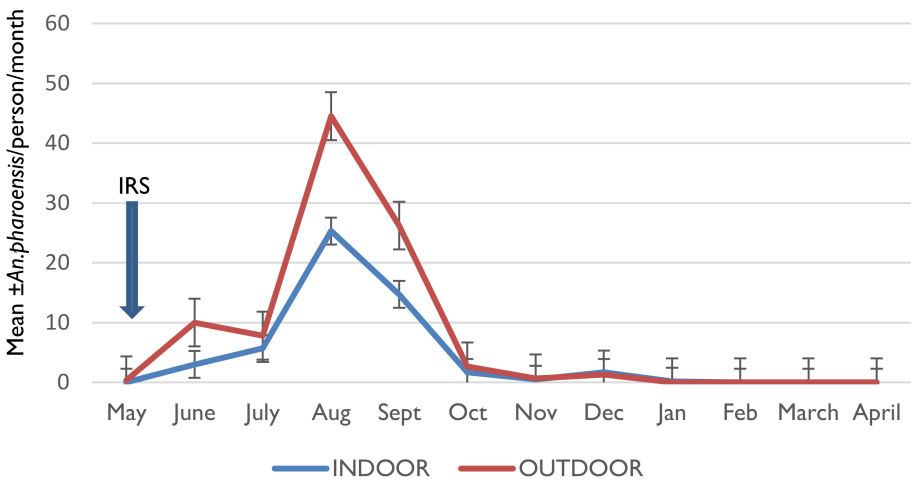


FIGURE 8: MONTHLY BITING DENSITY OF *AN. PHAROENSIS* IN LARE, MAY 2018-APRIL 2019



4.1.5.2 BITING DENSITITES OF *AN. GAMBIAE* S.L. IN BAMABASI AND ABAYA

In Bambasi, mosquito collections between May and September and in November revealed the short seasonality of *An. gambiae* s.l., from June and September, probably extending to October (Figure 9). *An. gambiae* s.l. was completely absent from HLC collections in May and November. In general, the night biting density of the vector population was low, ranging from 0 to 1.2 *An. gambiae* s.l. bites/person/night. In June, *An. gambiae* s.l. was collected from indoors only; in all other months, the outdoor collection was greater than the indoor (P<0.001). The outdoor biting densities in July, August, and September were 1.0, 1.2, and 1.2, respectively, whereas the indoor biting densities were 0.5, 0.8, and 0.3 *An. gambiae* s.l. bites/person/night.

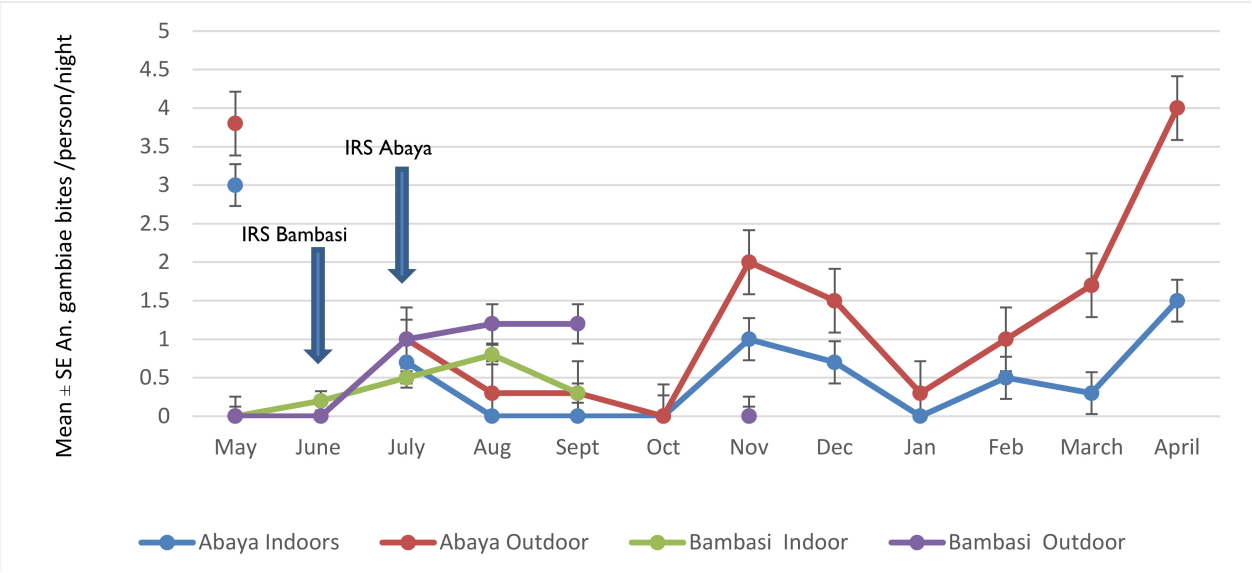
Mosquito sampling in Abaya was conducted for 11 months, and *An. gambiae* s.l. appeared in all months except October. During the 11 months, a total of 187 *An. gambiae* s.l. were collected, 75.9% of them from HLCs. Because of the small number from HLCs, the biting density in general was low when averaged over the 10 months. In Abaya, rain and water harvesting pools as well as small streams form the main breeding sites for *An. gambiae* s.l.

The mean human biting density of *An. gambiae* s.l. peaked in May, November, December, March, and April but it was very low from August to October probably because of the impact of IRS operation in July. The mosquitoes, specifically those indoors, seem to be more affected (Figure 9). The other likely reason for the reduction in density might be seasonality of the vector population. The area is characterized by two rainy seasons: February to April and September to November. The latter is considered a short rainy season; it may account for the creation of favorable breeding grounds and an increase in adult density in November and December. There was no rain from December through March; however, the water harvesting pools and streams might have supported breeding of larvae and consequent low densities of adults in those months. The adult increase in April might be attributed to the permanence of water harvesting pools and also rain pools since rain fell during that month.

As in Lare and Bambasi, the outdoor densities in Abaya were higher than the indoor ones, and the differences were significant in May, November, December, March, and April (P<0.001). The vector was caught only outdoors in August, September, and January. The outdoor peaks in May (3.8 *An. gambiae* s.l.

bites/person/night) and April (4.0 *An. gambiae* s.l. bites/person/night) were the highest of all months. Similarly the indoor densities in those months were 3.0 and 1.5 *An. gambiae* s.l bites./person/night.

FIGURE 9: *AN. GAMBIAE* S.L. MONTHLY BITING DENSITY IN BAMBASI AND ABAYA, MAY 2018-APRIL 2019



4.1.6 BITING PATTERN OF *ANOPHELES*

This section dealt on the night biting pattern of *An. gambiae* s.l., *An. funestus* s.l., and *An. pharoensis* from Lare and *An. gambiae* s.l. from Abaya and Bambasi.

4.1.6.1 BITING PATTERN OF *AN. GAMBIAE* S.L., *AN. FUNESTUS* S.L., AND *AN. PHAROENSIS* IN LARE

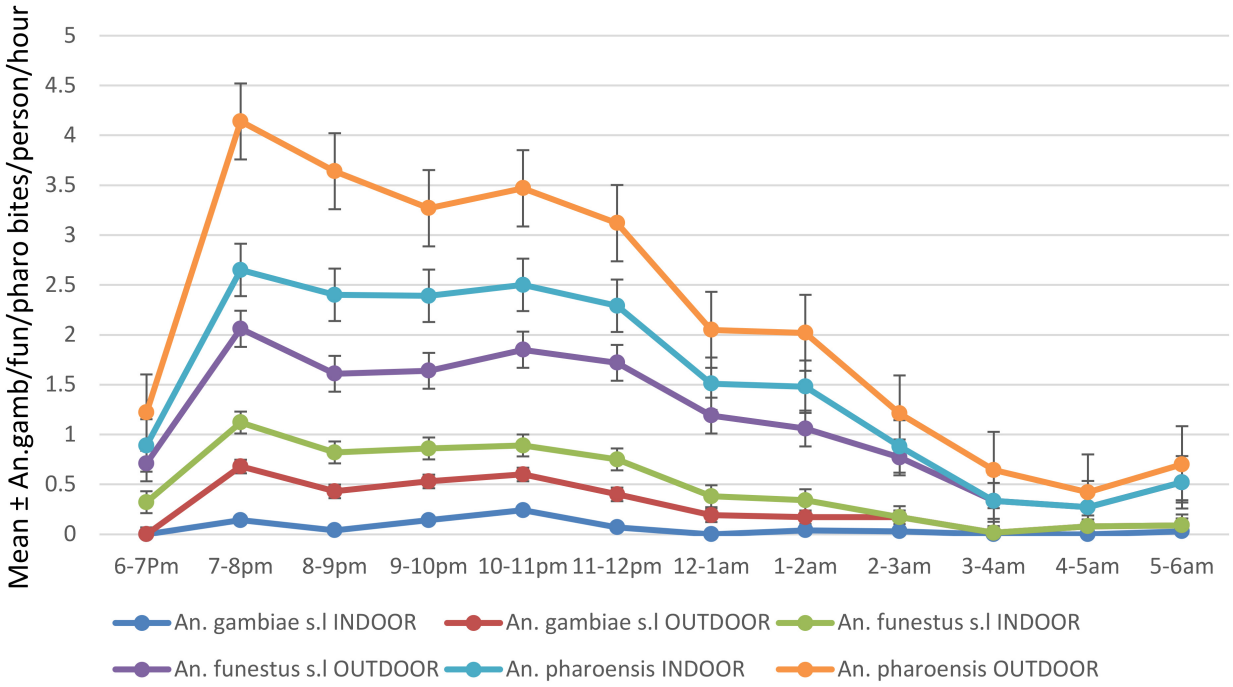
In Lare, *An. gambiae* s.l. actively searched indoors for human hosts from dusk to dawn. There were two indoor mean biting peaks, from 7:00 and 8:00 pm (0.14 *An. gambiae* s.l. bites/person/hour) and 10:00 and 11:00 pm (0.24 *An. gambiae* s.l bites./person/hour); the latter was the highest of all indoor mean densities. During the night, there were three breaks, from 12:00 to 1:00 am, 3:00 to 4:00 am, and 4:00 to 5:00 am, when no mosquitoes were collected (Figure 10). The outdoor mean human biting pattern was completely different: there was one high peak between 7:00 and 8:00 pm (0.5 *An. gambiae* s.l. bites /person/hour) and two lesser peaks, between 2:00 and 3:00 am and 4:00 and 5:00 am. Most biting occurred outdoors in the early hours of the evening and indoors before 11:00 pm when people were still outdoors.

Similar to *An. gambiae* s.l., the outdoor and indoor feeding patterns of *An. funestus* s.l. were clearly different (Figure 10). Early evening activity, between 6:00 and 7:00 pm, was noted both indoors and outdoors with respective hourly biting rate of 0.32 and 0.39 *An. funestus* s.l. bites/person/hour. The population exhibited three outdoor peaks, from 7:00 to 8:00 pm, 10:00 pm to 12:00 am, and 5:00 to 6:00 am; the indoor peaks were from 7:00 to 8:00 pm and 11:00 pm to 12:00 am, with hourly biting rates of 0.44 and 0.35 *An. funestus* s.l. bites/person/hour, respectively. As opposed to the outdoor biting pattern, from 6:00 pm to 6:00 am, *An. funestus* s.l. was consistently absent from indoors from 2:00 am to 6:00 am throughout the study period.

Past research studies in central Ethiopia (Abose et al. 1998; Kenea et al. 2016) established the natural outdoor biting behavior of *An. pharoensis*. The present study provides additional evidence from western

Ethiopia (Figure 10). Most of the biting activities in Lare took place outdoors before 10:00 pm, with one peak between 7:00 and 8:00 pm, when the hourly density was 1.5 *An. pharoensis* bites/person/hour. The indoor biting activities were limited, from 6:00 pm to 3:00 am only.

FIGURE 10: HOURLY BITING PATTERN OF *AN. GAMBIAE* S.L., *AN. FUNESTUS* S.L., AND *AN. PHAROENSIS* IN LARE, MAY 2018-APRIL 2019

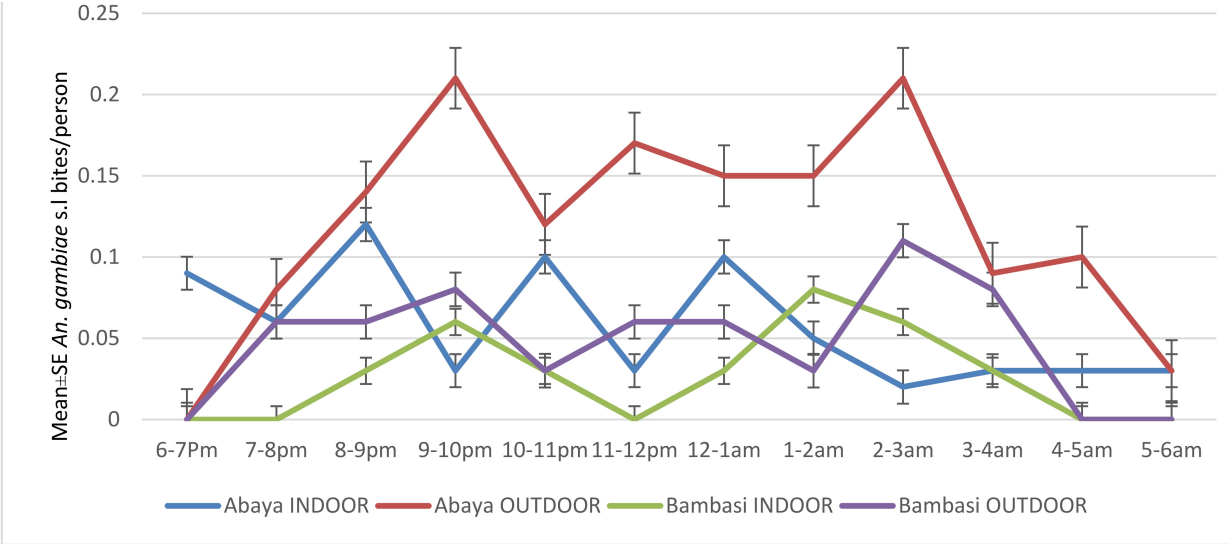


4.1.6.2 BITING PATTERN OF *AN. GAMBIAE* S.L. IN BAMBASI AND ABAYA

Unlike in Lare and Abaya, the mean hourly biting rates of *An. gambiae* s.l. in Bambasi did not show a distinct pattern between outdoor and indoor biting. This might be due to the small number of mosquitoes (N=31) collected from the site (Figure 11). The hourly night biting density was very low, remaining below 0.08 *An. gambiae* s.l. bites/person/hour except outdoors between 2:00 and 3:00 am, when the rate was 0.11 *An. gambiae* s.l. bites/person/hour. Four outdoor and two indoor peaks occurred between 6:00 pm and 4:00 am. There was no biting activity outdoors or indoors between 4:00 and 6:00 am.

In Abaya, biting activities of *An. gambiae* s.l. started early in the evening indoors; outdoors, the biting activities increased gradually, surpassing the indoor rates between 7:00 and 8:00 pm, and they remained high through the rest of the night and morning (Figure 11). There were four outdoor and three indoor peaks. The two highest outdoor peaks, both 0.21 *An. gambiae* s.l. bites/person/hour, were from 9:00 to 10:00 pm and from 2:00 to 3:00 am. The indoor peaks were 8:00 to 9:00 pm, 10:00 to 11:00 pm, and 12:00 to 1:00 am, at the hourly biting rates of 0.12, 0.1 and 0.1 *An. gambiae* s.l. bites/person/hour, respectively.

FIGURE 11: BITING PATTERN OF *AN. GAMBIAE* S.L. IN BAMBASI AND ABAYA, MAY 2018-APRIL 2019



4.1.7 DENSITY OF *AN. GAMBIAE* S.L. AND AN. FUNESTUS GROUP FROM CDC LIGHT TRAP COLLECTIONS

The density of *An. gambiae* s.l. from CDC light trap collections was very low (less than one mosquito per trap night). The highest density was from Bambasi in August and September, when nearly 0.9 and 1.0 *An. gambiae* s.l./trap/night were trapped, most likely due to the increase in breeding sites in August and September (Figure 12).

The mean peak density of *An. funestus* group in Lare was scored in November and December (2.2-2.3 *An. funestus* s.l/CDC light trap /night). In contrary, in Bambasi the densities were high from August to November although collection was interrupted in October (Figure 13). The mean densities in August, September and November were 4.4, 4.2 and 5.96 *An. funestus* s.l/CDC light trap /night.

FIGURE 12: MONTHLY DENSITY OF *AN. GAMBIAE* S.L. FROM CDC LIGHT TRAP COLLECTIONS, MAY 2018-APRIL 2019

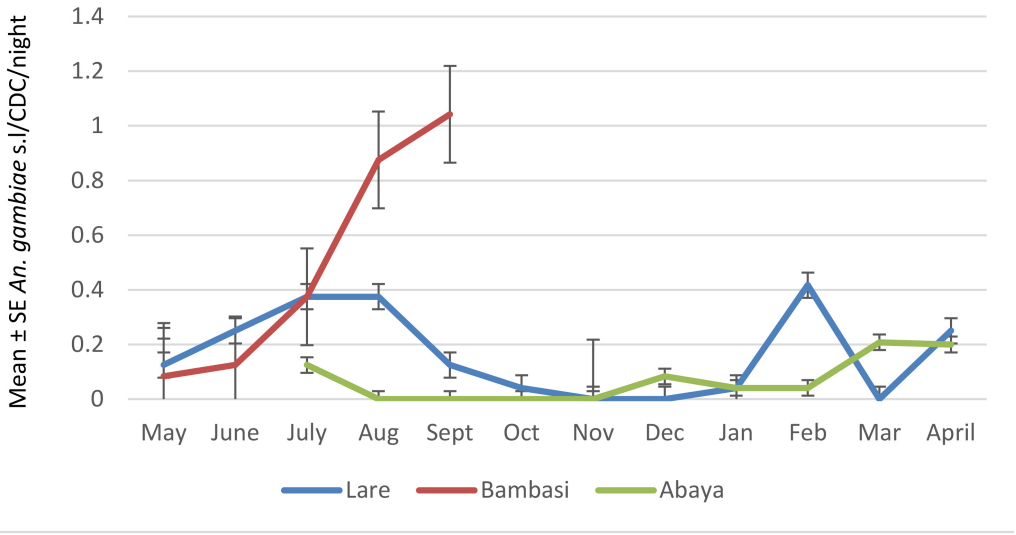
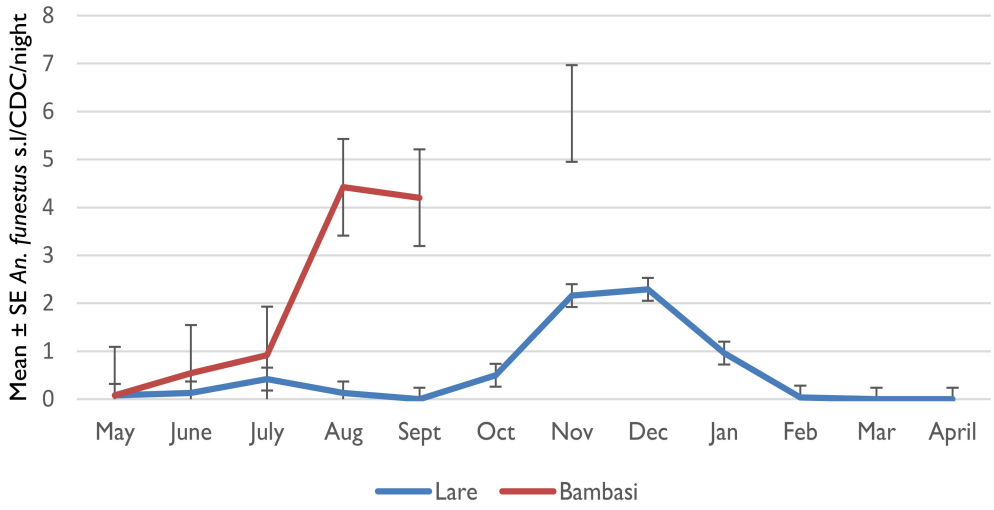


FIGURE 13: MONTHLY DENSITY OF *AN.. FUNESTUS* GROUP FROM CDC LIGHT TRAP COLLECTIONS, MAY 2018-APRIL 2019



4.2 RESULTS OF LABORATORY ANALYSIS

4.2.1 SPECIES ID

PCR was used for species identification of 154 *An. gambiae* s.l. specimens; of these, 146 (94.6%) amplified; all were identified as *An. arabiensis.* The other eight specimens did not amplify and it could be due to the low DNA quality or quantity of mosquito DNA.

TABLE 4: PCR IDENTIFIED *AN. ARABIENSIS* FROM ENTOMOLOGICAL MONITORING COLLECTIONS

Site	# *An. gambiae* s.l. tested	# *An. arabiensis* identified
Abaya	60	57
Bambasi	40	38
Gambela	54	51
Total	154	146

Of 37 specimens of the *An. funestus* group from Bambasi that were PCR tested, 34 were *An. rivulorum* and two were *An. funestus* s.s. The remaining one specimen failed to amplify. The majority (N=35) of the specimens were collections from CDC light traps while the remaining two specimens were from HLC and PSC. The two *An. funestus* s.s. mosquitoes were identified from samples collected by CDC light traps.

4.2.2 SPOROZOITE ELISA

A total of 4,168 *Anopheles* from the three entomological monitoring sites were tested for CSP, of which 70.2% (N=2,925) were *An. gambiae* s.l., *An. funestus* s.l and *An. pharoensis,* (Table 5 and Annex G). Of 308, 121, and 157 *An. gambiae* s.l. specimens from Lare, Bambasi, and Abaya tested, two (0.65%), one (0.83%), and one (0.64%) were positive for *P. falciparum*, respectively. Similarly, of 964 *An. funestus* s.l. and 999 *An. pharoensis* specimens from Lare, one specimen from each of the species was positive for *P. falciparum* (a 0.1% infection rate each). In addition, *An. funestus* s.l. from Lare and Bambasi was found with 0.1% and 0.27% *P. vivax* infections, respectively.

The five mosquito specimens from Lare that tested positive for CSP were collected in June, July, and October 2018 and January 2019. Two positive specimens were collected in Bambasi, one in September and the other in November 2018. The remaining single positive specimen of *An. gambiae* s.l., from Abaya, was from the February 2019 collections.

Based on sampling methods (Table 6), the *P. falciparum* infection rate *in An. gambiae* s.l. collected from HLCs was 0.41% in Lare, 0.88% in Abaya, and 3.0% in Bambasi. In Lare, the *P. falciparum* infection rate in *An. gambiae* s.l. from CDC light trap collections was 2.2%, and from PSC collections of *An. funestus* s.l. was 1.3%. *An. funestus* s.l. from PSCs in Lare and CDC light traps in Bambasi were found with *P. vivax* infection rates of 1.3% and 0.32%, respectively.

TABLE 5: ELISA RESULTS OF *AN. GAMBIAE* S.L., *AN. FUNESTUS* S.L., AND *AN. PHAROENSIS* COLLECTED FROM THE THREE ENTOMOLOGICAL MONITORING SITES, MAY 2018-APRIL 2019

Site	Species	No. tested	# Positive *Plasmodium falciparum* (%)	# Positive *Plasmodium vivax* (%)
Lare	*An. gambiae* s.l.	308	2 (0.65)	
	An. funestus s.l.	964	1 (0.1)	1 (0.1)
	An. pharoensis	999	1 (0.1)	
Bambasi	*An. gambiae* s.l.	121	1 (0.83)	
	An. funestus s.l.	376		1 (0.27)
Abaya	*An. gambiae* s.l.	157	1 (0.64)	

TABLE 6: PARASITE INFECTION RATES BY TYPE OF MOSQUITO SAMPLING METHODS

Site	Species	Method of collections	No. tested	# Positive *Plasmodium falciparum* (%)	# Positive *Plasmodium vivax* (%)
Lare	*An. gambiae* s.l.	HLC	242	1 (0.41)	
	An. funestus s.l.	CDC	46	1 (2.2)	
	An. pharoensis	PSC	77	1 (1.3)	1 (1.3)
Bambasi	*An. gambiae* s.l.	HLC	860	1 (0.12)	
	An. funestus s.l..	HLC	33	1 (3.0)	
Abaya	*An. gambiae* s.l.	CDC	315		1 (0.32)

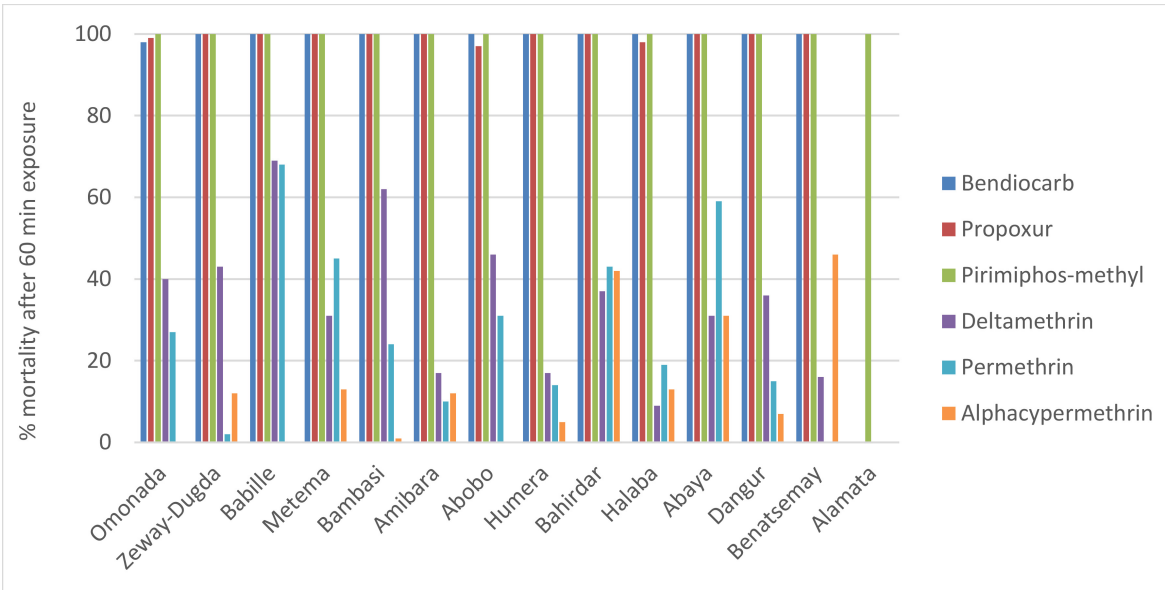
4.3 INSECTICIDE RESISTANCE MONITORING RESULTS

4.3.1 INSECTICIDE SUSCEPTIBILITY TEST RESULTS OF DISCRIMINATING DOSES

Populations of *An. gambiae* s.l. in the 13 sentinel sites were susceptible (mortality 98-100%) to bendiocarb and pirimiphos-methyl (Figure 14, Annex B). High susceptibility to propoxur was observed in 11 of the 13 sites. Possible resistance to propoxur was noted in Abobo and Abaya with 97% mortality. WHO recommends repeating the tests when mosquito mortality falls between 90% and 97%. However, it is becoming difficult to carry out this recommendation because of a shortage of mosquitoes in the field.

The 13 sites had very high deltamethrin resistance (9-69% mortality), and this was also true for permethrin in 12 sites and alpha-cypermethrin in eight sites. This low susceptibility of *An. gambiae* s.l. to the pyrethroid insecticides could be associated with cross-resistance of DDT and/or strong selection pressure from nets, and probably also from agricultural use.

FIGURE 14:: MORTALITY OF *AN. GAMBIAE* S.L. EXPOSED TO THE DIAGNOSTIC DOSE OF VARIOUS INSECTICIDES USED IN MALARIA CONTROL, JULY-DECEMBER 2018



Clothianidin susceptibility tests were conducted under two different test and holding temperature conditions, 16-22°C and 27-29°C. Mortality of *An. gambiae* s.l. tested from Ziway-Dugda and Amibara at 16-22°C was 96.7% and 86.2% respectively at the end of day 7 of the holding period (Table 7). If the WHO criterion for resistance classification is applied, these percentages are considered to show possible resistance and confirmed resistance, respectively. On the other hand, mortality of the susceptible colony of *An. arabiensis* tested simultaneously with *An. gambiae* s.l. was 97.6% and 98.3%, respectively.

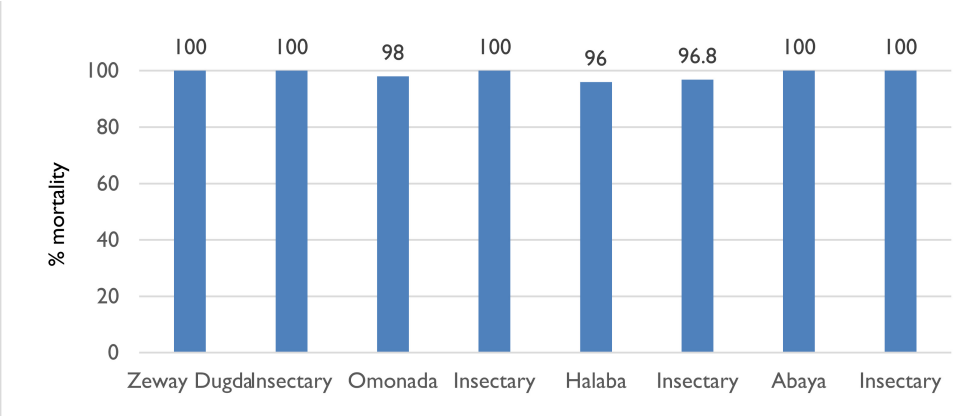
However, when the temperature was raised to 27-29°C, 100% mortality of *An. gambiae* s.l. from Abaya, Omonada, and Halaba was achieved within 3-4 days. Clothianidin caused 100% mortality to the insectary colony of *An. arabiensis* within 2-5 days. The difference in the test results obtained might be either due to the difference in the responses of the vectors from different sites or impact of temperature on mortality.

TABLE 7: CLOTHIANIDIN SUSCEPTIBILITY TEST RESULTS IN WILD AND INSECTARY MOSQUITOES FROM FIVE SENTINEL SITES, AUGUST-NOVEMBER 2018

Collection Site	Place Test Conducted	Mosquito Origin	% Mortality (cumulative)							Test Condition
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	
Ziway-Dugda	Ziway	*An. arabiensis*	40	56	75	73.3	94.3	97.7	97.6	During test in the field, temperature ranged from 16 to 22℃ and relative humidity ranged from 58% to 94%
		An. gambiae s.l.	24	29	41	62	87	95	96.7	
Amibara	Amibara	*An. arabiensis*	30	57	82.6	91.3	95	98.3	98.3	
		An. gambiae s.l.	17	39	51	74	81	83	86.2	
Abaya	Sokoru (insectary)	*An. arabiensis*	54	85	100					During test in the insectary, temperature ranged from 27 to 29℃ and relative humidity ranged from 57% to 80%
		An. gambiae s.l.	54	88	95	100				
Omonada	Sokoru (insectary)	*An. arabiensis*	52	78	92	99	100			
		An. gambiae s.l.	24	50	91	100				
Halaba	Sokoru (insectary)	*An. arabiensis*	82	100						
		An. gambiae s.l.	93	99	100					

An. gambiae s.l. was found to be susceptible to chlorfenapyr (98-100% mortality) in Zeway-Dugda, Omonada, and Abaya (Figure 15, Annex C). Parallel tests of the insectary colony of *An. arabiensis* produced 100% mortality. However 96% and 96.8% mortality of *An. gambiae* s.l. from Halaba and insectary *An. arabiensis* was recorded. The low mortality could be either due to problems in insecticide impregnation or a fall in temperature during the holding period.

FIGURE 15: MORTALITY OF *AN. GAMBIAE* S.L. AND INSECTARY *AN. ARABIENSIS* AFTER 72-HOUR HOLDING PERIOD OF CDC BOTTLE BIOASSAY OF CHLORFENAPYR TESTS AT A CONCENTRATION OF 100µG/BOTTLE, NOVEMBER 2018



4.3.2 INSECTICIDE RESISTANCE INTENSITY AND PBO ASSAYS

An. gambiae s.l. in Ziway-Dugda and Halaba exhibited low resistance intensity to deltamethrin (>98% mortality at 5X the diagnostic dose) (Figure 16, Annex D). Moderate resistance to deltamethrin was found in Metema, Bamabasi, and Amibara (>98% mortality at 10X), while in Abaya, there was high deltamethrin resistance intensity (97% mortality at 10X). Low permethrin intensity was recorded in Abaya, moderate intensity in Amibara. Permethrin at the 10X diagnostic dose killed less than 98% of *An. gambiae* s.l. in Zeway-Dugda, Metema, Bambasi, and Halaba, implying high resistance intensity (Figure 17, Annex D).

Pre-exposure PBO restored susceptibility of *An. gambiae* s.l. to deltamethrin in Ziway-Dugda (98.7% mortality), Metema (100% mortality), Amibara (100% mortality), and Halaba (100% mortality) and to permethrin in Metema (98.7% mortality), Amibara (100% mortality), and Halaba (98.7% mortality).

Pre-exposure to PBO partially restored susceptibility to deltamethrin in Bambasi (97.3% mortality) and Abaya (94.7% mortality) and to permethrin in Ziway-Dugda (45.3% mortality), Bambasi (66.7% mortality), and Halaba (93.3% mortality).

FIGURE 16: MORTALITY OF *AN. GAMBIAE* S.L. EXPOSED TO 1X, 5X, 10X, AND PBO +1X DELTAMETHRIN IN ZIWAY-DUGDA, BAMBASI, METEMA, AMIBARA, HALABA, AND ABAYA, JULY-DECEMBER 2018

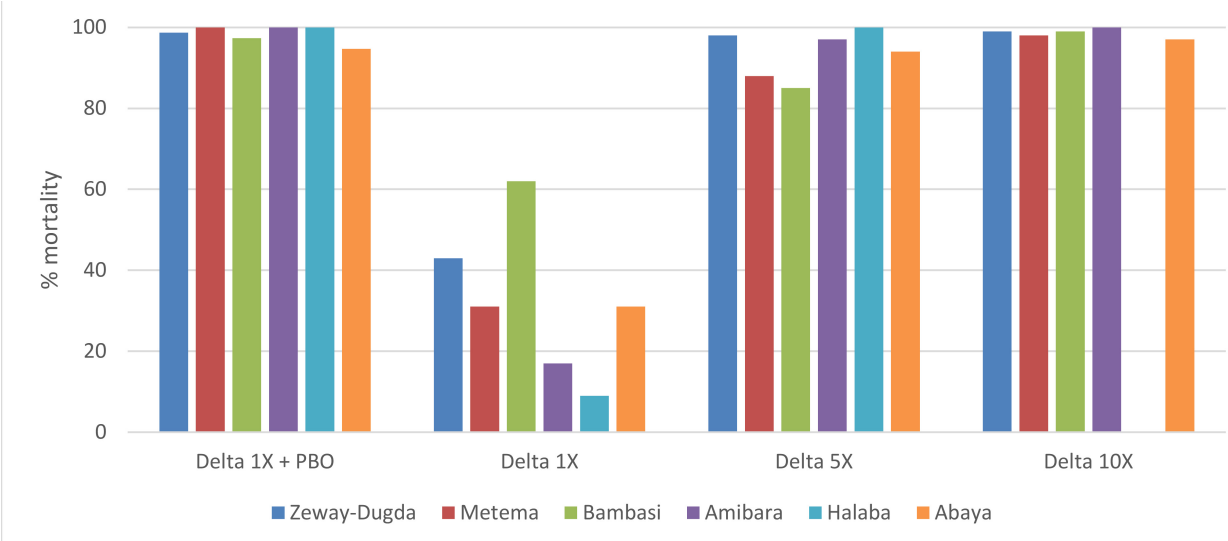
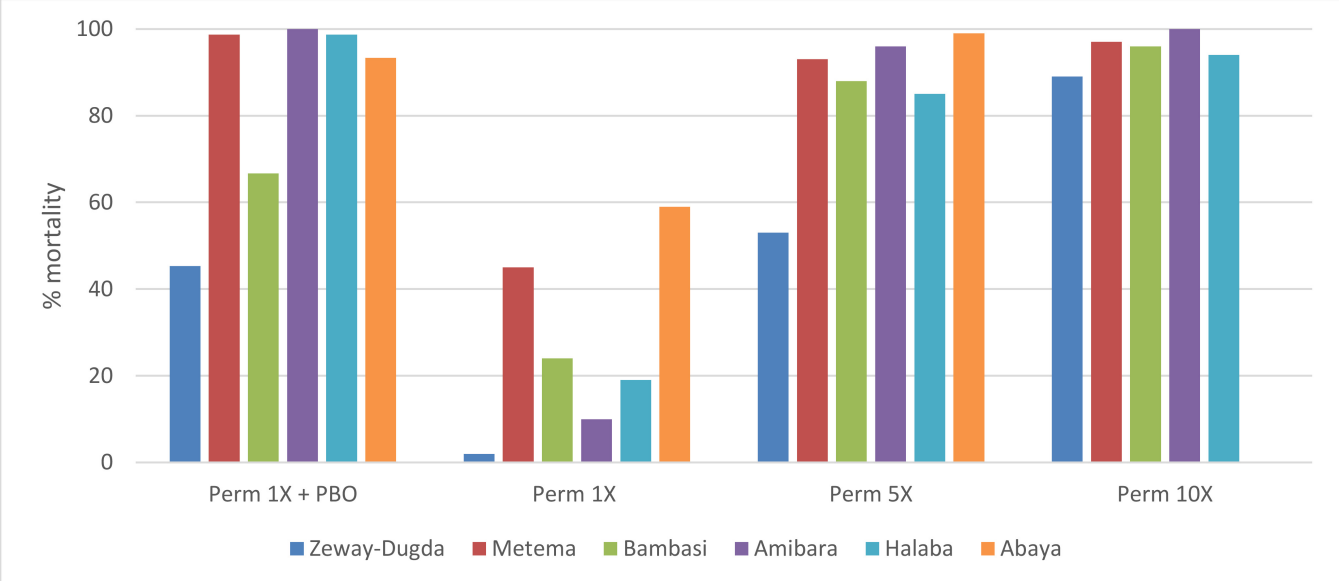


FIGURE 17: MORTALITY OF *AN. GAMBIAE* S.L. EXPOSED TO 1X, 5X, 10X, AND PBO +1X PERMETHRIN IN ZIWAY-DUGDA, BAMBASI, METEMA, AMIBARA, HALABA, AND ABAYA, JULY-DECEMBER 2018



4.3.3 DETECTION OF *KDR* ALLELE

A total of 392 PCR-identified *An. arabiensis* specimens from six sentinel sites were tested for *kdr* west (L1014F) using allele-specific PCR, of which 54.3%, 34.0%, and 11.7% were homozygote resistant, heterozygote resistant, and homozygote wild type susceptible, respectively (Table 8). The lowest resistant allelic frequency was 0.24 and the highest was 0.93.

TABLE 8: GENOTYPIC AND ALLELIC FREQUENCY OF KDR IN *AN. ARABIENSIS* SPECIMENS TESTED FOR DIFFERENT INSECTICIDES, ETHIOPIA

Site	Insecticide	Survival Status After Exposure	# Mosquitoes Tested	Homo-zygote Mutation (RR)	Hetero-zygote (RS)	Homo-zygote Wild Type (SS)	Kdr allele Freq.	
							R	S
	Deltamethrin	Alive	40	1	16	21	0.24	0.76
	Permethrin	Alive	37	29	6	2	0.86	0.14
Omonada	Permethrin	Dead	8	1	6	0	0.57	0.43
	Alpha-cypermethrin	Alive	31	13	14	4	0.65	0.34
		Dead	10	2	8	0	0.6	0.4
	Deltamethrin	Alive	40	33	4	1	0.92	0.08
Bambasi	Permethrin	Alive	32	26	5	1	0.89	0.11
	Deltamethrin	Alive	40	17	21	2	0.69	0.31
Abobo	Permethrin	Alive	40	31	5	0	0.93	0.07
	Alpha-cypermethrin	Alive	40	0	27	12	0.3	0.65
Amibara		Dead	10	1	7	2	0.45	0.55
Ziway-Dugda	Alpha-cypermethrin	Alive	40	28	12	0	0.9	0.1

Site	Insecticide	Survival Status After Exposure	# Mosquitoes Tested	Homo-zygote Mutation (RR)	Hetero-zygote (RS)	Homo-zygote Wild Type (SS)	Kdr allele Freq.	
							R	S
Metema	Alpha-cypermethrin	Alive	35	31	2	1	0.94	0.06
Total (%)			**392**	**213 (54.3)**	**133 (34)**	**46 (11.7)**		

RR= resistance homozygous, RS=resistance heterozygous, SS= Wild type susceptible, R= Resistance, S= Susceptible

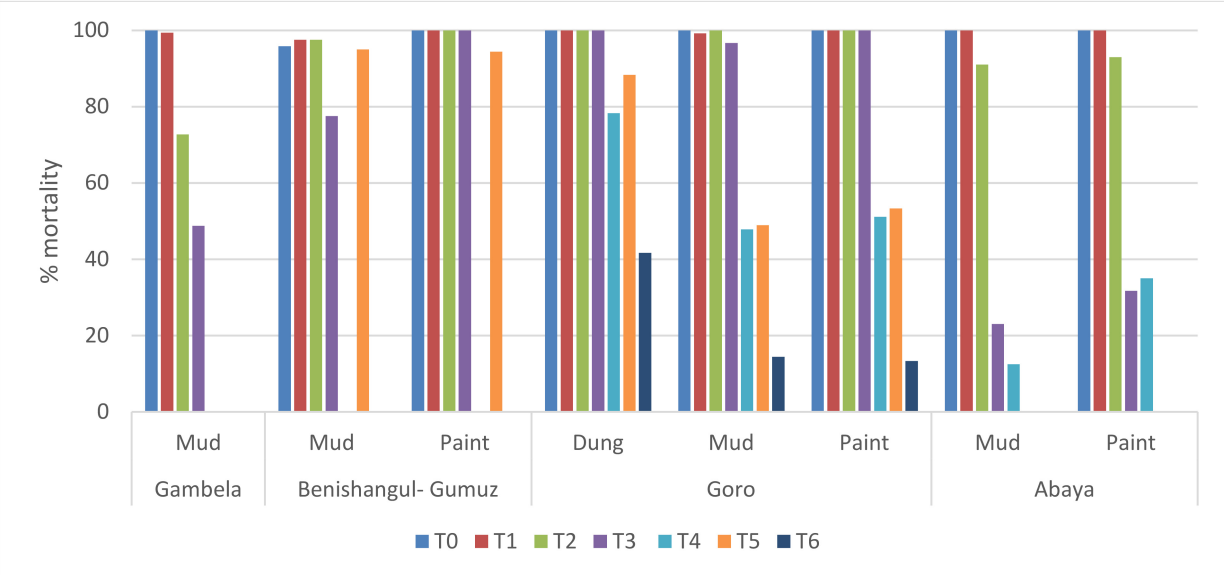
4.4 CONE BIOASSAY TEST RESULTS

4.4.1 SPRAY QUALITY ASSURANCE TEST AND INSECTICIDE DECAY RATE EVALUATION OF SUSCEPTIBLE *AN. ARABIENSIS*

Cone wall bioassays at T0 (within seven days after spraying) produced 100% mortality in *An. arabiensis* on all surfaces in the four sentinel sites except in Bambasi, where mortality on mud surfaces was 95.8%. At T1 (one month after spraying), mortality on mud surfaces was 97.5% in Bambasi but ranged from 99.2% to 100% in the other three sites (Figure 18, Annex E). At T2 (two months after spraying) and T3 (three months after spraying) in Lare, mortality of *An. arabiensis* declined to 72.7% and 48.8%, respectively. Mortality of *An. arabiensis* in Goro at T2 was 100% on all surfaces, but ranged from 97.5% to 100% in Bambasi and 91.1% to 98.3% in Abaya. At T3, the rate of mortality of *An. arabiensis* was 96.7% to 100% in Goro, 77.5% to 100% in Bambasi, and 23% to 31.7%. In Goro at T4, mortality on all surfaces declined to below 80%. At T5, dung surfaces produced an 88.3% mortality, and mud and paint surfaces 48.9% and 53%, respectively. In Bambasi at T5, mud surfaces killed more *An. arabiensis* (95%) than at T3 (77.5%) but paint surfaces caused lower mortality (94.4%) in T5 than in T3 (100%).

In general, the decay rate of Actellic 300CS was about two months in Lare and Abaya, 3-5 months in Goro, and five months or more in Bambasi.

FIGURE 18: CONE BIOASSAY TEST RESULTS OF AN INSECTARY COLONY OF *AN. ARABIENSIS* IN LARE, GORO, BAMBASI, AND ABAYA, MAY-NOVEMBER 2018

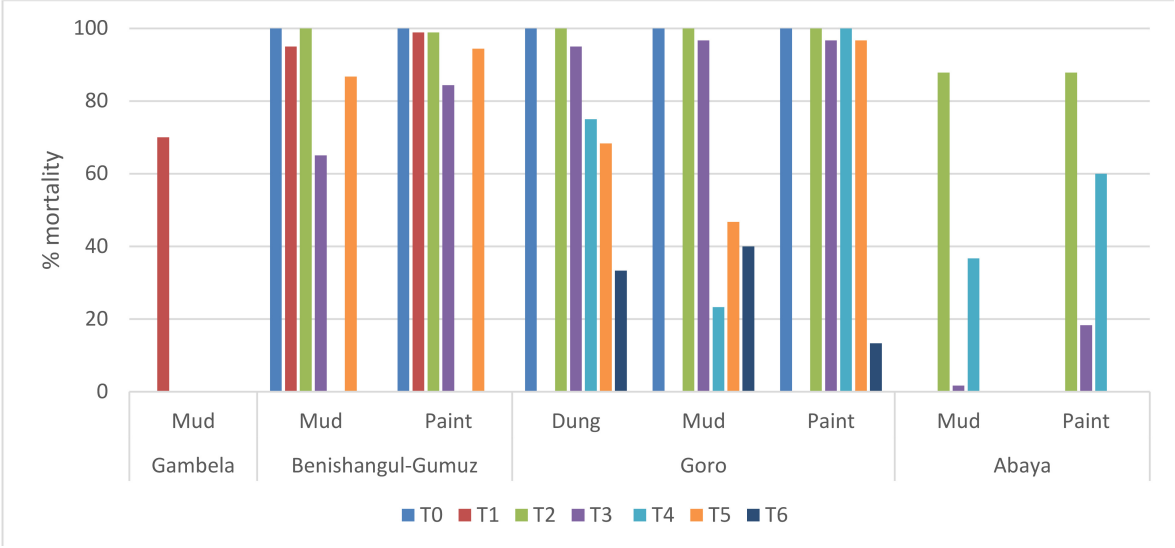


4.4.2 DECAY RATE EVALUATION OF WILD *AN. GAMBIAE* S.L.

Because of scarcity of *An. gambiae* s.l. larvae in the wild, cone bioassays were conducted in Lare at T1, Bambasi at T0-T5, Goro at T0 and T2-T6, and Abaya at T2-T4. In Lare, one month after spraying, cone wall bioassay tests resulted in 70% mortality of wild *An. gambiae* s.l. In Goro, cone bioassay test mortality was 100% at T0 and T2, 95-96.7% at T3, 100% on paint but 75% and 23.3% on dung and mud surfaces at T4, 96.7% on paint but lower than 80% on the rest of surfaces at T5, and 13-58% mortality at T6. In Bambasi, test mortality was 100% at T0 but declined to 95% and 98.9% on mud and painted surfaces at T1, respectively. At T2, the test mortality on painted surfaces remained 98.9% but increased to100% on mud surfaces. At T3, mortality on mud surface decreased to 65% but increased to 86.7% at T5. The corresponding mortality rates on paint surfaces were 84.4% and 94.4%. In Abaya, after the second month of spraying, the insecticide effect was similar on both mud and paint surfaces, killing 87.8% of *An. gambiae* s.l. In the third and fourth months, mortality was 1.7-60% for *An. gambiae* s.l. (Figure 19).

In conclusion, the results of the cone bioassay tests of *An. arabiensis* and *An. gambiae* s.l. were similar, showing a decay rate of Actellic 300CS over approximately 2-5 months in the study sites.

FIGURE 19: CONE BIOASSAY TEST RESULTS OF WILD *AN. GAMBIAE* S.L. IN LARE, GORO, BAMBASI, AND ABAYA, MAY-NOVEMBER 2018



4.4.3 FUMIGATION EFFECT: SUSCEPTIBLE COLONY *AN. ARABIENSIS* AND WILD *AN. GAMBIAE* S.L.

The fumigant effect of Actellic 300CS on mortality of *An. arabiensis* was high at T0 and T1 in Lare at 90.0% and 86.7% mortality and in Abaya at 83.3-100% and 68.3-75% mortality, respectively. Similarly, the fumigant effect was high for wild *An. gambiae* s.l. in Goro, with a test mortality of 100% in houses with dung and mud surfaces at T0 (Figure 20 and 21, Annex F). Actellic 300 CS continued to kill *An. gambiae* s.l. in Bambasi during all the test periods with variable mortality rates, the highest 100% at T1 and 80% at T2.

Based on mortality of *An. arabiensis*, the fumigant effect of Actellic 300 CS in Gambela was three months, in Bambasi five months, in Goro two months, and in Abaya one month. The insecticide killing effect of *An. gambiae* s.l. was lower than the cut of value of 22% in Lare at T1 (10% mortality), Bambasi at T5 (5% and 20% mortality), Goro at T3 (0% mortality), and Abaya at T2 (0% mortality).

FIGURE 20: FUMIGANT EFFECT OF PIRIMIPHOS-METHYL CS ON INSECTARY *AN. ARABIENSIS*, MAY-NOVEMBER 2018

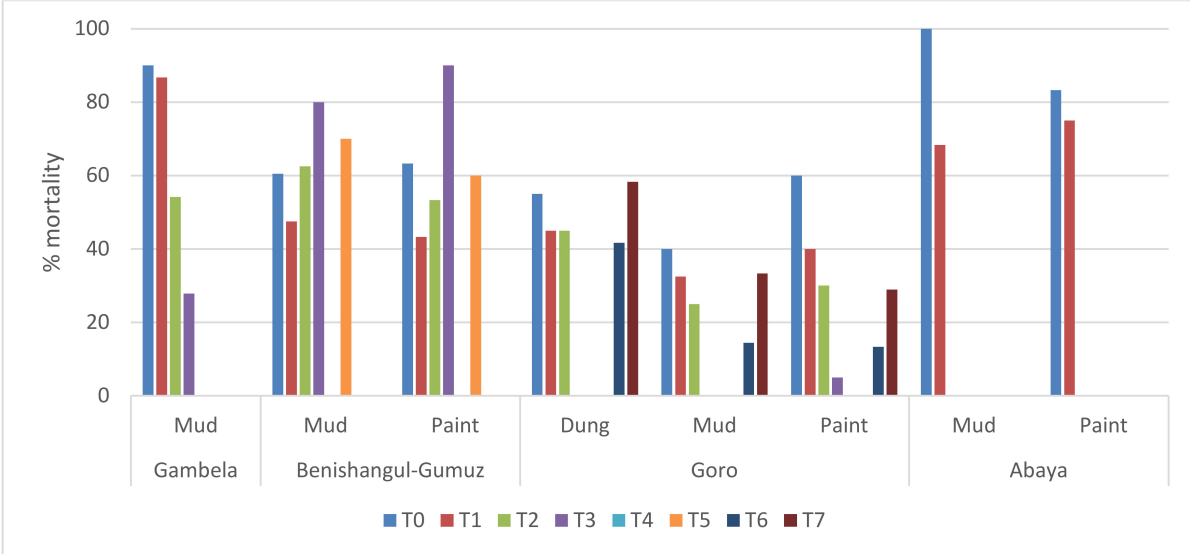
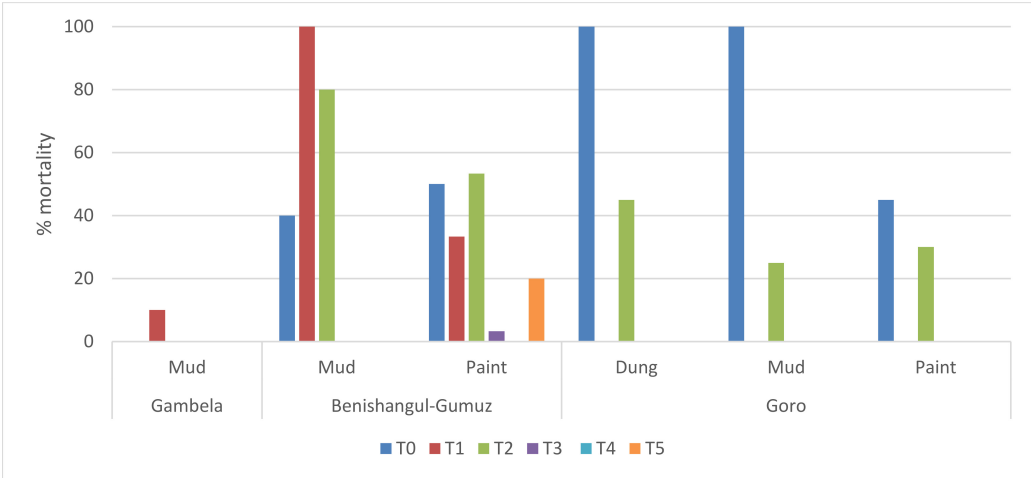


FIGURE 21: FUMIGANT EFFECT OF PIRIMIPHOS-METHYL CS ON WILD *AN. GAMBIAE* S.L., MAY-NOVEMBER 2018



4.5 SURVEY AND INSECTICIDE SUSCEPTIBILITY STATUS OF *AN. STEPHENSI*

4.5.1 *AN. STEPHENSI* SURVEY

Ninety adult *An. stephensi* were sampled in eight of the 10 survey sites. (The sites with non-adult collections were Awash Sebat Kilo and Bati.) Of the 90 adults, 74.4% were collected from 300 PSCs, 16.7% from 200 CDC light traps, and 8.9% from 45 HLCs (Table 9). The small number of adults collected implies that these mosquito sampling methods are not effective for *An. stephensi,* and this warrants finding alternative collection methods for this species.

TABLE 9: NUMBER OF *AN. STEPHENSI* COLLECTED FROM PSC, HLC, AND CDC LIGHT TRAPS IN 10 SURVEY SITES, AUGUST-NOVEMBER 2018

Site	PSC # (%)	HLC # (%)	CDC Light Traps # (%)	Total # (%)
Dire Dawa	3 (100)	0	0 (0)	3 (100)
Erer	14 (100)	ND	0 (0)	14 (100)
Awash Sebat Kilo	0 (0)	0 (0)	0 (0)	0 (0)
Bati	0 (0)	0 (0)	0 (0)	0 (0)
Gewane	6 (85.7)	0	1 (14.3)	7 (100)
Semera	38 (84.5)	6 (13.3)	1 (2.2)	45 (100)
Jigjiga	0	2 (100)	0	2 (100)
Kebridehar	3 (100)	0	0	3 (100)
Degehabour	1 (7.1)	0	13 (92.9)	14 9 (100)
Godey	2 (100)	0	0	2 (100)
Total (%)	67 (74.4)	8 (8.9)	15 (16.7)	90 (100)

ND= Not done

Larvae and pupae from all 10 sites were collected from cisterns, plastic sheets containing water for construction, barrels, water tanks, discarded water containers, and tires (Annex H). Adults were raised from larvae and pupa and identified to species using morphological keys. A total of 2,149 female *An. stephensi* were identified and its presence was confirmed in all the 10 sites (Table 10).

TABLE 10: *AN. STEPHENSI* IDENTIFIED FROM IMMATURE COLLECTIONS AND RAISED TO ADULTS

Site	Adults Raised from Larvae And Pupae
Dire Dawa	277
Erer	129
Awash Sebat Kilo	26
Bati	135
Gewane	102
Semera	260
Jigjiga	18
Kebridehar	700
Degehabour	166
Godey	336
Total	2149

4.5.2 MOLECULAR CONFIRMATION OF *AN. STEPHENSI*

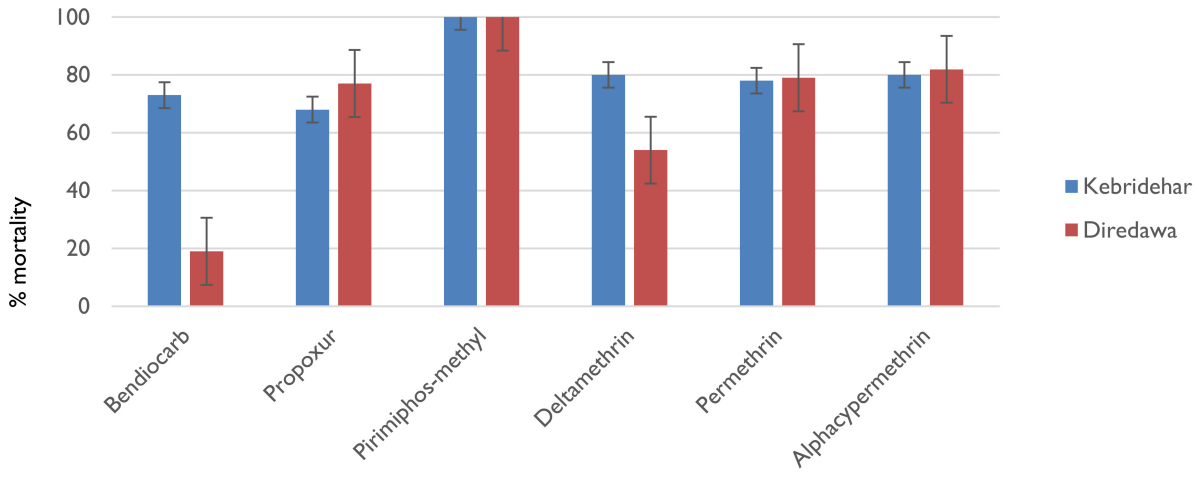
To confirm the morphological identification of *A. stephensi*, a molecular confirmatory investigation was conducted by Baylor University, USA, using PCR and sequence-based approaches. First, a PCR endpoint assay that involves amplification of a portion of the internal transcribed spacer 2 (ITS2) locus as previously described was used (Djadid et al. 2006). A species was determined with visualization of PCR products with gel electrophoresis. A band indicates the specimen is *An. stephensi* and absence of the

band indicates the specimen is another species. Using this method, we analyzed 140 *Anopheles* specimens, including all available wild-caught adults and some larvae collected from sites with no wild-caught adults. Successful PCR were obtained for 137 *Anopheles*: 128 *An. stephensi* and nine *An. gambiae s.l.* based on morphology. Overall, *An. stephensi* distribution was confirmed at all 10 study sites. The morphological identifications were compared with the ITS2 PCR endpoint assay results. Eight of the 128 (6.3%) morphologically identified *An. stephensi* were not confirmed to be *An. stephensi* with the PCR endpoint assay. Of these, five were from Semera and one each was from Erer, Awash, and Kebridehar. All morphologically identified *An. gambiae* s.l. that were successfully amplified were confirmed. Sequencing of the ITS2 and cytochrome oxidase subunit I loci was also completed. For the 34 specimens with available sequence data, the ITS2 endpoint assays were compared and sequencing BLAST results for consistency. Samples that were both morphologically identified as *An. stephensi* and *An. gambiae* s.l. were included. One specimen had inconsistent results (negative for *An. stephensi* via ITS2 endpoint PCR, positive via sequence BLAST). While ITS2 endpoint PCR was mostly consistent with sequence results (33/34) and can serve as preliminary insight into *An. stephensi* identification, sequencing remains the most reliable approach to identification. Sequencing of the remaining specimens are underway.

4.5.3 *AN. STEPHENSI* INSECTICIDE SUSCEPTIBILITY TESTS

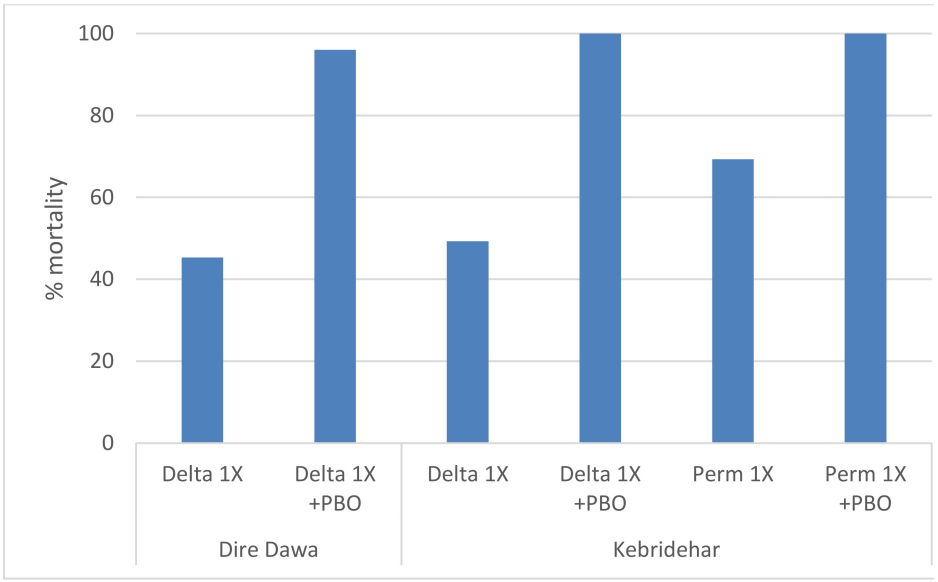
An. stephensi populations in Kebridehar and Dire Dawa were fully susceptible to pirimiphos-methyl (100% mortality) but resistant to bendiocarb, propoxur, deltamethrin, permethrin and alpha-cypermethrin (19-81% mortality) (Figure 22).

FIGURE 22: *AN. STEPHENSI* INSECTICIDE SUSCEPTIBILITY TEST RESULTS FROM KEBRIDEHAR AND DIRE DAWA, 2018



In Dire Dawa and Kebridehar, PBO+deltamethrin tests resulted in 96% and 100% mortality of *An. stephensi*, respectively. PBO+permethrin was tested only in Kebridehar; it resulted in 100% mortality (Figure 23).

FIGURE 23: PBO SYNERGIST TEST RESULTS OF *AN. STEPHENSI* FROM DIRE DAWA AND KEBRIDEHAR, SEPTEMBER AND NOVEMBER 2018



5. CONCLUSIONS

- *An. arabiensis* (species PCR confirmed) is the major malaria vector in Ethiopia. It was found in all the entomological monitoring sites at variable proportions.
- *An. funestus* s.l. in Lare and Bambasi as well as *An. pharoensis* in all sites could be secondary vectors.
- Preliminary molecular analysis on specimens identified morphologically as *An. funestus* s.l from Bambasi indicated the presence of two species, *An. funestus* s.s only 5 %(2/37) and *An. rivolurum about 95% (35/37)*. Although the density of *An. funestus* group in Bambasi was high from CDC trap collections, it is not yet known if *An. funestus* s.s is a primary vector because of the coexistence of the two species.
- All three species were more exophagic than endophagic; the proportion of the outdoor human biting density was two to three times higher than the indoor human biting density.
- The proportion of indoor resting density of *An. arabiensis* in the three sites was very low due to probably either the impact of the sprayed insecticide or the inherent/forced exophilic habit of the vector population. The same situation was also noted in *An. funestus* s.l. and *An. pharoensis*.
- The data presented in this report showed the hourly biting rhythm of *An. arabiensis*, *An. funestus* s.l., and *An. pharoensis* did not overlap.
- *An. funestus* s.l. feeding time started early in the evening both indoors and outdoors. It ceased in the early morning indoors, but continued outdoors until 6:00 am and probably beyond.
- Cirumsporozoite ELISA detected two *P. falciparum* infections in *An. arabiensis* from Lare, and one each from Bambasi and Abaya. In addition, each of a single sporozoite infections of the same species were found from *An. funestus* and *An. pharoensis* from Gambela. Two specimens of *An. funestus*, one from Bambasi and the other from Gambela, were found positive for *P. vivax* circumsporozoite proteins.
- Populations of *An. gambiae* s.l. were susceptible to bendiocarb, propoxur, and pirimiphos-methyl but resistant to the pyrethroids, deltamethrin, permethrin, and alpha-cypermethrin.
- Populations of *An. gambiae* s.l. were susceptible to clothianidin in four out of five sites. The vector was susceptible to chlorfenapyr in three of four sites.
- Resistance intensity assays indicated low to high resistance to deltamethrin and permethrin.
- PBO synergist either partially or fully restored susceptibility of *An. gambiae* s.l. to pyrethroids indicating the involvement of P450s as resistance mechanism.
- The low mortality response of *An. gambiae* s.l. in two sites after permethrin plus PBO tests might be explained by the presence of other resistance mechanisms.
- The decay rate of Actellic 300 CS could be more than five months in Bambasi and three to five months in Goro based on types of wall surfaces. For an unknown reason, the rate in Gambela and Abaya was two months.
- The geographical distribution of *An. stephensi* extended beyond the previously reported Somali region and found in Amhara and Afar regional sates.

- *An. stephensi* was susceptible to pirimiphos-methyl but resistant to bendiocarb, propoxur, deltamethrin, permethrin, and alpha-cypermethrin.
- The resistance mechanisms for deltamethrin and permethrin in *An. stephensi* might be linked to the family of P450 enzymes.

6. RECOMMENDATIONS

- Additional molecular studies are required on *An. funestus* s.l to determine the species composition both in Lare and Bambasi.
- Historically, *An. funestus* s.s in Ethiopia is considered as a secondary vector. Entomological studies in the late 1960s in Gambela documented very high natural infections in the population of *An. funestus*. Its occurrence in both Lare and Bambasi requires detailed studies on its bionomics to verify the degree of its vectorial capacity.
- The vectorial status of *An. rivolurum* in Bambasi needs to be investigated in view of its importance in the transmission of malaria in Tanzania (Wilkes et al. 1996).
- PBO nets might be considered for vector control interventions in Ethiopia since pre-exposure to PBO fully or partially restored susceptibility to deltamethrin and permethrin.
- Further studies on the bionomics and insecticide resistance mechanisms of *An. stephensi* are needed.

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ANNEX A. NUMBER OF *ANOPHELES* AND CULICINES COLLECTED FROM LARE, ABAYA, AND BAMBASI, MAY 2018-APRIL 2019

Site	Time	*An. gambiae* s.l.				*An. pharoensis*				*An. coustani*				*An. funestus*				*An. squamosus*				Culicines			
		PSC	CDC	HLC	Total	PSC	CDC	HLC	Total	PSC	CDC	HLC	Total	PSC	CDC	HLC	Total	PSC	CDC	HLC	Total	PSC	CDC	HLC	Total
Lare	May*	4	3	22	29	0	0	2	2	0	1	2	3	1	2	1	4	0	2	0	2	136	94	194	424
	June	2	6	19	27	0	9	78	87	0	37	58	95	4	3	1	8	0	1	2	3	22	297	332	651
	July	4	9	11	24	0	42	81	123	0	32	23	55	1	10	2	13	0	3	1	4	85	370	382	837
	Aug	1	9	25	35	0	56	419	475	0	48	12	60	0	3	0	3	0	9	1	10	82	441	633	1156
	Sept	2	3	24	29	2	32	245	279	1	37	75	113	3	0	2	5	0	4	2	6	74	258	522	854
	Oct	1	1	1	3	2	2	26	30	1	80	24	105	19	12	18	49	0	0	0	0	69	214	179	462
	Nov	0	0	1	1	0	0	7	7	0	35	58	93	17	52	126	195	0	0	0	0	6	136	221	363
	Dec	0	0	10	10	0	1	18	19	0	2	2	4	30	55	528	613	0	0	5	5	43	101	369	513
	Jan	4	1	41	46	0	0	1	1	0	0	0	0	5	23	69	97	0	0	0	1	15	51	106	172
	Feb	0	10	66	76	0	0	0	0	0	0	0	0	0	1	1	2	0	0	0	0	5	13	30	48
	March	1	0	13	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	12	16
	April	0	6	8	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	7	15	26
Subtotal		**19**	**48**	**241**	**308**	**4**	**142**	**877**	**1023**	**2**	**272**	**254**	**254**	**80**	**161**	**748**	**989**	**0**	**19**	**11**	**30**	**538**	**1978**	**2980**	**5522**

Site	Time	*An. gambiae* s.l.				*An. pharoensis*				*An. coustani*				*An. funestus*				*An. squamosus*				Culicines			
		PSC	CDC	HLC	Total	PSC	CDC	HLC	Total	PSC	CDC	HLC	Total	PSC	CDC	HLC	Total	PSC	CDC	HLC	Total	PSC	CDC	HLC	Total
Abaya	May*	14	6	41	61	0	11	50	61	0	0	1	1	0	0	0	0	0	0	0	0	153	243	399	795
	June	NOT DONE																							
	July	1	3	10	14	0	16	48	64	0	0	1	1	0	0	0	0	0	0	0	0	26	171	315	512
	Aug	0	0	2	2	0	1	3	4	0	0	1	1	0	0	0	0	0	0	0	0	10	85	83	178
	Sept	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	19	112	61	192
	Oct	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	7	86	61	154
	Nov	0	0	18	18	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	28	157	93	278
	Dec	1	0	13	14	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0	12	109	78	199
	Jan	2	2	2	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	32	41	85
	Feb	4	1	9	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	38	33	59	130
	March	5	1	12	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	15	83	103
	April	0	5	33	38	0	0	3	3	0	0	0	0	0	0	0	0	0	0	0	0	5	80	243	328
Subtotal		**27**	**18**	**142**	**187**	**0**	**28**	**104**	**132**	**0**	**0**	**10**	**10**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**157**	**80**	**1117**	**2159**
Bambasi	May*	0	2	0	2	0	0	0	0	1	3	0	4	0	2	0	2	0	1	0	1	1	19	4	24
	June	1	3	1	5	0	0	0	0	0	8	0	8	0	13	0	13	0	1	0	1	0	40	1	41
	July	3	9	9	21	0	1	0	1	0	46	15	61	13	22	1	36	0	14	1	15	0	72	26	98
	Aug	17	21	12	50	1	5	0	6	1	89	42	132	7	106	1	114	1	36	42	79	0	131	45	176
	Sept	6	25	9	40	0	3	0	3	1	105	22	128	23	101	3	127	0	48	0	48	5	168	19	192
	Oct	NOT DONE																							
	Nov	1	1	0	2	0	5	1	6	1	42	11	54	27	143	6	176	0	21	1	22	7	134	17	158
	Dec	NOT DONE																							
	Jan	NOT DONE																							
	Feb	NOT DONE																							
	March	NOT DONE																							
	April	NOT DONE																							
Subtotal		**28**	**6**	**31**	**120**	**1**	**14**	**1**	**16**	**4**	**293**	**90**	**387**	**70**	**387**	**12**	**469**	**1**	**121**	**44**	**166**	**13**	**564**	**112**	**690**

* Collections conducted in May were pre-IRS. All subsequent collections were post-IRS.

Annex B. Insecticide Susceptibility Test Results from 13 Sentinel Sites, August-November 2018

Type of Assay	Insecticide	Percent Mortality of Wild *An. gambiae* s.l., by Region and Site												
		Oromia				Gambela	Afar	Amhara		Tigray	SNNPR		Benishangul Gumuz	
		Abaya	Omonada	Ziway-Dugda	Babile	Abobo	Amibara	Metema	Bahirdar	Humera	Halaba	Benatsemay	Bambasi	Dangur
Discriminating dose	Bendiocarb	100 (100/100)	98 (98/100)	100 (100/100)	100 (75/75)	100 (100/100)	100 (100/100)	100 (100/100)	100 (100/100)	100 (100/100)	100 (100/100)	100 (100/100)	100 (100/100)	100 (100/100)
		S	S	S	S	S	S	S	S	S	S	S	S	S
	Propoxur	97 (97/100)	99 (99/100)	100 (100/100)	100 (75/75)	97 (97/100)	100 (100/100)	100 (100/100)	100 (100/100)	100 (100/100)	98 (98/100)	100 (100/100)	100 (100/100)	100 (100/100)
		POR	S	S	S	POR	S	S	S	S	S	S	S	S
	Pirimiphos-methyl	100 (100/100)	100 (100/100)	100 (100/100)	100 (75/75)	100 (100/100)	100 (100/100)	100 (100/100)	100 (100/100)	100 (100/100)	100 (100/100)	100 (100/100)	100 (100/100)	100 (100/100)
		S	S	S	S	S	S	S	S	S	S	S	S	S
	Deltamethrin	31 (31/100)	40 (40/100)	43 (43/100)	69 (52/75)	46 (46/100)	17 (17/100)	31 (31/100)	37 (37/100)	17 (17/100)	9 (9/100)	16 (16/100)	62 (62/100)	36 (36/100)
		R	R	R	R	R	R	R	R	R	R	R	R	R
	Permethrin	59 (59/100)	27 (27/100)	2 (2/100)	68 (51/75)	31 (31/100)	10 (10/100)	45 (45/100)	43 (43/100)	14 (14/100)	19 (19/100)	ND	24 (24/100)	15 (15/100)
		R	R	R	R	R	R	R	R	R	R		R	R
	Alpha-cypermethrin	31 (31/100)	ND	12 (12/100)	ND	ND	12 (12/100)	13 (13/100)	42 (42/100)	5 (5/100)	13 (13/100)	46 (46/100)	1 (1/100)	7 (7/100)
		R		R			R	R	R	R	R	R	R	R

SNNPR=Southern Nations, Nationalities and Peoples Region, S=susceptible, POR=possible resistance, R=resistance

ANNEX C. CHLORAFENAPYR SUSCEPTIBILITY TEST RESULTS, 2018

Sentinel Site	Mosquito	No. tested	% knocked down after 60 min	% Mortality			Status
				24 hr.	48 hr.	72 hr.	
Zeway-Dugda	*An. gambiae* s.l.	100	8	91	99	100	Susceptible
Insectary	*An. arabiensis*	100	8	100	100	100	Susceptible
Omonada	*An. gambiae* s.l.	100	3	83	93	98	Susceptible
Insectary	*An. arabiensis*	100	4	93	100	100	Susceptible
Halaba	*An. gambiae* s.l.	100	8	91	95	96	Possible resistance
Insectary	*An. arabiensis*	100	5	90	92.6	96.8	Possible resistance
Abaya	*An. gambiae* s.l.	100	6	86	99	100	Susceptible
Insectary	*An. arabiensis*	100	20	91	100	100	Susceptible

ANNEX D. INTENSITY AND SYNERGIST RESULTS, 2018

Insecticide concentration and synergist	Sentinel sites					
	Zeway-Dugda	**Metema**	**Bambasi**	**Amibara**	**Halaba**	**Abaya**
Deltamethrin 1X + PBO	98.7 (74/75)	100 (75/75)	97.3 (73/75)	100 (75/75)	100 (75/75)	94.7 (71/75)
Deltamethrin 1X	64 (48/75)	42.6 (32/75)	10.7 (8/75)	34.7 (26/75)	13.3 (10/75)	29.3 (22/75)
Deltamethrin 5X	98 (98/100)	88 (88/100)	85 (85/100)	97 (97/100)	100 (100/100)	94 (94/100)
Deltamethrin 10X	99 (99/100)	98 (98/100)	99 (99/100)	100 (100/100)		97 (97/100)
Permethrin 1X + PBO	45.3 (34/75)	98.7 (74/75)	66.7 (50/75)	100 (75/75)	98.7 (74/75)	93.3 (70/75)
Permethrin 1X	4 (3/75)	42.6 (32.75)	16 (12/75)	53.3 (40/75)	49.3 (37/75)	53.3 (40/75)
Permethrin 5X	53 (53/100)	93 (93/100)	88 (88/100)	96 (96/100)	85 (85/100)	99 (99/100)
Permethrin 10X	89 (89/100)	97 (97/100)	96 (96/100)	100 (100/100)	94 (94/100)	

ANNEX E. CONE BIOASSAY TEST RESULTS, 2018

Test Site	Spray Date	Wall Surfcae		T0	T1	T2	T3	T4	T5	T6	T7
				% 24 hrs test mortality (N)	% 24 hrs test mortality (N)	% 24 hrs test mortality (N)	% 24 hrs test mortality (N)	% 24 hrs test mortality (N)	% 24 hrs test mortality (N)	% 24 hrs test mortality (N)	% 24 hrs test mortality (N)
Lare (Gambela)	**May-2018**	Mud	*An. arabiensis (Susceptible colony)*	100 %(360)	99.4 %(180)	**72.7(395)**	48.8 (379)				
			Wild mosquitoes *(An. gambiae* s.l.)	ND	70 (180)	ND	ND				
Bambasi (Benishangul-gumz)	**Jun-18**	Mud	*An. arabiensis (Susceptible colony)*	95.8 % (120)	97.5 % (120)	97.5 % (120)	77.5 (120)	ND	95 (120)	ND	ND
			Wild mosquitoes *(An. gambiae* s.l.)	100%(60)	95% (60)	100 (60)	65 (60)	ND	86.7 (60)	ND	ND
		Painted	*An. arabiensis (Susceptible colony)*	100%(90)	100%(90)	100%(90)	100 (90)	ND	94.4 (90)	ND	ND
			Wild mosquitoes *(An. gambiae* s.l.)	100% (90)	98.9(90)	98.9(90)	84.4 (90)	ND	94.4 (90)	ND	ND
Goro (Oromia)	**Jun-18**	Dung	*An. arabiensis (Susceptible colony)*	100%(60)	100% (120)	100% (60)	100 (60)	78.3 (60)	88.3 (60)	41.7 (60)	58.3 (60)
			Wild mosquitoes *(An. gambiae* s.l.)	100%(60)	ND	100% (60)	95 (60)	75 (60)	68.3 (60)	33.3 (60)	30 (60)
		Mud	*An. arabiensis (Susceptible colony)*	100% (60)	99.2 (120)	100 (60)	96.7 (60)	47.8 (90)	48.9 (90)	14.4 (90)	33.3 (90)
			Wild mosquitoes *(An. gambiae* s.l.)	100%(60)	ND	100% (60)	96.7 (60)	23.3 (30)	46.7 (30)	40.0(30)	16.7 (30)
		Painted	*An. arabiensis (Susceptible colony)*	100%(60)	100 (120)	100% (60)	100 (60)	51.1 (90)	53.3 (90)	13.3 (90)	28.9 (90)
			Wild mosquitoes *(An. gambiae* s.l.)	100%(60)	ND	100% (60)	96.7 (60)	100 (30)	96.7 (30)	13.3 (30)	56.7 (30)
Abaya (Oromia)	**Jul-18**	Mud	*An. arabiensis (Susceptible colony)*	100% (180)	100% (180)	91.1 (90)	23.3 (120)	12.5 (120)			
			Wild mosquitoes *(An. gambiae* s.l.)	ND	ND	87.8 (90)	1.7 (60)	36.7 (60)			
		Painted	*An. arabiensis (Susceptible colony)*	100%(180)	100%(180)	93.3 (90)	31.7 (120)	35 (120)			
			Wild mosquitoes *(An. gambiae* s.l.)	ND	ND	87.8 (90)	18.3 (60)	60 (60)			

Tests were terminated after two data tests showed less than 80% mortality.

ANNEX F. FUMIGATION BIOASSAY TEST RESULTS, 2018

Test Site	Spray Date	Wall Surface		T0	T1	T2	T3	T4	T5	T6	T7
Lare (Gambela)	18-May	Mud	*An. arabiensis (Susceptible colony)*	90(120)	86.7(60)	54.2(120)	27.8(126)				
			Wild mosquitoes *(An. gambiae s.l.)*	ND	10(60)	ND	ND				
Bambasi (Benishangul-Gumuz)	18-Jun	Mud	*An. arabiensis (Susceptible colony)*	60.5(40)	47.5(40)	62.5(40)	80(40)	ND	70(40)	ND	ND
			Wild mosquitoes *(An. gambiae s.l.)*	40(20)	100(20)	80(20)	0(20)	ND	5(20)	ND	ND
		Painted	*An. arabiensis (Susceptible colony)*	63.3(30)	43.3(30)	53.3(30)	90(30)	ND	60(30)	ND	ND
			Wild mosquitoes *(An. gambiae s.l.)*	50(30)	33.3(30)	53.3(30)	3.3(30)	ND	20(30)	ND	ND
Goro (Oromia)	18-Jun	Dung	*An. arabiensis (Susceptible colony)*	55(20)	45(40)	45(20)	0(20)				
			Wild mosquitoes *(An. gambiae s.l.)*	100(20)	ND	45(20)	0(20)				
		Mud	*An. arabiensis (Susceptible colony)*	40(20)	32.5(40)	25(20)	0(20)				
			Wild mosquitoes *(An. gambiae s.l.)*	100(20)	ND	25(20)	0(20)				
		Painted	*An. arabiensis (Susceptible colony)*	60(20)	40(40)	30(20)	5(20)				
			Wild mosquitoes *(An. gambiae s.l.)*	45(20	ND	30(20)	0(20)				
Abaya (Oromia)	18-Jul	Mud	*An. arabiensis (Susceptible colony)*	100	68.3	0(20)					
			Wild mosquitoes *(An. gambiae s.l.)*	ND	ND	0(20)					
		Painted	*An. arabiensis (Susceptible colony)*	83.3	75	0(20)					
			Wild mosquitoes *(An. gambiae s.l.)*	ND	ND	0(20)					