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The PMI Africa IRS (AIRS) Project

Indoor Residual Spraying (IRS 2) Task Order Six

AIRS MADAGASCAR: ENTOMOLOGICAL MONITORING FINAL REPORT

JULY 2015 – MAY 2016

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ACRONYMS

AIRS	Africa Indoor Residual Spraying
CDC	U.S. Centers for Disease Control and Prevention
CHL	Central Highlands
DDT	Dichlorodiphenyltrichloroethane
HLC	Human Landing Catches
IRS	Indoor Residual Spraying
KD	Knock-Down
LLIN	Long-Lasting Insecticidal Net
MBR	Man Biting Rate
ODC	Outdoor Collection
PMI	President's Malaria Initiative
PSC	Pyrethrum Spray Collections
RBM	Roll-Back Malaria
USAID	United States Agency for International Development
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme

EXECUTIVE SUMMARY

Background

In Madagascar, indoor residual spraying (IRS) is an important component of the malaria control strategy, as noted in the current National Strategic Plan. Madagascar currently receives donor support for implementing IRS from the U.S. President's Malaria Initiative (PMI) and the Global Fund.

During the 2015 spray round from August 3 – 26 in the South East and from August 31 - September 26 in the East Coast, the PMI Africa Indoor Residual Spraying (AIRS) Project in Madagascar covered 32 communes in the South East and 33 communes in the East Coast with blanket IRS. Pirimiphos-methyl CS, an organophosphate insecticide, was used for the campaign. Entomological monitoring is an integral component of the PMI AIRS Project. The 2015-2016 entomological monitoring activities included collection of comprehensive entomological data on vector density, species composition, seasonal patterns, biting behavior, insecticide resistance and parity of anopheline mosquitoes from six sentinel sites, four intervention (IRS) and two control sites (non-IRS). Data on vector species composition, density and behavior was collected using various mosquito sampling methods that included pyrethrum spray catches (PSC), human landing catches (HLC) and sucking tube (aspirator). One data point was collected prior to the spray campaign to serve as a baseline from both the intervention and control sites, and subsequent monthly data were collected post-spray to help understand if there was any change in the species composition, density and behavior following IRS. The PMI AIRS Project conducted wall bioassay tests to assess the quality of spraying within 24 hours of application and monthly thereafter to monitor the decay rate of the insecticide sprayed. Insecticide susceptibility data was also collected from eleven sentinel sites including the six sentinel sites used to collect comprehensive entomological data to inform insecticide-based malaria vector control program (IRS and LLINs). The presence of target site mutations (*kdr and Ace-1*^{R}) and metabolic resistance mechanism was assessed using molecular methods and by pre-exposing mosquitoes to synergists, respectively. This final report covers entomological monitoring activities performed from July 2015 to May 2016.

Results

Vector density and seasonality: A total of 3,700 female anopheline and 3,736 culicine mosquitoes were collected during the monitoring period. The most abundant vector species was *An. gambiae* s.l. that constituted 51.8% (n=1,914) of the total anopheline mosquitoes collected. The two other anophelines that are vectors of malaria in Madagascar, *An. funestus* and *An. mascarensis,* accounted only for 4.1% (n=153) and 2.6% (n=956), respectively. Only 13 *An. gambiae* s.l., two *An. mascarensis,* and five *An. funestus were* collected resting indoors with PSC. At the same time 186, four and ten *An. gambiae* s.l., *An. funestus* and *An. mascarensis*, respectively, were collected from artificial pit shelters resting outdoors with aspirators. 6,986 (93.9%) mosquitoes were collected while seeking human blood through HLC method: 3,385 female anopheline - of which 1,914 were *An. gambiae s.l.* - and 3,601 culicine.

Feeding time and location: At the baseline before IRS, *An. gambiae* s.l. human biting rates ranged from 0.0 bites per person per night in Mahambo to 3.8 bites per person per night in Ambodifaho indoors, and from 0.17 bites per person per night in Mahambo to 15.2 per person per night in Vohitrambato

outdoors. In all sentinel sites but Ambodifaho, *An. gambiae* s.l. exhibited exophagic tendencies pre-IRS. No change in the feeding habit of *An. gambiae* s.l. was noted after IRS when compared to pre-IRS. It is apparent that the vector prefers feeding outdoors as compared to indoors in both the intervention and control sites (Table 1). The low mean biting rates noted at baseline as compared to after spray could most probably be explained by the limited availability of breeding sites before the rainy season when the baseline data was collected.

TABLE I. COMPARISON OF INDOOR AND OUTDOOR BITING RATES BEFORE & AFTER IRS

Intervention status	Spray Status	MBR		P=value
		Indoor	Outdoor	
Intervention	Pre-IRS	1.8	4.5	p<0.001
Control	Pre-IRS	0.6	2.7	p<0.001
Intervention	Post-IRS	2.57	6.00	p<0.001
Control	Post-IRS	1.68	2.33	P=0.0067

An. gambiae s.l. engaged in biting throughout the night but peak biting was variable between sites. In some sites like Ambodifaho (Brickaville) and Vohitrambato (Toamasina II) significant mosquito biting was noted between 20:00pm and 04:00 am. In Mahambo (Fenerive Est) the peak biting time of *An. gambiae* s.l. was recorded from 12:00am and 01:00 am. In the two control villages, Vavatenina and Lopary, most human-vector contact occurred in the first half of the night. Similarly, in Manambotra Sud site a higher proportion of host seeking *An. gambiae* s.l. was collected before midnight (22:00pm – 01:00 am).

Quality of spraying and residual life: The results of wall bioassays indicated that the spray quality, both in the East Coast and in the South East, was good; mortality was 100% for all the structures sampled at T0 (24 hours after spraying) and T1 (one month after spray). In the South East and in the East Coast, three months after spraying, pirimiphos-methyl CS retained 100% effectiveness. However, four months after spray a diminution of residual efficacy of pirimiphos-methyl CS on thatch and wood surfaces was observed with mortality rates of 95.8% and 95%, respectively, in the South East. Pirimiphos-methyl CS remained effective for seven months on both types of surfaces in the two monitoring sites.

Susceptibility tests: The results of the vector susceptibility tests indicated full susceptibility of *An. gambiae* s.l. to bendiocarb and pirimiphos-methyl in all areas where the tests were conducted. The test results also showed that *An. gambiae* s.l. had developed resistance to DDT in Imerina Imady, Vohimarina, and Ankafina Tsarafidy, to permethrin in Mahambo, Vavatenina, Bekily and Ankafina Tsarafidy, and to lambda-cyhalothrin in Bekily.

Suspected resistance was noted for DDT in Mahambo and Ambodifaho, for deltamethrin in Vohitrambato and Vavatenina, for permethrin in Ankafina Tsarafidy, Ambodifaho and Vohitrambato, for lambda-cyhalothrin in Imerina Imady and for alphacypermethrin in Vohitrambato, Mahambo and Imerina Imady. *An.funestus* and *An.mascarensis* were fully susceptible to deltamethrin, permethrin and pirimiphos-methyl where the test was done.

Mechanism of resistance: No knockdown resistance (*kdr*) mutations were found in 1,006 *An. gambiae* s.l. samples genotyped for L1014F and L1014S alleles. Molecular analysis results of 248 *An. gambiae* s.l. samples also indicated absence of G119S mutations.

Generally pre-exposing *An. ga*mbiae s.l. to synergists fully restored susceptibility to pyrethroids and partially restored susceptibility to DDT. There were exceptions where pre-exposure to synergists fully restored susceptibility to DDT at three sites and in one site where pre-exposure to PBO only partially resorted susceptibility to permethrin.

1. INTRODUCTION

In Madagascar, malaria is endemic across 90% of the country; however, the entire population is considered to be at risk for the disease. In 2013, it was the second leading cause of death among children under five as reported by district hospitals.

Madagascar's national malaria strategy from 2008 - 2012 recommended blanket IRS. In 2012, there was a change in the vector control strategy to include focal IRS and epidemic alert reporting in addition to blanket IRS. Villages in the Central Highlands were selected for the focal spraying based on health facility malaria cases and rapid diagnostic test positivity rates. PMI supported spraying in the Central Highlands (CHL) and the Fringe areas in 2008 and 2009. In 2010, the South was added. Blanket IRS was conducted in the CHL, Fringe areas, and the South in 2011. However, in 2012 and 2013, spraying in the CHL and Fringe areas were switched from blanket spraying to focal spraying. In 2014, the CHL received focal spray but the Fringe districts were moved back to blanket spray with three districts in the East Coast (Brickaville, Toamasina II and Fenerive Est) also receiving blanket coverage.

In 2015, the annual IRS campaign was performed between August 3rd – August 26th in the South East and August 31st - September 26th in the East Coast with pirimiphos-methyl CS. The National Malaria Control Programme (NMCP) implemented IRS in 16 districts of the Central High Lands (CHL), between December 7th and December 31st using pyrethroids.

This report presents the results of the entomological monitoring activities completed by the PMI AIRS Madagascar project between July 2015 and May 2016, including data on the residual efficacy of insecticides, insecticide susceptibility and mechanism of resistance, mosquito density, and mosquito behavior.

2. OBJECTIVES

The objectives of the entomological surveillance were:

- To identify the vector species, composition, and density;
- To determine vector biting and resting behavior;
- To determine the quality of spraying and insecticide decay rate following spray operation; and
- To ascertain vector susceptibility to the four classes of insecticides approved by the World Health Organization Pesticide Evaluation Scheme (WHOPES) for IRS and determine resistance mechanisms.

Entomological surveillance will continue to play a critical role in informing vector control programs, including the impact of IRS on vector density, resting and feeding behavior. It also identifies insecticides that are effective against local vectors.

3. MATERIALS AND METHODS

3.1. STUDY SITES

In April 2015, AIRS Madagascar and the vector control committee of the NMCP selected entomological monitoring sentinel sites for 2015. The committee decided to keep one of the sentinel sites located in the South and four others in the Central Highlands for insecticide resistance monitoring to help the country obtain insecticide resistance data from representative areas and monitor trends in resistance over time. Some sentinel sites monitored during the 2014-2015 IRS campaign were dropped due to a change in PMI's supported IRS target districts in 2015. Three intervention sites, two from the East Coast and one from the Southeast, were selected and used for comprehensive entomological monitoring activities in 2015.

Ankafina Tsarafidy (district of Ambohimahasoa), Vavatenina (district of Vavatenina) and Lopary (district of Vangaindrano) were selected as control sentinel sites, respectively, for the Central Highlands, the East Coast and the South East. All sentinel sites where entomological surveillance was performed during the 2015 IRS campaign are listed in Table 2.

TABLE 2. LIST OF SENTINEL SITES

Region	District	Sentinel Site Location	Notes
Antsinanana (East Coast)	Toamasina II	Vohitrambato	Used as a sentinel site during the 2014-2015 IRS campaign
Analanjirofo (East Coast)	Fenerive Est	Mahambo	Used as a sentinel site during the 2014-2015 IRS campaign
Antsinanana (East Coast)	Brickaville	Ambodifaho	Used as a sentinel site during the 2014-2015 IRS campaign
Analanjirofo (East Coast)	Vavatenina	Vavatenina (control site)	Used as a control sentinel site during the 2014-2015 IRS campaign
Atsimo Antsinanana (South East)	Farafangana	Manambotra Sud	New sentinel site
Atsimo Antsinanana (South East)	Vangaindrano	Lopary	New control site
Amoron'I Mania (CHL)	Fandriana	Milamaina	Non IRS area for susceptibility test (old site for 2014/2015 round)
Amoron'I Mania (CHL)	Ambositra	Imerina Imady	Non IRS area for susceptibility test (old site for 2014/2015 round)
Haute Matsiatra (CHL)	Ambohimahasoa	Ankasina Tsarafidy	Non IRS area for susceptibility test (old site for 2014/2015 round)
Haute Matsiatra (CHL)	Fianarantsoa II	Vohimarina	Non IRS area for susceptibility test (old site for 2014/2015 round)
Androy (South)	Bekily	Bekily	Non IRS area for susceptibility test (old site for 2014/2015 round)

3.2. ADULT MOSQUITO COLLECTIONS

Baseline entomological data was collected one month before the start of the IRS campaign in two spray zones (i.e., in July 2015 in the South East and in August 2015 in the East Coast). Data was collected monthly through February 2016. The East Coast has three entomological sentinel sites that were used for comprehensive entomological data collection: Ambodifaho (Brickaville district), Vohitrambato (Toamasina II district), Mahambo (Fenerive Est district) and one control site, Vavatenina. The South East had two sites, one control and one intervention site, used for entomological monitoring: Manambotra Sud (intervention site in Farafangana district) and Lopary (control site in Vangaindrano district).

Data on species composition, vector densities, and vector behavior were gained via collecting adult mosquitoes using human landing catches (HLC), pyrethrum spray collections (PSC) and outdoor resting collection (ODC) using sucking tube.

3.2.1. Human Landing Catches (HLC)

HLC was intended to determine vector biting location, time and frequency (man biting rate (MBR)). The HLC was conducted indoors and outdoors in three houses per sentinel site, for two nights per month. Collections were made over a period of 12 hours (18:00 – 6:00) indoors and outdoors. One mosquito collector was seated indoors and another seated outdoors from 6 p.m. to 6 a.m in six hour shifts to collect blood-seeking mosquitoes. Outdoor and indoor collectors switched sites every hour. Collectors adjusted their clothing so that the legs were exposed up to the knees. When a mosquito was felt, collectors quickly turned on the torch (flashlight), collected the mosquito with the sucking tube and transferred it to a paper cup. One cup was used for each hour of collection. Hourly temperature and humidity were recorded. At the end of the collection, mosquitoes were transported to the field lab and were identified using taxonomic keys (Gilles and Coetzee, 1987).

3.2.2. Pyrethrum Spray Collection (PSC)

PSC was used to estimate the room resting density and to measure indirectly the MBR. PSC activity was completed in the morning between 06:00am and 09:00am, once a month. AIRS Madagascar entomology staff conducted PSC at ten houses per sentinel site per month. Before the PSC was performed, all occupants were cordially asked to move out of the house. AIRS Madagascar entomology staff then covered the floor of a room in the house with white sheets and closed all other openings that would allow the mosquito to escape from the house. The walls and roof space inside the houses then sprayed with insecticide that knocks down the mosquitoes. Knocked-down mosquitoes were collected using forceps and kept separately in pill-boxes until species identification could be performed along with the determination of blood digestion stage. Identification of all mosquitoes was done using morphological keys (criteria of Gillies and de Meillon).

3.2.3. Outdoor Resting Collection (ODC)

Mosquitoes resting outdoors were collected from natural resting places and pit shelters using aspirators. Four pit shelters (about 1m x 1m x 1m) per sentinel site and natural resting places such as tree holes,

vegetation, cattle sheds, and ground holes were used to collect outdoor resting mosquitoes each month. The productivity of artificial and natural resting places varied temporally and by sites. Mosquitoes collected outdoors were kept in paper cups separately labeled for each collection sites and were morphologically identified to species.

For all collection types, after species identification, malaria vector mosquitoes were preserved individually in Eppendorf tubes with silica gel for ELISA tests, and molecular identification to be completed by the University of Witwatersrand in South Africa.

3.2.4 Parity rates

All unfed and freshly fed vectors species collected using PSC, HLC and ODC were dissected and examined for parity.

3.3. INSECTICIDE SUSCEPTIBILITY TESTS

3.3.1. Bioassay Tests

Vector susceptibility was tested for all four classes of insecticides recommended for public health use in Madagascar (i.e., carbamates, pyrethroids, organochlorines, and organophosphates). The PMI AIRS Madagascar team performed the tests using both World Health Organization (WHO) tube assays with insecticide-impregnated papers and U.S. Centers for Disease Control and Prevention (CDC) bottle assays at eleven sentinel sites. All the three vectors of malaria (i.e., *An. gambiae* s.l., *An. funestus* and *An. mascarensis*) found in Madagascar were included in the resistance testing. With regards to *An. gambiae* s.l., two- to five-day old adult, non-blood-fed female mosquitoes (reared from field-collected larvae) were used for both the WHO tube test and CDC bottle assay. In contrast, due to the difficulty of obtaining sufficient numbers of aquatic stages of *An. funestus* and *An. mascarensis* from the breeding sites, wild adult mosquitoes resting in unsprayed houses were collected using Prokopack aspirators and used for the resistance testing. However, this might underestimate resistance status if it were to be compared with using age standardized young mosquitoes for the test. Nevertheless, the result may not deviate from what actually happens under natural conditions in the field. Mortality was recorded after a 24-hour holding period for the WHO tube test. For CDC bottle bioassays, a diagnostic time of 30 and 45 minutes for non-DDT and DDT insecticides were used respectively.

If resistance to one insecticide was observed or suspected, the tested samples were sent to a specialized lab for *kdr* mutation analysis. Since the previous analysis last year did not show any *kdr* mutation, synergist tests were also conducted. The determination of the resistance intensity was also done when resistance was detected.

3.3.2. Synergist bioassays

To get an indication of the presence of a metabolic resistance mechanism, non-blood-fed, two-to-five-day-old female *An. gambiae* s.l. mosquitoes reared from larvae and pupae collected from areas with

suspected or confirmed resistance to DDT and pyrethroids were pre-exposed to the synergists piperonyl-butoxide (PBO), S.S.S-tributyl phosphorotrithioate (DEF), and/or Ethacrynic acid (E), Diethyl maleate (D), and Chlorfenethol (C) (together, EDC) for one hour. They then were exposed to diagnostic concentrations of insecticide: DDT 4% or pyrethroids (permethrin, deltamethrin, lambda-cyhalothrin, and alpha-cypermethrin) using WHO and CDC bioassay methods respectively due to the absence of DDT technical grade that could be used in the CDC bottle bioassay. Synergists' concentrations used for the tests were prepared according to the CDC protocol (38): 100µg/ bottle for PBO, 125µg/ bottle for DEF, and 80µg/ bottle for EDC, and used individually in all sites but Ankafina-Tsarafidy and Vohimarina where combinations of more than one synergist were used. In Ankafina-Tsarafidy PBO + EDC was used pre-DDT exposure and in Vohimarina PBO + DEF were pre-permethrin exposure. One hundred test mosquitoes (four replicates) and 50 control mosquitoes (two replicates) were used for the synergist bioassay. Mortality was recorded after 30 minutes of exposure to the pyrethroids (CDC bottle bioassay) and a 24-hour holding period for DDT (WHO bioassay). Test results of each insecticide with and without mosquitoes' pre-exposure to a synergist, pre-exposure to combinations of individual synergists, and/or without pre-exposure to synergists were compared.

3.3.3. Vector Molecular Characterization and Infection Rate

A sub-sample of mosquito specimens from resistance testing was sent to the University of Witwatersrand in South Africa for vector molecular characterization, specifically to conduct the following analyses:

- Identification of sibling species of *An. gambiae* s.l.: All *An. gambiae* s.l. were identified to species by using PCR as described by Scott et al. (1993). Samples identified as *An. gambiae* after the species-specific assay were further amplified to differentiate between *An. coluzzii* and *An. gambiae* s.s., formerly called M and S molecular forms, respectively, by PCR according to Favia et al. (2001).

- Detection of the knock down resistance (*kdr*) and *ace-1*^{R} mutation: A sample of *An. gambiae* s.s. and *An. arabiensiss* were tested for the Leu-Phe *kdr* mutation according to the protocol of Martinez-Torres et al. (1998) in live and dead mosquitoes. The *ace-1*^{R} mutation was diagnosed by PCR- RFLFP as described by Weill et al. (2004) in a sample of the vectors.

A sub-sample of mosquitoes collected using HLC during the routine monthly monitoring was sent to Institut Pasteur of Madagascar to determine infection rate.

3.4. RESIDUAL EFFICACY METHODOLOGY

The WHO cone bioassay tests were used to determine the residual efficacy of an insecticide on sprayed surfaces. Since PMI AIRS Madagascar does not have access to a susceptible colony in Madagascar, wild-caught mosquitoes reared from larvae at sentinel sites were used to determine the quality of spraying and subsequently to monitor the residual efficacy of insecticides sprayed. The susceptibility of the local vector to the insecticide sprayed in the area was determined before mosquitoes from the same

population were used for the cone bioassay testing. Bioassays were used to evaluate the quality of spraying by spray operators during the first two weeks of the start of IRS campaigns. The residual bio-efficacy of the insecticides was then monitored on monthly intervals. Two common surface types were selected from each of the different sites: thatch (Falafa) and wood or bamboo, were used for the cone bioassay data collection.

The mosquitoes were exposed to the sprayed surfaces for 30 minutes and the "knock-down" rate was recorded at 30 minutes and 60 minutes post exposure. The vector mortality was observed after a 24-hour recovery period.

3.5. DATA ANALYSIS METHODS

PSC data was used to calculate the density of vectors in a room using the formula:

Vector Density = Total number of vectors collected by species / Total number of rooms surveyed

The mean biting rate was computed from HLCs using the formula:

Mean Biting Rate = Total number of mosquitoes collected by species / Total number of collectors/ Total number of collection nights

The parity rate of identified *An. gambiae* s.l. vector species collected during PSC was calculated using the formula:

*Parity Rate = Total number of vectors parous / Number of vectors dissected *100*

WHO 2013 criteria was used to interpret susceptibility test results, as it was noted that:

- Susceptibility= Mortality rate of the exposed vector greater than 98%
- Possible Resistance= Mortality rates that are between 90% to 97%
- Resistance= Mortality rate after 24-recovery period is less than 90%.

When the control mortality was between 5% and 20%, observed mortality was corrected using Abbott's formula. An experiment was repeated when control mortality was more than 20%.

4. RESULTS AND DISCUSSION

4.1. SPECIES COMPOSITION, VECTOR DENSITIES AND VECTOR BEHAVIOR, JULY-FEBRUARY 2016

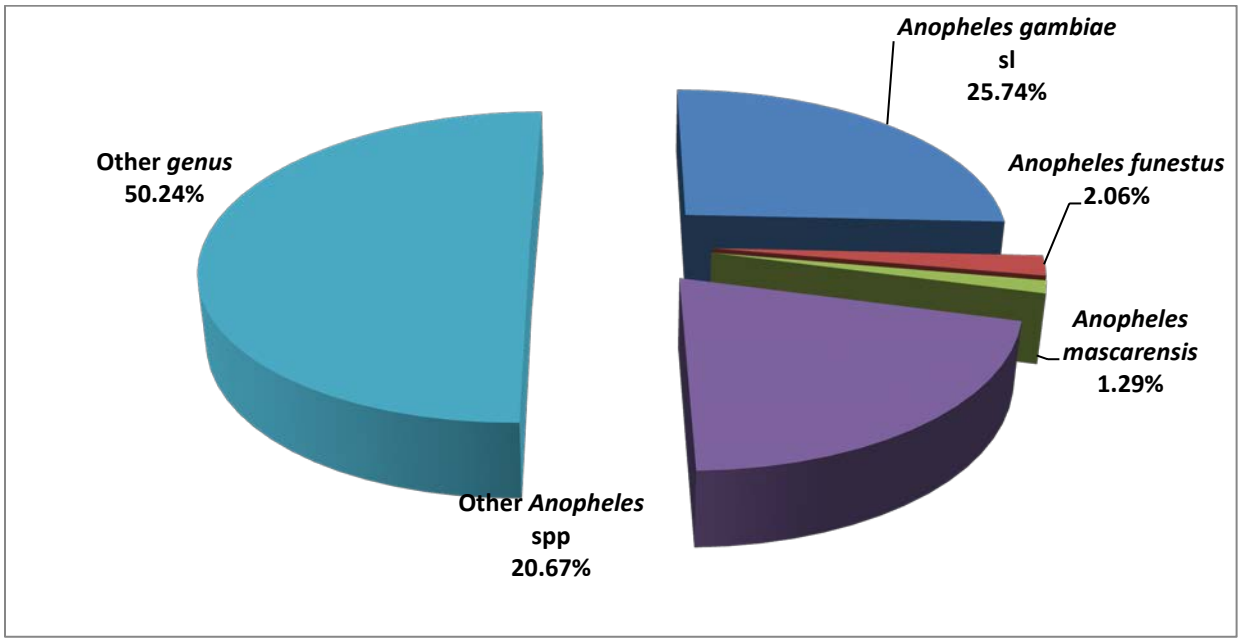
PMI AIRS Madagascar entomological teams collected 7,436 mosquitoes in total from all the sentinel sites between July 2015 – February 2016 using HLC, PSC, and outdoor collection (ODC) with aspirators. Listed below were the number and proportion of mosquitoes collected via each mosquito sampling method:

- HLC: 6986 (93.9%)
- PSC: 60 (0.8%)
- ODC: 390 (5.3%)

The results clearly indicate that HLC was the most productive sampling method in the collection of mosquitoes in Madagascar.

Species composition of the mosquitoes collected from July 2015 to February 2016 is noted in Figure 1, below, 49.8% of the mosquitoes collected were anopheline species and 29.1% of the *Anopheles* were vectors (*An. gambiae* s.l., *An. funestus* group and *An. mascarensis*).

FIGURE 1. VECTOR SPECIES AS A PERCENTAGE OF TOTAL MOSQUITOES COLLECTED



Vector species, *An. gambiae* s.l., *An. funestus* group and *An. mascarensis*, distribution varied by sentinel site. Overall, *An. gambiae* s.l. was collected from all sentinel sites and was noted as the primary and predominant vector species in the PMI-supported spray areas. Additionally, *An. gambiae* s.l. was the

only vector species collected from Ambodifaho (Brickaville). *An. funestus* was collected from Vohitrambato (Toamasina II), Vavatenina, Manambotra Sud (Farafangana) and Lopary (Vangaindrano). *An. mascarensis* was collected from Vohitrambato (Toamasina II), Vavatenina, Mahambo (Fenerive Est) and Manambotra Sud (Farafangana) (Table 3).

An. gambiae s.l., *An. funestus* and *An. mascarensis* were found co-existing in three districts, Toamasina II (Vohitrambato), Vavatenina (control site of the East) and Farafangana districts. The highest vector density was recorded in Vohitrambato during the surveillance period. *An. gambiae* s.l. was the most prevalent anopheline species found and accounted for 88.4% of the three vectors, followed by *An. funestus* (7.1%) (Table 3). *An. mascarensis* constituted about 4.5% only.

At baseline, the proportion of *An. gambiae* s.l., *An. funestus and An. mascarensis* was 69.7%, 21.2% and 9.1%, respectively. At the baseline, non-anopheline mosquitoes accounted for more than 60.4% of all the mosquitoes collected in the East Coast and 41.6% of mosquitoes collected in the South East.

TABLE 3. NUMBER OF MOSQUITOES COLLECTED AT EACH SENTINEL SITE, DISAGGREGATED BY VECTOR SPECIES

	Ambodifaho Brickaville	Vohitram bato (Toamasi na II)	Mahambo (Fenerive Est)	(Vavateni na)	Manambotra Sud (Farafangana)	Lopary (Vangain drano)	Total
An. gambiae s.l.	738	456	169	176	183	192	1,914
An. funestus	0	17	0	22	41	73	153
An. mascarensis	0	29	36	30	2	0	97
Other *Anopheles* sp.	6	658	145	223	245	259	1,536
Other *Genus*	1,023	416	631	519	325	822	3,736
Total	1,767	1,576	981	970	796	1,346	7,436

4.2. RESULTS OF HUMAN LANDING COLLECTION

From July to February, 2016, 6,986 female mosquitos were collected from six sentinel sites using human landing catches. Among these, 542 *An. gambiae* s.l. (7.8%) were collected indoors and 1,173 (16.8%) were collected outdoors.

Regarding the other malaria vectors, the numbers collected were low compared to *An. gambiae* s.l. 53 *An. funestus* were collected indoors and 91 outdoors, while 34 *An. mascarensis* were collected indoors

and 51 outdoors. No statistically significant difference was observed in biting location in *An. mascarensis* (p=0.082). *An. funestus* had an exophagic tendency (p=0.0019).

An. gambiae s.l. human biting rates were very low before spraying, except in Vohitrambato and Ambodifaho, most likely related to environmental factors (e.g., low or no rainfall and hence few breeding sites before IRS). In most spray areas, the vector biting rates inside houses decreased post spraying in comparison with the baseline. *An. gambiae* s.l. appeared to have exophagic tendencies both in the East Coast and in the South East (Table 4). Post IRS, the overall proportion of *An. gambiae* s.l. caught while seeking a blood meal indoors was lower than those caught outdoors. Results were statistically significant (p<0.001). Owing to the small number of mosquitoes collected, village by village comparison in feeding location did not result in statistically significant differences between outdoor and indoor feeding.

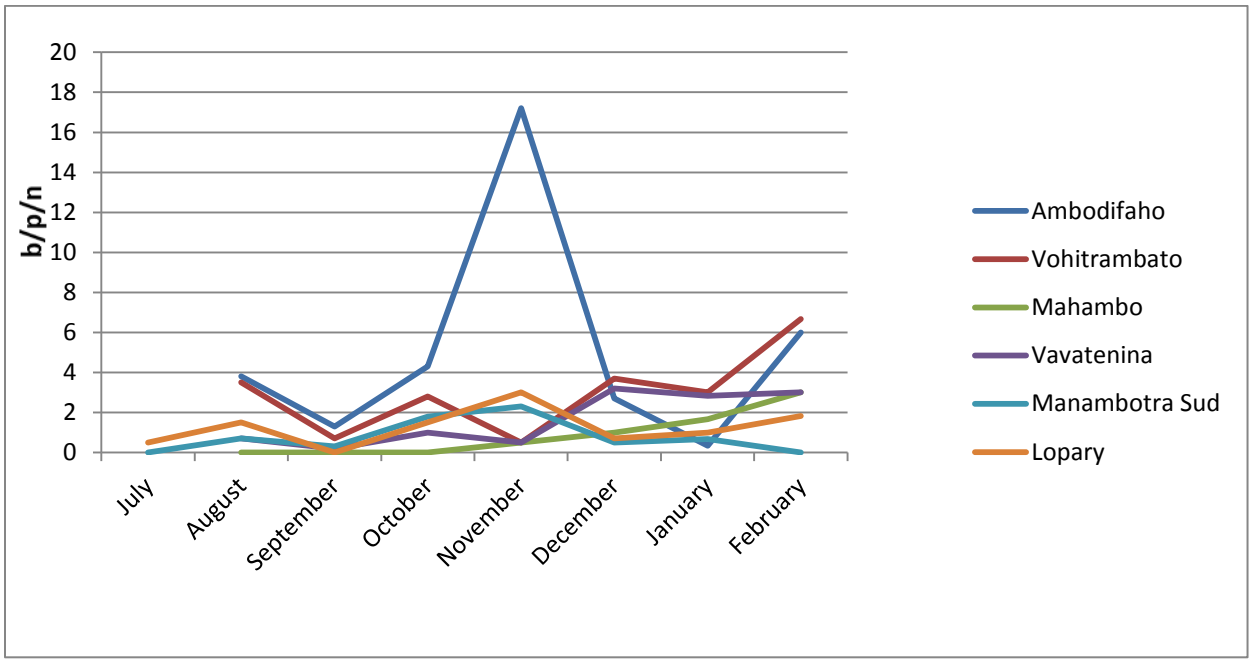
The vectors showed an exophagic tendency in all sites. When HLC data from all the villages were combined, the proportion of *An. gambiae* s.l. caught while seeking human blood outdoors was significantly higher than indoors (p<0.001) (Table 4).

TABLE 4. NUMBER OF MOSQUITOS COLLECTED BY HLC AND MAN BITING RATES (BITES/PERSON/NIGHT = B/P/N), JULY 2015 - FEBRUARY 2016

Sites	Month	*An.gambiae s.l.*				*An. funestus*				*An. mascariensis*				Other *Anopheles*			
		Indoor	Indoor (b/p/n)**	Outdoor	Outdoor (b/p/n)**	Indoor	Indoor (b/p/n)**	Outdoor	Outdoor (b/p/n)**	Indoor	Indoor (b/p/n)**	Outdoor	Outdoor (b/p/n)**	Indoor	Indoor (b/p/n)**	Outdoor	Outdoor (b/p/n)**
Ambodifaho, Brickaville	August*	23	3.8	15	2.5	0	0	0	0	0	0	0	0	1	0.17	1	0.2
	September	8	1.3	23	3.8	0	0	0	0	0	0	0	0	0	0	1	0.2
	October[1]	26	4.3	85	14.2	0	0	0	0	0	0	0	0	0	0	1	0.2
	November	103	17.2	212	35.2	0	0	0	0	0	0	0	0	0	0	1	0.2
	December	16	2.7	47	7.8	0	0	0	0	0	0	0	0	0	0	1	0.2
	January	2.0	0.3	27.0	4.5	0	0	0	0	0	0	0	0	0	0	0	0
	February	36.0	6.0	40.0	6.7	0	0	0	0	0	0	0	0	0	0	0	0
Vohitrambato, Toamasina II	August*	21	3.5	91	15.2	0	0	3	0.5	7	1.2	14	2.3	9	1.5	62	10.3
	September	4	0.7	10	1.2	0	0	1	0.2	2	0.3	2	0.3	11	1.8	116	19.3
	October	17	2.8	64	10.7	0	0	5	0.8	1	0.17	2	0.3	21	3.5	76	12.7
	November	3	0.5	13	2.2	0	0	1	0.2	0	0.0	0	0.0	2	0.3	14	2.3
	December	22	3.7	44	7.3	2	0.3	4	0.7	0	0.0	0	0.0	10	1.7	102	17
	January	18.0	3.0	44.0	7.3	0	0	1	0.2	0	0	0	0	8	1.3	55	9.2
	February	40.0	6.7	50.0	8.3	0	0	0	0	0	0	0	0	47	7.8	93	15.5
Mahambo, Fenerive Est	August*	0	0.0	1	0.2	0	0	0	0	18	3	15	2.5	0	0	0	0
	September	0	0	7	1.2	0	0	0	0	0	0	0	0	0	0	3	0.5
	October	0	0.0	14	2.3	0	0	0	0	0	0	0	0	3	0.5	4	0.7.
	November	3	0.5	2	0.3	0	0	0	0	0	0	0	0	1	0.17	0	0
	December	6	1.0	13	2.2	0	0	0	0	0	0	0	0	2	0.3	3	0.5
	January	10.0	1.7	22.0	3.7	0	0	0	0	0	0	0	0	23	3.8	40	6.7
	February	18.0	3.0	25.0	4.2	0	0	0	0	0	0	0	0	25	4.2	25	4.2
Vavatenina, control East (control site for east)	August*	4	0.7	10	1.7	1	0.2	1.0	0.2	1	0.2	2.0	0.3	3	0.5	8	1.3
	September	1	0.2	0	0	3	0.5	5.0	0.8	0	0.2	0.0	0.3	8	1.3	15	2.5
	October	6	1.0	15	2.5	0	0.0	1.0	0.2	1	0.2	1.0	0.17	14	2.3	10	1.7
	November	3	0.5	14	2.3	0	0.0	0.0	0.2	2	0.3	4.0	0.7	20	3.3	28	4.7
	December	19	3.2	20	3.3	1	0.2	7.0	1.2	2	0.3	7.0	1.2	7	1.2	42	7.0
	January	17.0	2.8	16.0	2.7	1	0.2	1	0.2	0	0	2	0.3	10	1.7	17	2.8
	February	18.0	3.0	15.0	2.5	0	0	0	0	0	0	0	0	7	1.2	9	1.5
Manambotra Sud, Farafangana	July*	0	0.0	2	0.3	1	0.2	11	1.8	0	0	1	0.17	6	1	18	3.0
	August	4	0.7	2	0.3	2	0.3	2	0.3	0	0	0	0	7	1.2	13	2.2

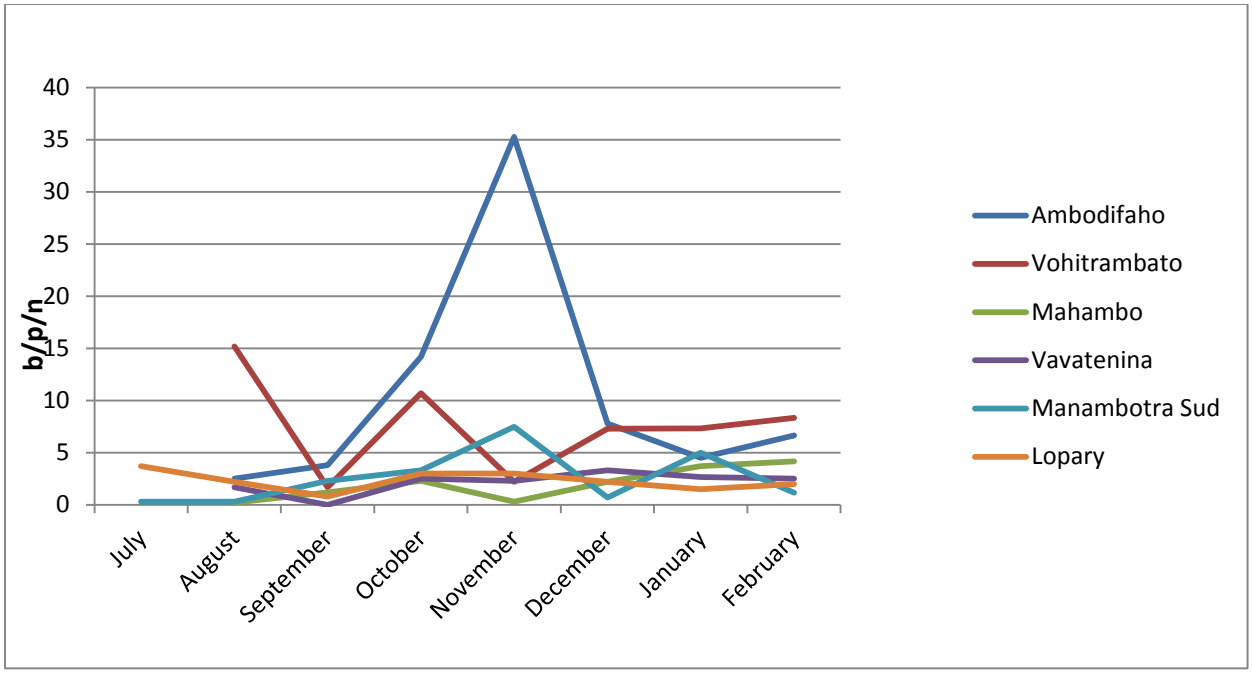
	September	2	0.3	14	2.3	1	0.2	1	0.2	0	0	1	0.17	5	0.8	23	3.8
	October	11	1.8	20	3.3	1	0.2	2	0.3	0	0	0	0	6	1	17	2.8
	November	14	2.3	45	7.5	2	0.3	1	0.17	0	0	0	0	10	1.7	24	4.0
	December	3	0.5	4	0.7	1	0.2	3	0.5	0	0	0	0	2	0.3	5	0.8
	January	4.0	0.7	30.0	5.0	0	0	8	1.3	0	0	0	0	1	0.2	63	10.5
	February	0.0	0.0	7.0	4.7	0	0	0	0	0	0	0	0	17	2.8	14	2.3
Lopary, Vangaindrano, (control site for south East)	July*	3	0.5	22	3.7	0	0.0	1	0.2	0	0	0	0	1	0.2	11	1.8
	August	9	1.5	13	2.2	2	0.3	0	0.0	0	0	0	0	5	0.8	6	1.0
	September	0	0.0	5	0.8	13	2.2	14	2.3	0	0	0	0	15	2.5	33	5.5
	October	9	1.5	18	3.0	17	2.8	11	1.8	0	0	0	0	24	4.0	61	10.2
	November	18	3.0	18	3.0	4	0.7	1	0.2	0	0	0	0	2	0.3	1	0.2
	December	4	0.7	13	2.2	1	0.2	1	0.2	0	0	0	0	10	1.7	18	3.0
	January	6.0	4.0	9.0	1.5	6.0	0	0	0	0	0	0	0	22	3.7	19	3.2
	February	11.0	7.3	12.0	2.0	11.0	0	0	0	0	0	0	0	0	0	0	0

FIGURE 2. MONTHLY DISTRIBUTION OF INDOOR MAN BITING RATE (BITES/PERSON/NIGHT: B/P/N) AT SENTINEL SITES



Note: July was baseline for the South East districts: Farafangana, Vangaindrano; August was baseline for Ambodifaho, Vohitrambato, Mahambo and Vavatenina.

FIGURE 3. MONTHLY DISTRIBUTION OF OUTDOOR MAN BITING RATE (BITES/PERSON/NIGHT: B/P/N) AT SENTINEL SITES

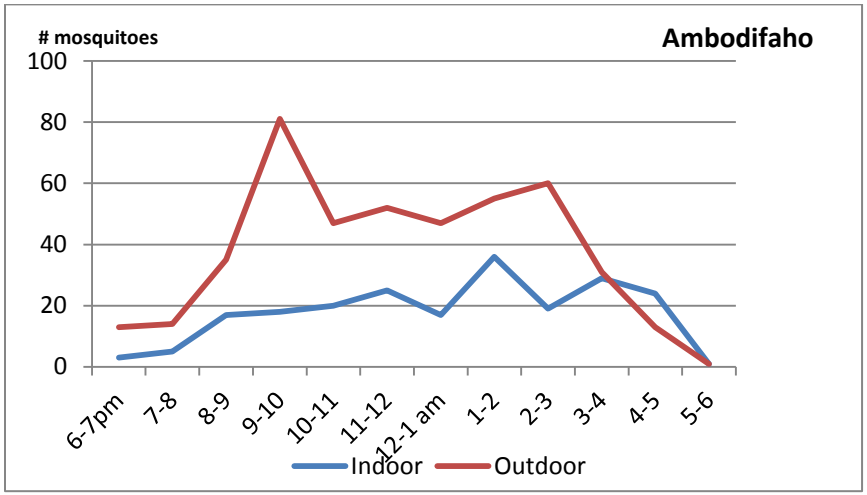


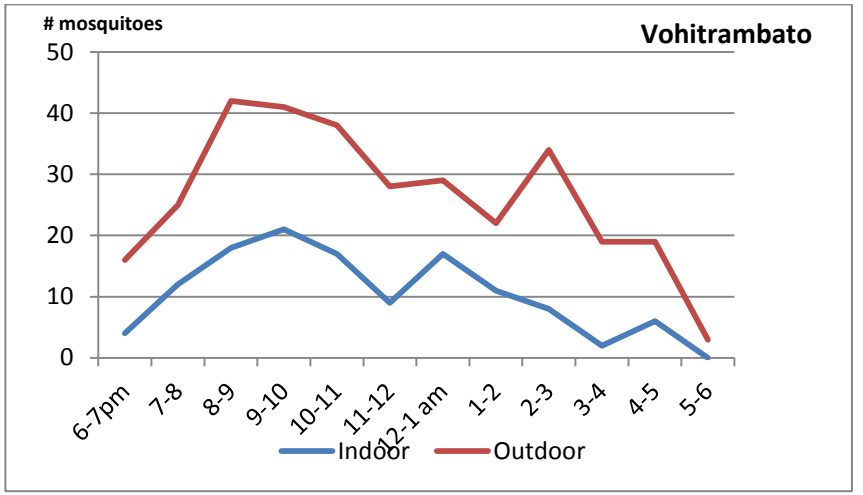
Note: July was baseline for the South East districts: Farafangana, Vangaindrano; August was baseline for Ambodifaho, Vohitrambato, Mahambo and Vavatenina.

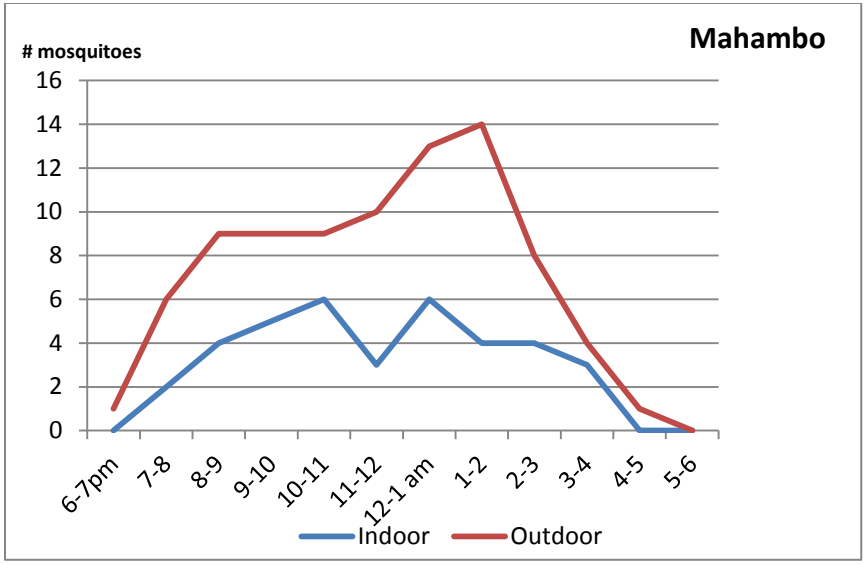
4.3 PEAK BITING TIME

From the entomological monitoring data collected during the period that this report covers, it was noted that the peak biting time for mosquitoes seemed to vary by sentinel site (Figure 4 and Figure 5). In some sites like Ambodifaho (Brickaville) and Vohitrambato (Toamasina II) significant mosquito biting started as early as 20:00 and continued until 04:00. In Mahambo (Fenerive Est) most *An*. *gambiae* s.l. mosquitoes seem to bite in the second half of the night with the peak biting time recorded from 12:00 to 02:00. In the control villages, Vavatenina and Lopary, *An*. *gambiae* s.l. was caught more when seeking human blood in the first half of the night. It was difficult to draw a conclusion on the feeding habit of the vector based on the current data for some of the sites. For the other vectors (i.e., *An. funestus* and *An. mascarensis*), either due to the absence of consistent biting pattern or the small number of mosquitoes collected. Additional data is required to fully understand their biting habits in the country.

FIGURE 4. *An. gambiae* s.l. BITING HOURS AT EAST COAST SENTINEL SITES AND CONTROL






Mahambo
mosquitoes
16
14
12
10
8
6
4
2
0
6-7pm
7-8
8-9
9-10
10-11
11-12
12-1 am
1-2
2-3
3-4
4-5
5-6
Indoor
Outdoor

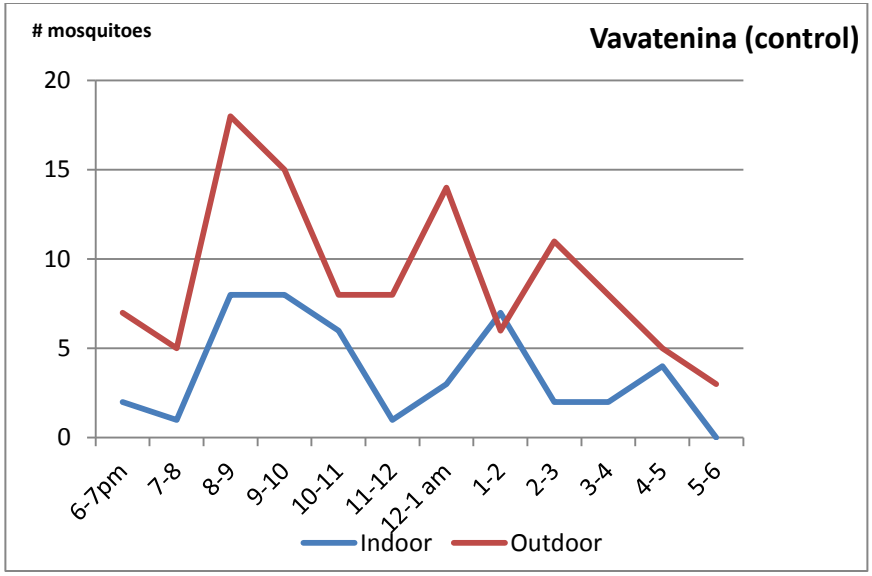
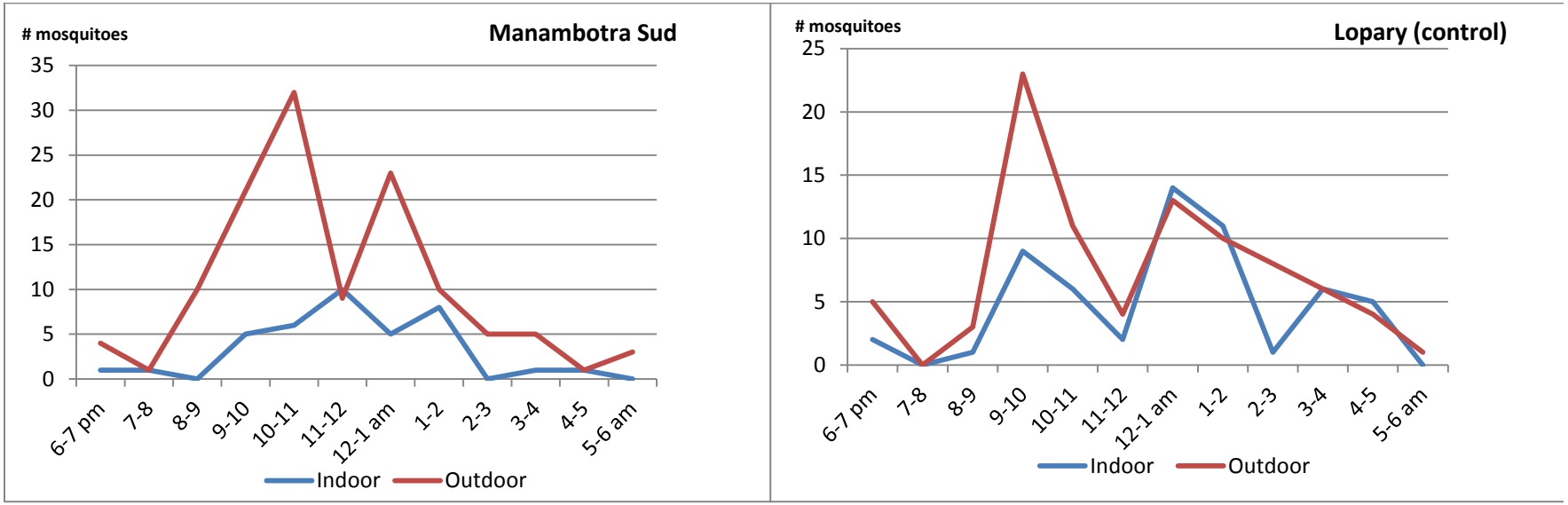

Vavatenina (control)
mosquitoes
20
15
10
5
0
6-7pm
7-8
8-9
9-10
10-11
11-12
12-1 am
1-2
2-3
3-4
4-5
5-6
Indoor
Outdoor

FIGURE 5. *AN. GAMBIAE* S.L. BITING HOURS AT SOUTH EAST SENTINEL SITES AND CONTROL



4.4. RESULTS OF OUTDOOR COLLECTION

A total of 186 *An. gambiae* s.l. were collected resting outdoors in natural and pit shelters using aspirators from all six sites in the South East and East Coast of Madagascar. Only four *An. funestus* were collected via outdoor collection from two sites: Vohitrambato and Lopary. Ten *An. mascarensis* were collected from three sites (Vohitrambato, Mahambo and Vavatenina) via outdoor collection (Table 5).

TABLE 5. TOTAL NUMBER OF MOSQUITOS COLLECTED BY OUTDOOR COLLECTION WITH ASPIRATOR (ODC) METHOD, JULY 2015 - FEBRUARY 2016

Species	Ambodifaho Brickaville	Vohitrambato Toamasina II	Mahambo Fenerive Est	Vavatenina	Manambotra Sud Farafangana	Lopary Vangaindrano	Total
An. gambiae s.l.	75	13	48	13	21	16	186
An. funestus	0	0	0	1	0	3	4
An. mascariensis	0	1	2	7	0	0	10
Other *Anopheles* sp.	0	36	6	32	8	7	89
Other *Genus*	0	25	17	31	17	15	105
Total	75	75	73	84	46	41	394

4.5. INDOOR RESTING ANOPHELINE DENSITIES

From July to February, 2016, PMI AIRS Madagascar determined indoor resting density using PSC in six sentinel sites. The indoor vector density was low (0.0 to 0.1 vector per room per day) at baseline, and remained low (at or close to zero) throughout the collection period.

4.6 PARITY RATES

At the baseline, the parity rate of *An. gambiae* s.l. was high in Toamasina II (86%; n=112), Brickaville (100%; n=38) and Vavatenina (control site) (68.7%; n=16) but was low in Lopary (13.7%; n=51). The number of mosquitoes collected and dissected from Farafangana and Fenerive Est was small with a parous rate of 16.7% (n=12) and 100% (n=1), respectively. After IRS, parity rates reduced to 5.6% (n=179), 61.4% (n=700), 46.8 %(n=347), and 61.3 %(n=169) in Farafangana, Brickaville, Toamasina II, and Fenerive Est, respectively. Two-sided McNemar's chi-square test of two paired sample test was used

to assess if the observed parity rate reduction was statistically significant when pre spray data was compared with post spray. In all four spray areas where vector surveillance was conducted, post-spray reduction of the proportion of parous mosquitoes compared to pre spray was statistically significant (Table 6). In the South East control site, Lopary, an increase in parity rate was observed post spray when compared to pre-spray and the difference was statistically significant ($p< 0.001$). In the other control site, Vavatenina, post IRS parity rates were significantly lower that pre-spray ($p<0.001$).

Chi-square test was used to compare parity rate data between the intervention and control sites. No statistically significant difference was observed between Farafangana (intervention) and Lopary (control) when pre spray data was compared ($p=0.79$). However, post spray parity rate in Farafangana was significantly lower than Lopary ($p=0.002$). This reduction in parity might at least partially be attributed to the impact of IRS. Similarly, we used data obtained from the East Coast and Vavatenina to compare the parity rate between the intervention and control site. At the baseline, before spray, the parity rate was higher in the intervention villages as compared to the control site ($p=0.018$). However, post IRS, the parity rate in the intervention villages was reduced, narrowing down the difference between the two arms. Hence, no statistically significant difference was observed between the two sites post spray. It appears that the impact of IRS was obscured due to higher parity rates in the intervention areas at the baseline (Table 6 and Table 14 in Annex).

At baseline, *An. funestus* was collected from three out of the six sentinel sites, namely Lopary, Toamasina II and Vavatenina. The proportion of parous recorded in these three sites were 0% (n=1), 86.4 %(n=22), 62.55(n=8) in Lopary, Toamasina II and Vavatenina, respectively). Post-spray this species was collected from the same sites and parity rates ranged from 34.4% in Lopary to 77.8% in Vohitrambato (Table 6 and Table 14 in Annex).

We compared parity rates of *An. funestus* between the intervention and control villages for the East Coast. There was no significant difference before and post IRS ($p=0.148$ and 0.608) (data not shown).

TABLE 6. PARITY RATE COMPARISON

Sentinel sites	*An. gambiae* s.l.						P value(pre and post IRS parity comparison)
	Pre-IRS			Post IRS			
	# dissected	# parous	% parous	# dissected	# parous	% parous	
Manambotra Sud (Farafangana) (Intervention South East (SE))	12	2	16.7	179	10	5.6	**< 0.001***
Lopary (Vangaindrano): Control(SE)	51	7	13.7	163	26	16	<.001*
Comparison of Farafangana VS Lopary	p=0.79			p=0.002*			
Ambodifaho (Brickaville)	38	38	100.0	700	430	61.4	<.001*

Vohitrambato (Toamasina II)	112	96	85.7	347	157	45.2	<.001*
Mahambo (Fenerive Est)	1	1	100.0	117	76	65	<.001*
Total: East Coast (Intervention)	151	135	89.4	1164	663	57	<.001*
Vavatenina: Control(East Coast)	16	11	68.8	160	90	56.3	<.001*
Comparison between intervention and control(East Coast)	p=0.018*			p=0.865			

NOTE: *SIGNIFICANT AT P<0.05.

4.7. Infection Rates

Among the 500 *An gambiae* s.l. mosquito samples processed to assess the rate of their infection with malaria parasites only one sample was positive for *Plasmodium* giving a sporozoite rate of 0.2%.

4.8. CONE BIOASSAY TEST RESULTS

During the first week of IRS campaigns in the East Coast and in the South East, PMI AIRS Madagascar conducted cone bioassay tests to assess whether the quality of the spraying was satisfactory. The results indicated that the spray quality, both in the East Coast and in the South East, was good, mortality being 100% for all the structures sampled at T0 and T1. T1 was used as baseline for the subsequent monitoring due to the airborne effect of pirimiphos-methyl, as mortality at T0 may be due to this effect and not necessarily due to the insecticide applied on the wall. PMI AIRS Madagascar subsequently collected monthly cone bioassay data using the World Health Organization (WHO) procedure to assess the residual bio-efficacy of insecticides sprayed during the 2015 IRS campaign. The tests were conducted in the following sentinel sites: Ambodifaho (district of Brickaville), Vohitrambato (district of Toamasina II), and Mahambo (district of Fenerive Est) in the East Coast; and Manambotra Sud (district of Farafangana) in the South East.

In the East Coast, pirimiphos-methyl CS 300 remained effectiveness for seven months (Figure 6). Similar results were obtained in the South East (Figure 7).

FIGURE 6. RESIDUAL EFFECTIVENESS OBSERVED FOR PIRIMIPHOS-METHYL (ORGANOPHOSPHATES) IN THE EAST COAST

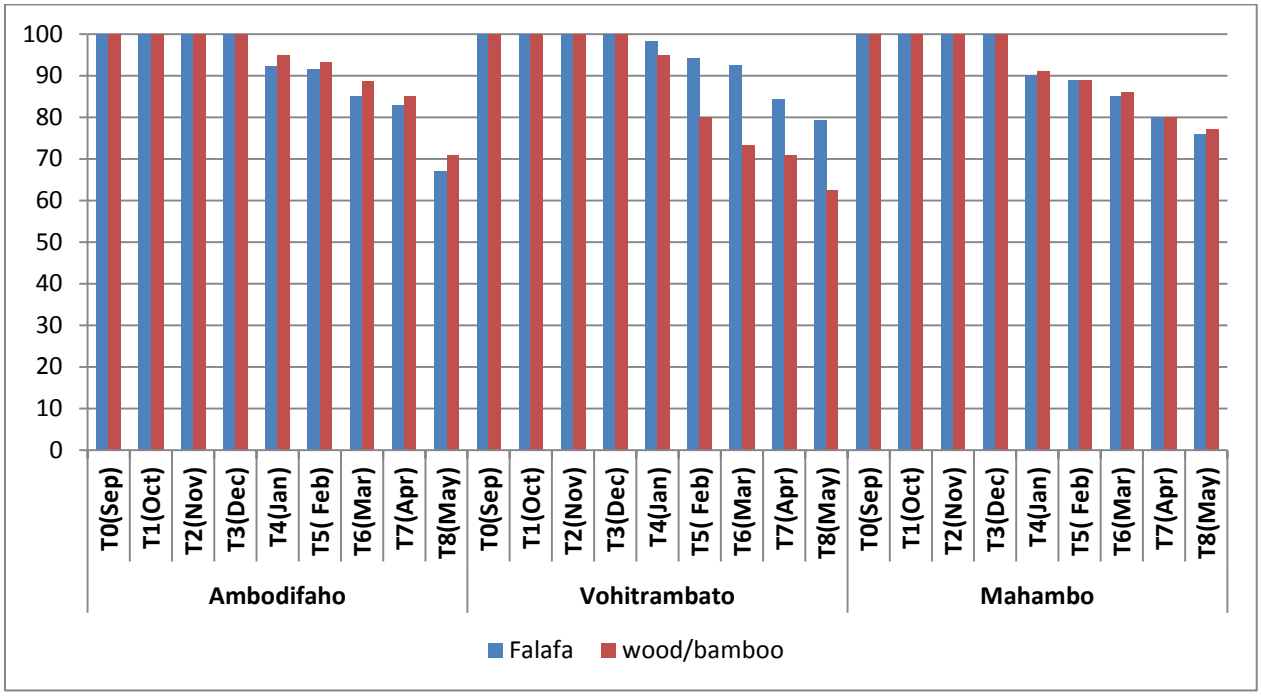
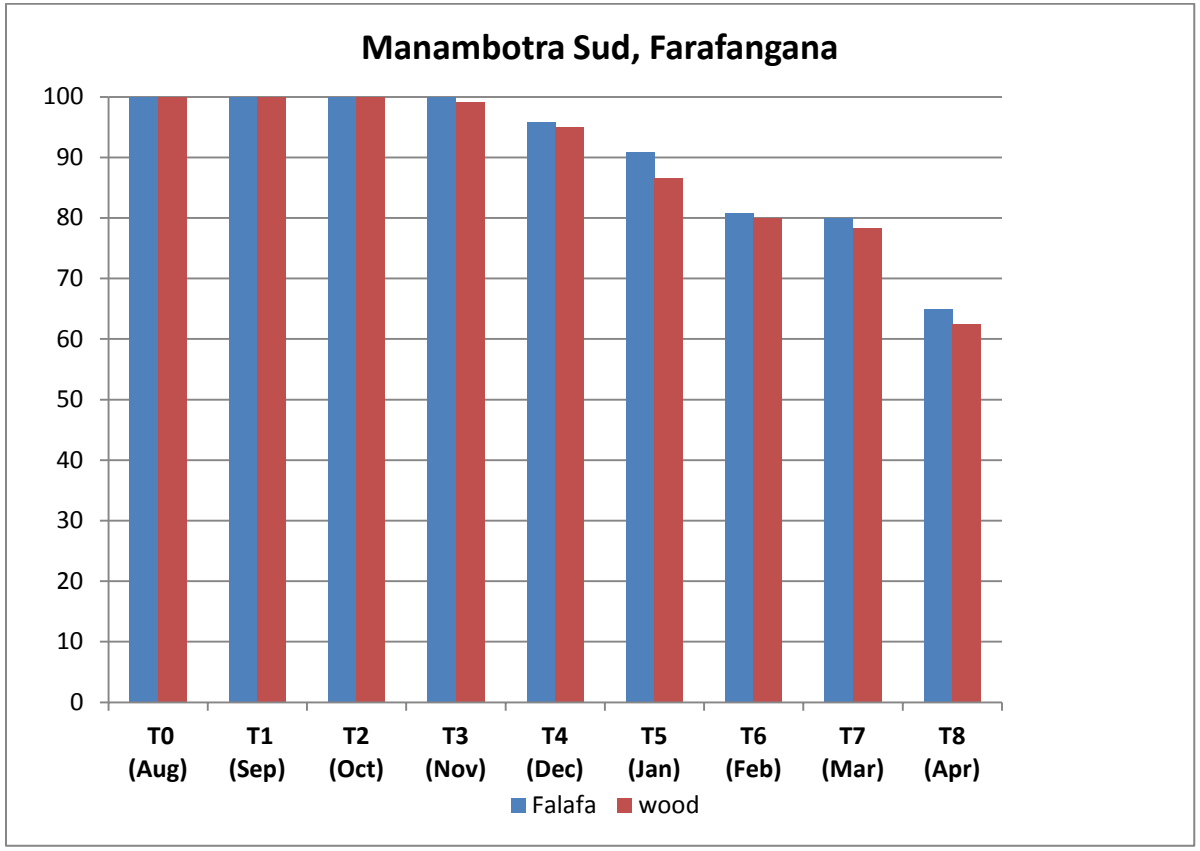


FIGURE 7. RESIDUAL EFFECTIVENESS OBSERVED FOR PIRIMIPHOS-METHYL (ORGANOPHOSPHATES) IN THE SOUTH EAST



4.9. INSECTICIDE SUSCEPTIBILITY TEST RESULTS

4.9.1 WHO tube tests showed:

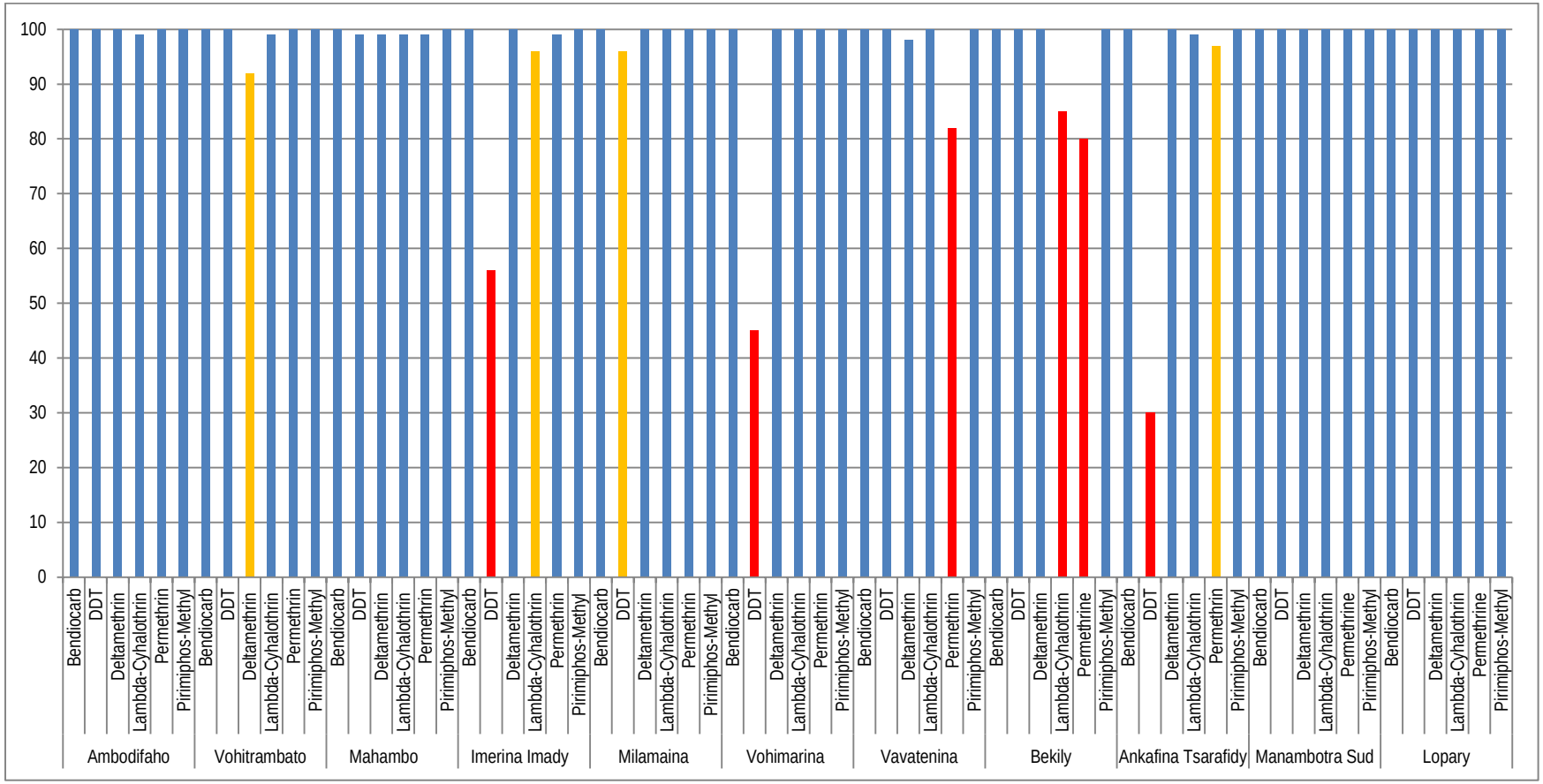
An. gambiae s.l. has developed resistance to:

- DDT in Imerina Imady, Vohimarina and Ankafina Tsarafidy;
- Permethrin in Mahambo, Vavatenina and Bekily; and
- Lambda- cyhalothrin in Bekily.

Possible resistance was observed for:

- DDT in Mahambo;
- Deltamethrin in Vohitrambato;
- Permethrin in Ankafina Tsarafidy; and
- Lambda-cyhalothrin in Imerina Imady.

FIGURE 8. RESULTS OF INSECTICIDE SUSCEPTIBILITY TESTS USING WHO TUBE TEST FOR *AN. GAMBIAE* S.L.



4.9.2. CDC bioassays showed that:

An. gambiae s.l. has developed resistance to:

- Permethrin in Bekily, Vavatenina and Ankafina Tsarafidy.

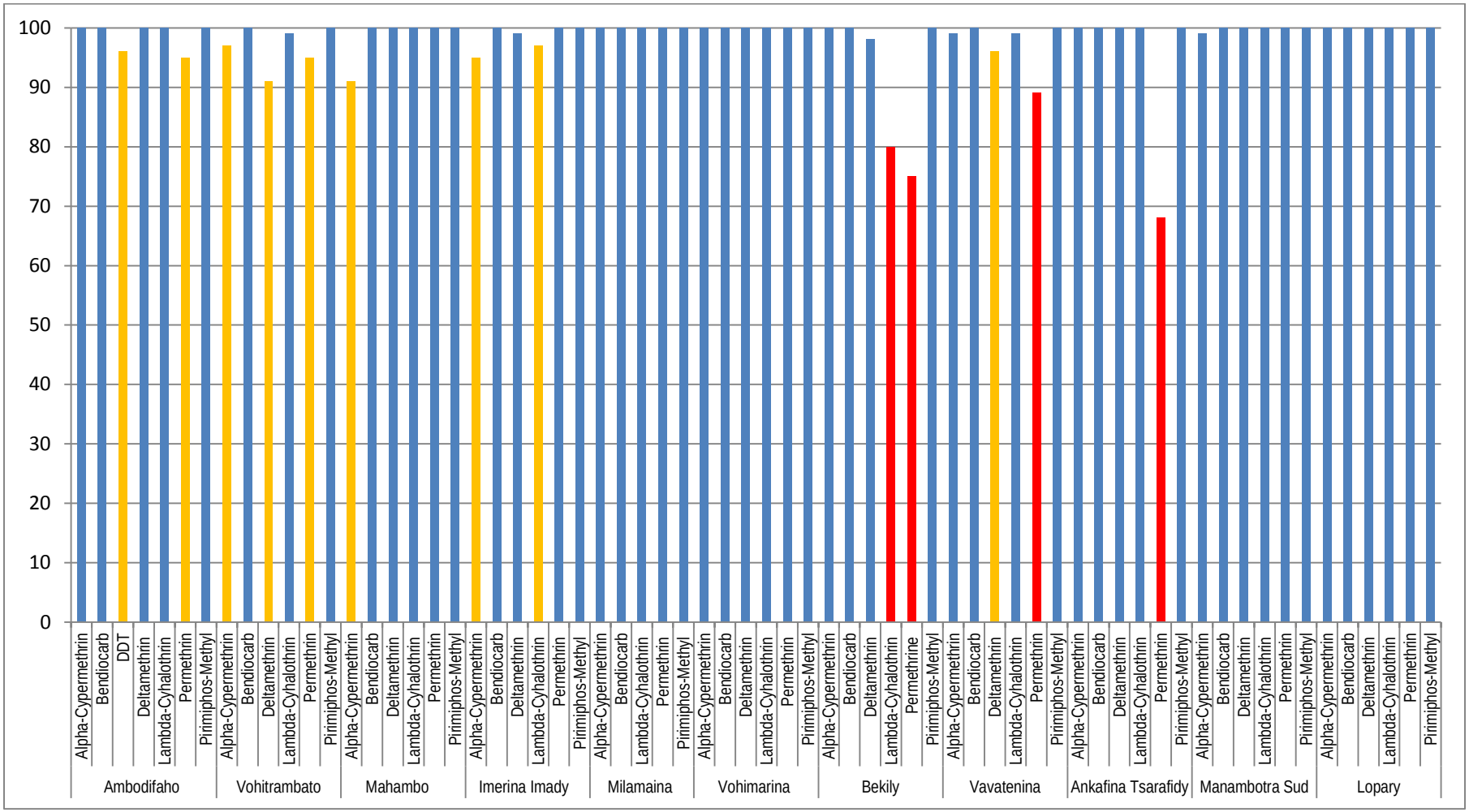
Possible resistance was observed for:

- DDT in Ambodifaho;
- Deltamethrin in Vohitrambato and Vavatenina;
- Permethrin in Ambodifaho and Vohitrambato;
- Lambda-cyhalothrin in Imerina Imady; and
- Alphacypermethrin in Vohitrambato, Mahambo and Imerina Imady.

The emergence of insecticide resistance to permethrin and other pyrethroids might be due to the wider use of long-lasting insecticide-treated nets (LLINs) for several years in the East Coast and the South East, IRS with alpha-cypermethrin /deltamethrin for several years in the Central Highlands (Imerina Imady, Ankafina Tsarafidy, Milamaina and Vohimarina), as well as use of similar insecticides in agriculture, or a combination of those factors.

The results of the susceptibility tests using the WHO tube and CDC bottle methods are shown in Figures 8 and 9 below and Table 8.

FIGURE 9. RESLTS OF INSECTICIDE SUSCEPTIBILITY TESTS USING CDC BOTTLE BIOASSY FOR *AN. GAMBIAE* S.L.



4.8.3 An. funestus and *An. mascarensis*

Table 7 summarizes the susceptibility level where tested of *An. funestus* and *An. mascarensis* in Madagascar.

An. funestus is susceptible to:

- Pirimiphos-methyl and deltamethrin in Farafangana; and
- Pirimiphos-methyl in Vohitrambato Toamasina.

An. mascarensis is susceptible to:

- Pirimiphos-methyl and deltamethrin in Mahambo, Fenerive Est;
- to permethrin in Brickaville; and
- deltamethrin in Farafangana and Vavatenina.

TABLE 7. *An. funestus* AND *An. mascarensis* CDC INSECTICIDE SUSCEPTIBILITY TEST RESULTS BY SITE/VILLAGE

District	Site / Village	Vector mosquito tested	Insecticide tested	% Mortality(N)	Resistance status
Farafangana	Manambotra Sud (SE)	*An. funestus*	Pirimiphos-methyl	100(30)	S
		An. funestus	Deltamethrin	100(75)	S
		An. mascarensis	Deltamethrin	100(20)	S
Toamasina II	Vohitrambato (EC)	*An. funestus*	Pirimiphos-methyl	100(35)	S
Fenerive Est	Mahambo (EC)	*An. mascarensis*	Pirimiphos-methyl	100(18)	S
		An. mascarensis	Deltamethrin	100(50)	S
Brickaville	Sahamatevina (EC)	*An. mascarensis*	Permethrin	100(50)	S
Vavatenina	Vavatenina (EC)	*An. mascarensis*	Deltamethrin	100(65)	S

4.9.4. Assessment of resistance intensity

- The susceptibility test performed with permethrin at the 2x diagnostic dose killed 94% of the mosquitos tested in Vohitrambato, Toamasina II.
- Permethrin 2x killed 100% of the mosquitos tested in Vavatenina, Ankafina Tsarafidy and Ambodifaho, Brickaville.
- Deltamethrin 2x killed 100% of the mosquitos tested in Vavatenina.
- Alphacypermethrin 2x killed 100% of the mosquitos tested in Ankafina Tsarafidy

TABLE 8. INTENSITY RESISTANCE

Site	species tested	INSECTICIDES	2x diagnostic dosage		1X diagnostic dosage	
			# mosquitos tested (N)	% mortality	# mosquitos tested (N)	% mortality
Vohitrambato, Toamasina II	*An. gambiae* s.l.	Permethrin	100	94%	100	95%
Vavatenina	*An. gambiae s.l.*	Permethrin	100	100%	100	89%
Vavatenina	*An. gambiae s.l.*	Deltamethrin	100	100%	100	96%
Ankafina Tsarafidy	*An. gambiae s.l.*	Permethrin	100	100%	100	68%
Ambodifaho, Brickaville	*An. gambiae s.l.*	Permethrin	100	100%	100	95%

4.9.5. Synergists

Results of synergist bioassays are summarized in Figures 10 to 15. Pre-exposure to PBO and EDC either fully or partially restored susceptibility to DDT. Pre-exposure to PBO restored complete susceptibility to all three insecticides from the pyrethroid class except in one site, Ambodifaho, where PBO only partially restored susceptibility to permethrin. Pre-exposure to PBO fully eliminated *An. gambiae* s.l. resistance to DDT in three of four sites but restored susceptibility only partially in one of the four sites.

Pre-exposure to the synergist DEF fully synergized *An. gambiae* s.l. susceptibility to deltamethrin and alpha-cypermethrin. It also partially and fully restored *An. gambiae* s.l. susceptibility to permethrin in two of three, and one of three sites, respectively.

FIGURE 10. MORTALITY OF AN. GAMBIAE S.L. POPULATIONS OBSERVED DURING TWO-HOUR EXPOSURE TO CDC BOTTLES TREATED WITH LAMBDA-CYHALOTHRIN WITH AND WITHOUT PRE-EXPOSURE TO SYNERGISTS IN IMERINA IMADY

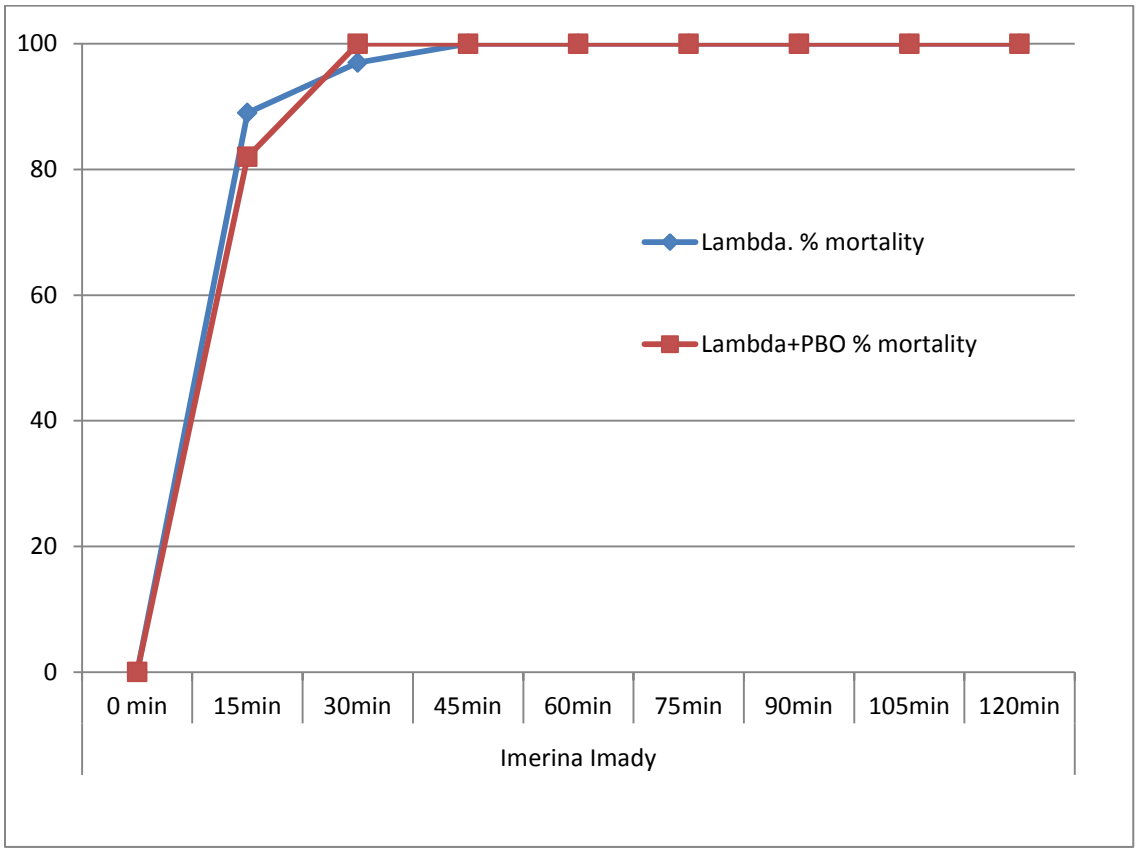


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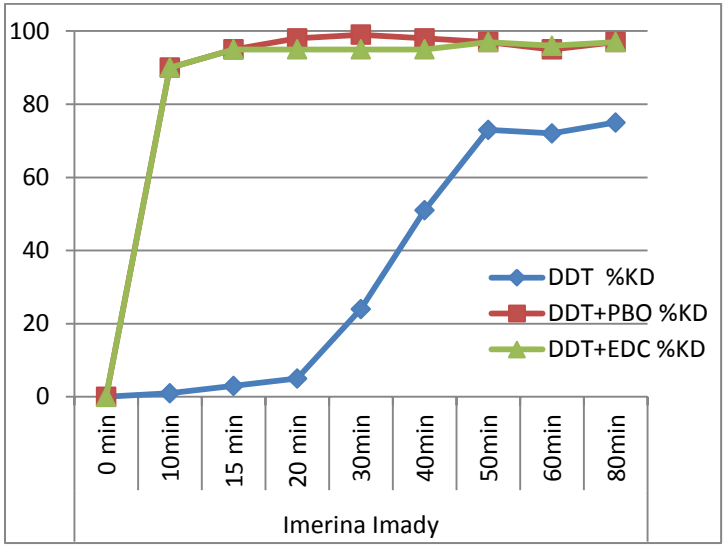
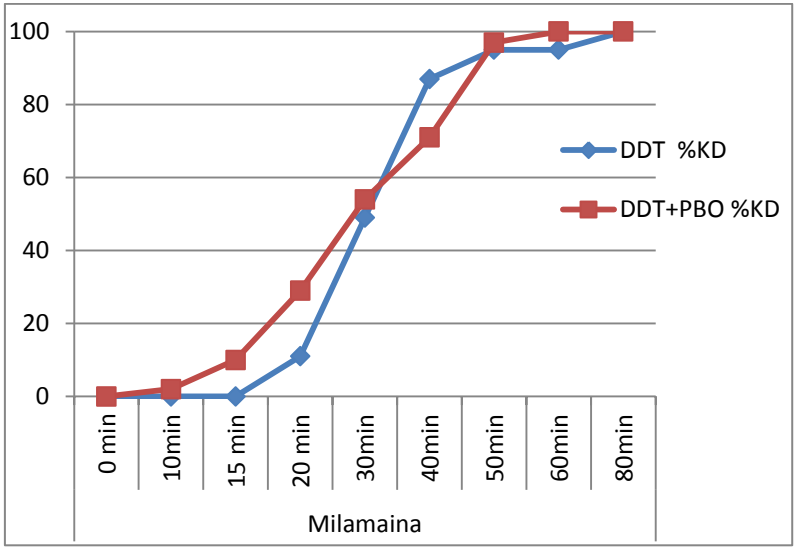
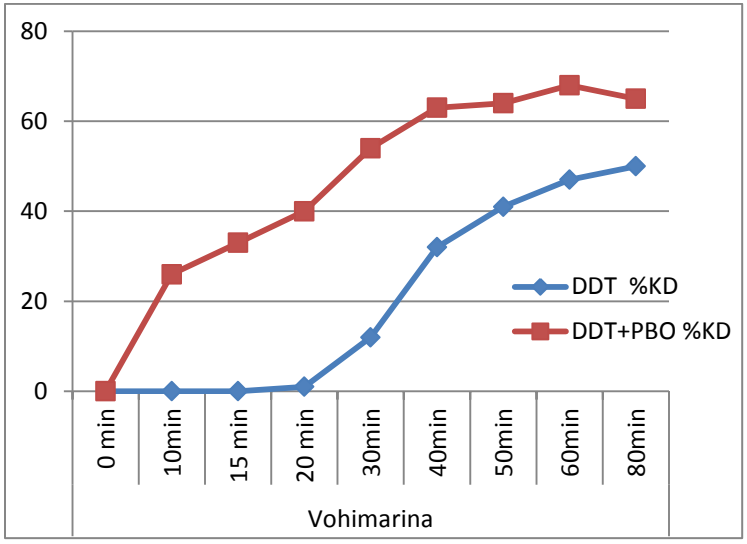
FIGURE 11. PERCENT *KDR* OF *AN. GAMBIAE* S.L. TESTED AGAINST 4% DDT IMPREGNATED PAPERS USING WHO TUBE BIOASSAY WITH AND WITHOUT PRE-EXPOSURE TO SYNERGISTS IN FOUR SITES IN MADAGASCAR







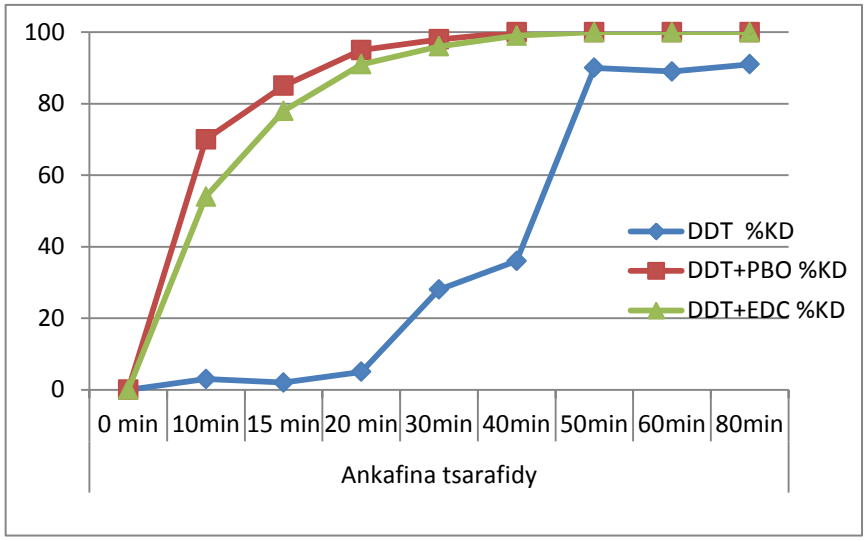
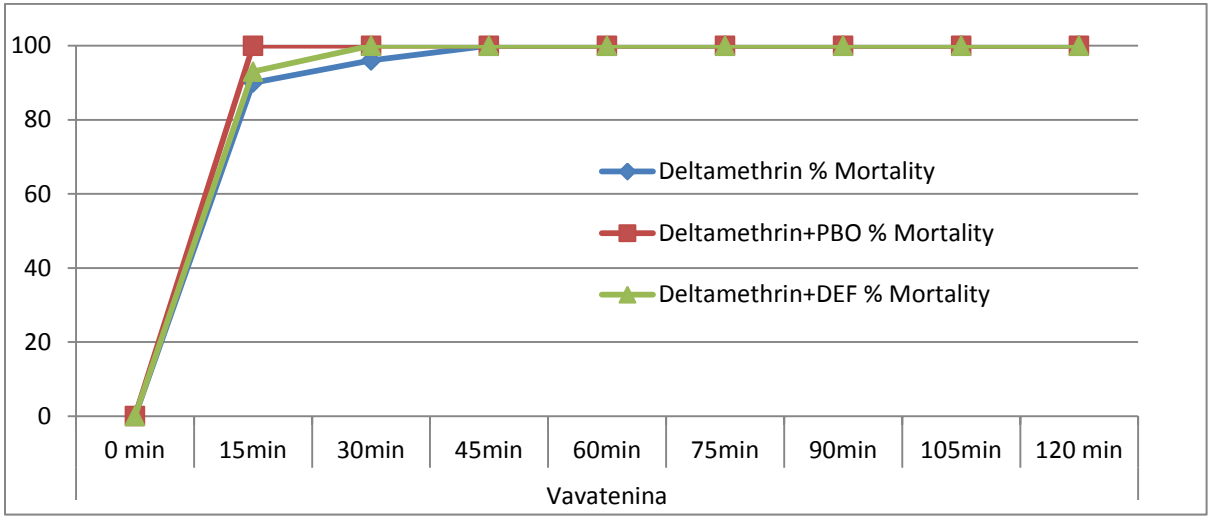


FIGURE 12. MORTALITY OF *AN. GAMBIAE* S.L. POPULATIONS OBSERVED DURING TWO-HOUR EXPOSURE TO CDC BOTTLES TREATED WITH DELTAMETHRIN WITH AND WITHOUT PRE-EXPOSURE TO SYNERGISTS IN TWO SITES IN MADAGASCAR



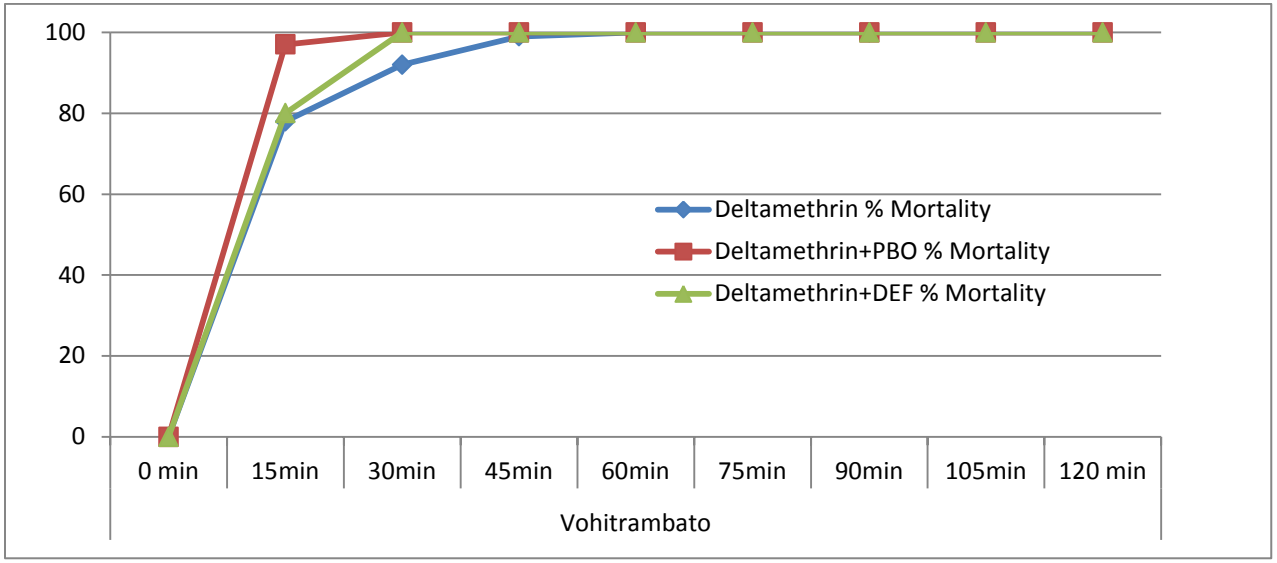
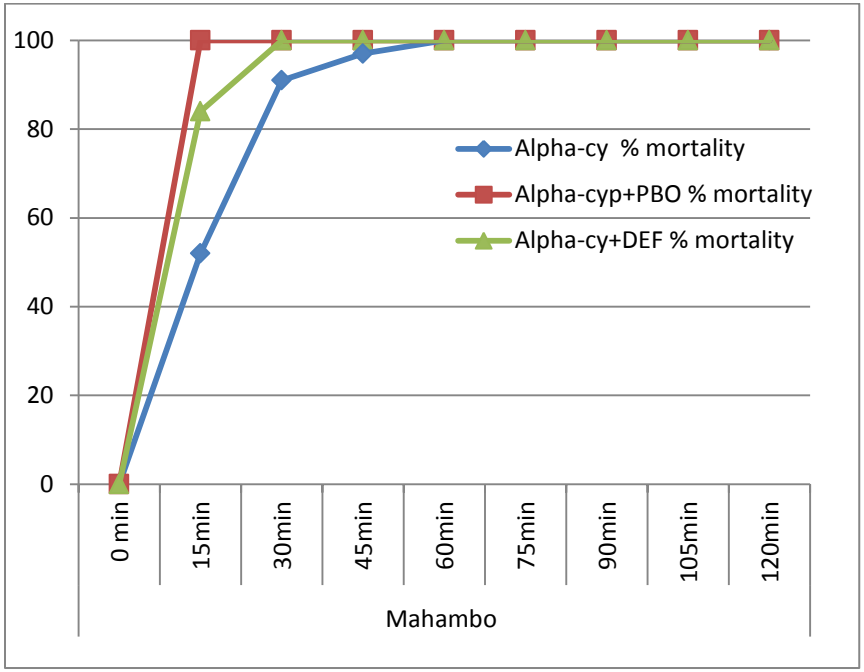


FIGURE 13. MORTALITY OF *AN. GAMBIAE* S.L. POPULATIONS OBSERVED DURING TWO-HOUR EXPOSURE TO CDC BOTTLES TREATED WITH ALPHA-CYPERMETHRIN WITH AND WITHOUT PRE-EXPOSURE TO SYNERGISTS IN TWO SITES IN MADAGASCAR



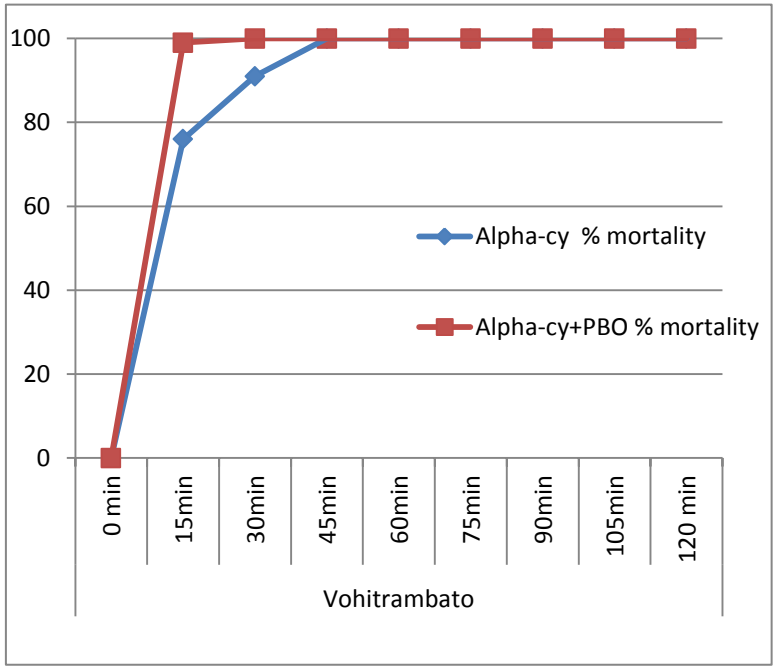


FIGURE 14. MORTALITY OF *AN. GAMBIAE* S.L. POPULATIONS OBSERVED DURING TWO-HOUR EXPOSURE TO CDC BOTTLES TREATED WITH PERMETHRIN WITH AND WITHOUT PRE-EXPOSURE TO SYNERGISTS IN FOUR SITES IN MADAGASCAR

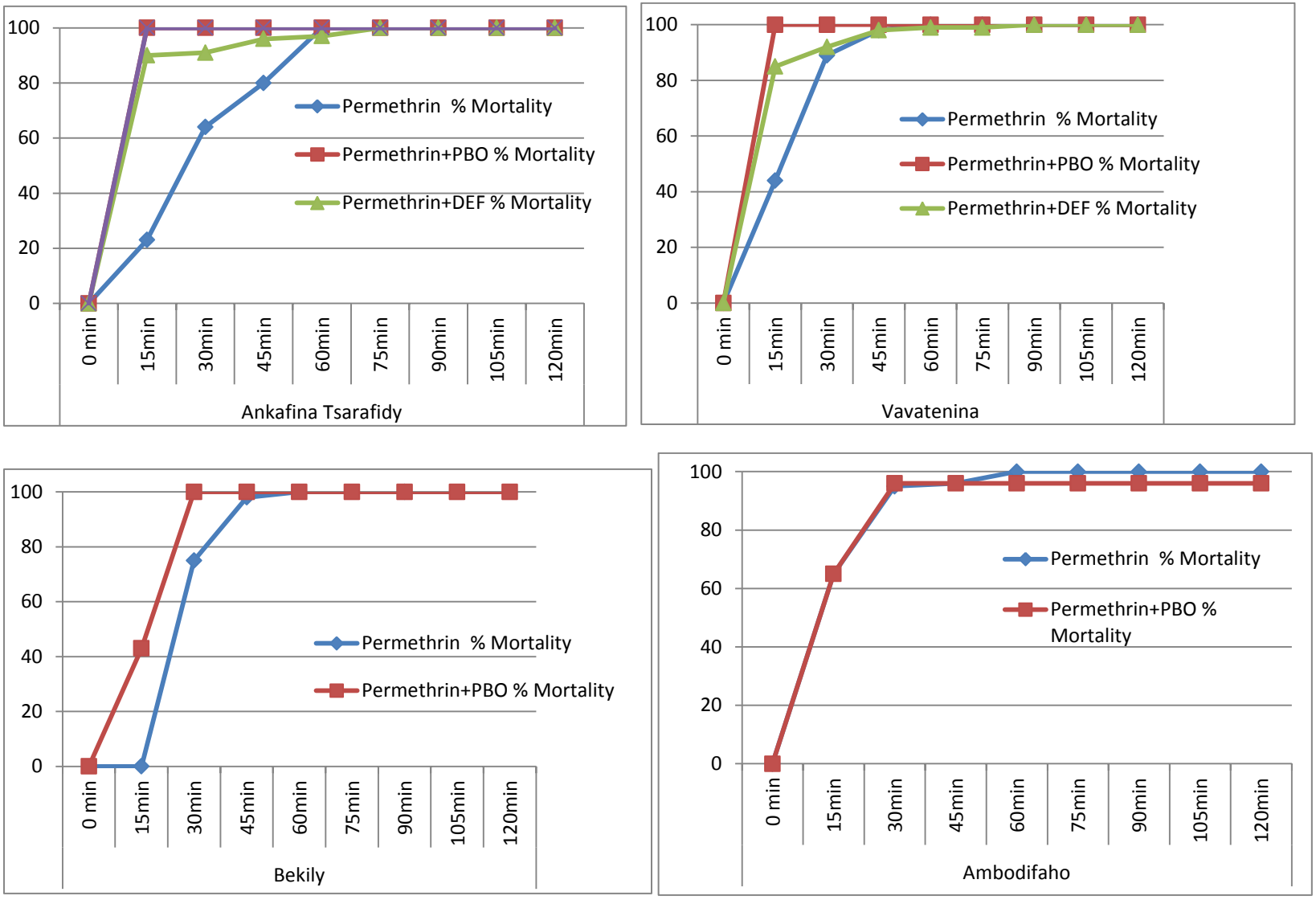
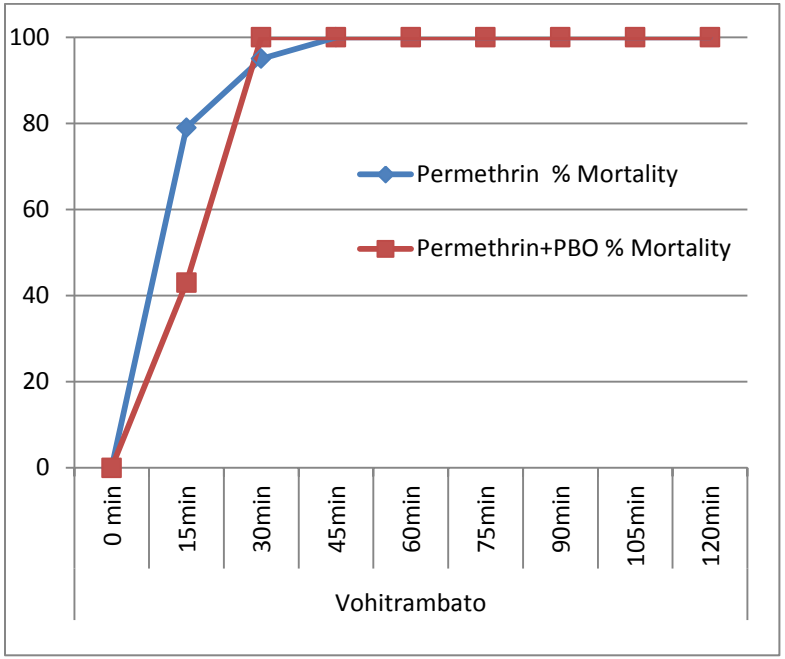
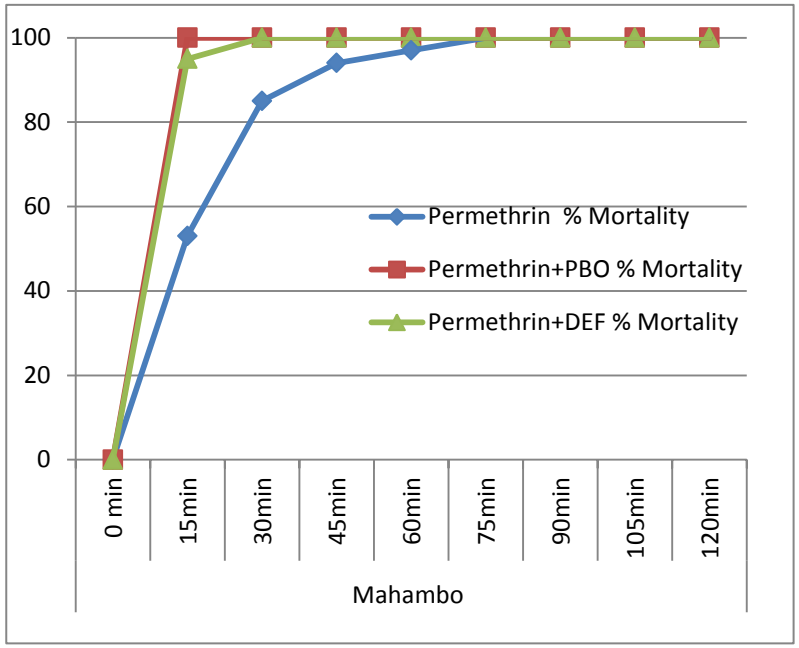


FIGURE 15. MORTALITY OF *AN. GAMBIAE* S.L. POPULATIONS OBSERVED DURING TWO-HOUR EXPOSURE TO CDC BOTTLES TREATED WITH PERMETHRIN WITH AND WITHOUT PRE-EXPOSURE TO SYNERGISTS IN TWO SITES IN MADAGASCAR





4.10. MOLECULAR ANALYSIS

A subsample of 986 and 1,006 *An. gambiae* s.l. mosquitoes preserved post insecticide resistance testing from the eight sites were analyzed for species identification and presence of *kdr* mutation. A more limited sample from three sites was also genotyped for detection of the G119S mutation (n=248). These mosquito samples were randomly selected from the mosquitoes exposed to DDT and pyrethroids (alpha-cypermethrin, deltamethrin, permethrin, and lambda-cyhalothrin) to include surviving and deceased specimens. All mosquito samples analyzed from the three sites in the CHL—Imerina Imady, Milamaina, and Vohimarina—were found to be exclusively *An. arabiensis* (n=465). Specimens from the East Coast and South sub-desert were mainly An. gambiae and An. *arabiensis*. Fourteen An. coluzzi and one An. merus also were identified from samples analyzed from Vavatenina and Bekily, respectively. No *kdr* L1014F or L1014S alleles, or G119S mutation were found among the 1,006 specimens analyzed (Table 9).

TABLE 9. MOLECULAR ANALYSIS RESULTS OF MOSQUITO SPECIMENS FROM THREE DIFFERENT ECO-EPIDEMIOLOGICAL ZONES IN MADAGASCAR

Study site (eco-epidemiological zone)	***An. gambiae* s.s.**	***An. arabiensis***	***An. coluzzi***	***An. merus***	**Total**	***Kdr*-e(L1014S) genotype**		***Kdr*-w(L1014F) genotype**		***Ace-1^R* (G119S) genotype**	
						# genotyped	% SS**	# genotyped	% SS	# genotyped	% SS
Imerina Imady (CHL)	0	150 (100%)	0	0	150	150	100	150	100	48	100
Vohitrambato (EC)	75 (93%)	6 (7%)	0	0	81	91	100	92	100	10	100
Vavatenina (EC)	4 (11.5%)	20 (52.6%)	14 (36.8%)	0	38	46	100	42	100	0	N/A
Bekily (South)	94 (66%)	47 (33%)	0	1 (0.7%)	142	140	100	142	100	0	N/A
Ambodifaho (EC)	46 (92%)	4 (8%)	0	0	50	50	100	50	100	0	N/A
Mahambo (EC)	144 (69%)	66 (31%)	0	0	210	217	100	218	100	146	100
Milamaina (CHL)	0	216 (100%)	0	0	216	212	100	212	100	44	100
Vohimarina (CHL)	0	99 (100%)	0	0	99	100	100	100	100	0	N/A
Total	363 (36.8%)	608 (61.7%)	14 (1.4%)	1 (0.1%)	986	1006	100	1006	100	248	100

Note: %SS**=% homozygous susceptible, NA=Not applicable

4. CONCLUSIONS

Data collected indicates that *An. gambiae* s.l., *An. funestus* group, and *An. mascarensis* vector species are present with varying prevalence in various sentinel sites. *An. gambiae* s.l. is the most common mosquito caught seeking human blood in the East Coast and the South East. High coverage with LLINs for more than three years might have contributed to the outdoor biting behavior.

Results of the vector susceptibility tests indicate susceptibility of *An. gambiae* s.l. to bendiocarb and pirimiphos-methyl in all spray areas. *An. gambiae* s.l. phenotypic resistance to DDT is widespread followed by resistance to permethrin (a type-I pyrethroid). Resistance to type–II pyrethroids (deltamethrin, lambda-cyhalothirn, and alpha-cypermethrin) was limited. *An. funestus*. and *An. masacrensis* were fully susceptible to the insecticides tested, the organophosphate pirimiphos-methyl and the pyrethroids deltamethrin and permethrin. Based on the insecticide susceptibility data collected following the 2014-2015 IRS campaign, technically three of the four insecticide classes (except for organochlorines) approved by the WHO for IRS are potentially eligible for selection and use in Madagascar. In areas where LLINs coverage is still low and pyrethroid insecticide is still efficacious, there is a possibility that this class of insecticide can be considered for use.

No knockdown resistance (kdr) mutations were found in 1,006 *An. gambiae* s.l. samples genotyped for L1014F and L1014S allele. Molecular analysis results of 248 *An. gambiae* s.l. samples also indicated absence of G119S mutations.

Generally pre-exposing *An. gambiae* s.l. to synergists fully and partially restored resistance to pyrethroids and DDT, respectively, though there were exceptions where pre-exposure to synergists fully restored susceptibility to DDT in three sites and one site where pre-exposure to PBO only partially restored susceptibility to permethrin.

Cone bioassay tests conducted during the first week of the IRS campaign indicated that the quality of spraying in the South East and East Coast was good with test mortality rates of 100 percent for all structures sampled and used for the testing within 24 hours and one month after structures were sprayed. The monthly monitoring of the insecticide decay rate, for the insecticide used (Actellic® CS 300) showed pirimiphos-methyl lasted seven months on all surface types in Madagascar.

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6. ANNEXES

ANNEX 1: NUMBER OF MOSQUITOES VECTORS COLLECTED BY PSC AND INDOOR RESTING DENSITIES IN THE STUDY SITES, 2015/2016

Species	Month	Ambodifaho		Vohitrambato		Mahambo		Vavatenina		Manambotra Sud		Lopary	
		#	Vector Density	#	Vector Density	#	Vector Density	#	Vector Density	#	Vector Density	#	Vector Density
An. gambiae s.l.	July									0	0	0	0
	August	0	0	0	0	0	0	1	0.1	0	0	0	0
	September	0	0	0	0	0	0	0	0	0	0	0	0
	October	0	0	0	0	0	0	0	0	0	0	0	0
	November	0	0	0	0	0	0	1	0.1	0	0	2	0.2
	December	0	0	0	0	0	0	0	0	0	0	0	0
	January	0	0	0	0	0	0	0	0	0	0	2	0.2
	February	0	0	2	0.2	0	0	3	0.3	0	0	4	0.4
	Total	0	0	2	0.2	0	0	5	0.5	0	0	0	0
An. funestus	July	0	0	0	0	0	0	0	0	0	0	0	0
	August	0	0	0	0	0	0	0	0	0	0	0	0
	September	0	0	0	0	0	0	0	0	0	0	3	0.3
	October	0	0	0	0	0	0	0	0	0	0	2	0.2
	November	0	0	0	0	0	0	0	0	0	0	0	0

	December	0	0	0	0	0	0	0	0	0	0	0	0
	January	0	0	0	0	0	0	0	0	0	0	0	0
	February	0	0	0	0	0	0	0	0	0	0	0	0
	Total	0	0	0	0	0	0	0	0	0	0	5	0.5
An. mascarensis.	July	0	0	0	0	0	0	0	0	0	0	0	0
	August	0	0	0	0	1	0.1	0	0	0	0	0	0
	September	0	0	0	0	0	0	0	0	0	0	0	0
	October	0	0	0	0	0	0	0	0	0	0	0	0
	November	0	0	0	0	0	0	0	1	0.1	0	0	0
	December	0	0	0	0	0	0	0	0	0	0	0	0
	January	0	0	0	0	0	0	0	0	0	0	0	0
	February	0	0	0	0	0	0	0	0	0	0	0	0
	Total	0	0	0	0	1	0.1	0	1	0.1	0	0	0

Note: In July, collections happened only in Manambotra Sud (Farafangana district) and Lopary (control site in Vangaindrano district

ANNEX 2: RESULTS OF SUSCEPTIBILITY TESTS FOR *An. gambiae* s.l.

Sentinel site	Insecticide tested	WHO tube tests		CDC bottles assay	
		N# mosquitos tested	24h % Observed mortality	N# mosquitos tested	% Observed mortality at diagnostic dose and time
Ambodifaho (Brickaville)	Alpha-Cypermethrin			100	100
	Bendiocarb	100	100	100	100
	DDT	100	100	100	96
	Deltamethrin	100	100	100	100
	Lambda-Cyhalothrin	100	99	100	100
	Permethrin	100	100	100	95
	Pirimiphos-Methyl	100	100	100	100
Vohitrambato (Toamasina II)	Alpha-Cypermethrin			100	97
	Bendiocarb	100	100	100	100
	DDT	100	100		
	Deltamethrin	100	92	100	91
	Lambda-Cyhalothrin	100	99	100	99
	Permethrin	100	100	100	95
	Pirimiphos-Methyl	100	100	100	100
Mahambo (Fenerive Est)	Alpha-Cypermethrin			100	91
	Bendiocarb	100	100	100	100
	DDT	100	99		
	Deltamethrin	100	99	100	100
	Lambda-Cyhalothrin	100	99	100	100
	Permethrin	100	99	100	100
	Pirimiphos-Methyl	100	100	100	100
Imerina Imady	Alpha-Cypermethrin			100	95
	Bendiocarb	100	100	100	100
	DDT	100	56		
	Deltamethrin	100	100	100	99
	Lambda-Cyhalothrin	100	96	100	97
	Permethrin	100	99	100	100
	Pirimiphos-Methyl	100	100	100	100

Sentinel site	Insecticide tested	WHO tube tests		CDC bottles assay	
		N# mosquitos tested	24h % Observed mortality	N# mosquitos tested	% Observed mortality at diagnostic dose and time
Milamaina (Fandriana)	Alpha-Cypermethrin			100	100
	Bendiocarb	100	100	100	100
	DDT	100	96		
	Deltamethrin	100	100	100	100
	Lambda-Cyhalothrin	100	100	100	100
	Permethrin	100	100	100	100
	Pirimiphos-Methyl	100	100	100	100
Vohimarina (Fianarantsoa II)	Alpha-Cypermethrin			100	100
	Bendiocarb	100	100	100	100
	DDT	100	45		
	Deltamethrin	100	100	100	100
	Lambda-Cyhalothrin	100	100	100	100
	Permethrin	100	100	100	100
	Pirimiphos-Methyl	100	100	100	100
Bekily	Alpha-Cypermethrin			100	100
	Bendiocarb	100	100	100	100
	DDT	100	100		
	Deltamethrin	100	100	100	98
	Lambda-Cyhalothrin	100	85	100	80
	Permethrine	100	80	100	75
	Pirimiphos-Methyl	100	100	100	100
Vavatenina	Alpha-Cypermethrin			100	99
	Bendiocarb	100	100	100	100
	DDT	100	100		
	Deltamethrin	100	98	100	96
	Lambda-Cyhalothrin	100	100	100	99
	Permethrin	100	82	100	89
	Pirimiphos-Methyl	100	100	100	100
Ankafina Tsarafidy	Alpha-Cypermethrin			100	100
	Bendiocarb	100	100	100	100

Sentinel site	Insecticide tested	WHO tube tests		CDC bottles assay	
		N# mosquitos tested	24h % Observed mortality	N# mosquitos tested	% Observed mortality at diagnostic dose and time
	DDT	100	30		
	Deltamethrin	100	100	100	100
	Lambda-Cyhalothrin	100	99	100	100
	Permethrin	100	97	100	68
	Pirimiphos-Methyl	100	100	100	100
Manambotra Sud, Farafangana)	Alpha-Cypermethrin			100	100
	Bendiocarb	100	100	100	100
	DDT	100	96		
	Deltamethrin	100	100	100	100
	Lambda-Cyhalothrin	100	100	100	100
	Permethrin	100	100	100	100
	Pirimiphos-Methyl	100	100	100	100
Lopary (Vangaindrano)	Alpha-Cypermethrin			100	100
	Bendiocarb	100	100	100	100
	DDT	100	96		
	Deltamethrin	100	100	100	100
	Lambda-Cyhalothrin	100	100	100	100
	Permethrin	100	100	100	100
	Pirimiphos-Methyl	100	100	100	100

ANNEX 3: RESULTS OF SUSCEPTIBILITY TESTS FOR WILD ADULT *An. gambiae* s.l.

Sentinel site	Insecticide tested	CDC bottles assay	
		N# mosquitos tested	% Observed mortality at diagnostic dose and time (30mn)
Manambotra Sud, Farafangana (Permanet area)	Deltamethrin	60	100
Ambodifaho, Brickaville (Olyset area)	Permethrin	75	100
Mahambo, Fenerive Est (Permanet area)	Deltamethrin	100	100

ANNEX 4: RESULTS OF SYNERGISTS TESTS FOR RESISTANT OR POSSIBLE RESISTANT *An. gambiae* s.l.*(CDC Bottles Assay)

Sentinel site	Insecticide tested	CDC bottles assay	
		N# mosquitos tested	% Observed mortality at diagnostic dose and time (30mn)
Ankafina Tsarafidy	Permethrin	100	64
	Permethrin + PBO	100	100
	Permethrin + DEF	100	91
	Permethrin + DEF + PBO	100	100
	DDT	100	30
	DDT + Ethacrynic	100	100
	DDT + PBO	100	100
Ambodifaho, Brickaville	Permethrin	100	95
	Permethrin + PBO	100	96
Mahambo, Fenerive Est	Permethrin	100	85
	Permethrin + PBO	100	100
	Alphacypermethrin	100	91
	Alphacypermethrin + PBO	100	100

Sentinel site	Insecticide tested	CDC bottles assay	
		N# mosquitos tested	% Observed mortality at diagnostic dose and time (30mn)
	Alphacypermethrin + DEF	100	100
Vavatenina	Permethrin	100	89
	Permethrin + PBO	100	100
	Permethrin + DEF	100	92
	Deltamethrin	100	96
	Deltamethrin + PBO	100	100
	Deltamethrin + DEF	100	100
Vohitrambato	Permethrin	100	95
	Permethrin + PBO	100	100
	Deltamethrin	100	92
	Deltamethrin + PBO	100	100
	Deltamethrin + DEF	100	100
	Alphacypermethrin	100	91
	Alphacypermethrin + PBO	100	100
Imerina Imady	Lambdacyhalothrin	100	97
	Lambdacyhalothrin + PBO	100	100
	DDT	100	56
	DDT + Ethacrynic	100	90
	DDT + PBO	100	100
Bekily	Permethrin	100	75
	Permethrin + PBO	100	100
Vohimarina	DDT	100	45
	DDT + PBO	100	84
	DDT + PBO+ Ethacrynic	100	65
Milamaina	DDT	100	95
	DDT + PBO	100	100

ANNEX 5: PARITY RATE

Month	Species	Manambonitra Sud Farafangana			Lopary Vangaindrano			Ambodifaho Brickaville			Vohitrambato Toamasina II			Mahambo Fenerive Est			Vavatenina		
		# dissected	# parous	Parity rate	# dissected	# parous	Parity rate	# dissected	# parous	Parity rate	# dissected	# parous	Parity rate	# dissected	# parous	Parity rate	# dissected	# parous	Parity rate
July*	*An. gambiae* s.l.	4	1	1/4	29	4	13.8%												
	An. funestus	0	0	0	1	0	0/1												
	An. mascarensis	0	0	0	0	0	0												
August*	*An. gambiae* s.l.	8	1	1/8	22	3	13.6%	38	38	100%	112	96	85.7%	1	1	1/1	16	11	68.8%
	An. funestus	0	0	0	2	1	1/2	0	0	0	22	19	86.4%	0	0	0	8	5	5/8
	An. mascarensis	0	0	0	0	0	0	0	0	0	3	3	3/3	36	19	53%	20	15	75.0%
September	*An. gambiae* s.l.	18	1	5.6%	5	1	1/5	49	22	44.9%	15	8	53.3%	11	3	3/11	1	1	1/1
	An. funestus	0	0	0	31	9	29.0%	0	0	0	1	1	3/3	0	0	0	3	3	3/3
	An. mascarensis	0	0	0	0	0	0	0	0	0	4	3	3/4	0	0	0	3	3	3/3
October	*An. gambiae* s.l.	38	3	7.9%	30	5	16.7%	122	53	43.4%	84	34	40.5%	0	0	0	23	16	69.6%
	An. funestus	0	0	0	32	11	34.4%	0	0	0	5	2	2/5	17	1	5.9%	8	7	7/8
	An. mascarensis	0	0	0	0	0	0	0	0	0	4	3	3/4	0	0	0	7	6	6/7
November	*An. gambiae* s.l.	61	1	1.6%	42	4	9.5%	344	216	62.8%	17	8	47.1%	0	0	0	20	10	50.0%
	An. funestus	3	0	0	5	0	0	0	0	0	1	1	1/1	8	6	6/8	1	1	1/1
	An. mascarensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	6	6/6
December	*An. gambiae* s.l.	8	1	1/8	21	9	42.9%	66	60	90.9%	68	33	48.5%	0	0	0	44	25	56.8%
	An. funestus	0	0	0	2	1	1/2	0	0	0	6	5	5/6	26	20	76.9%	0	0	0.00
	An. mascarensis	0	0	0	0	0	0	0	0	0	0	0	0.00	0	0	0	0	0	0.00
January	*An. gambiae* s.l.	37	1	2.7%	17	3	4.7%	33	27	81.8%	66	36	54.6%	47	29	61.7%	33	21	63.6%
	An. funestus	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	2	2/2
	An. mascarensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	2/2
February	*An. gambiae* s.l.	9	2	2/9	26	1	3.9%	86	52	60.5%	97	38	39.2%	59	44	74.6%	39	17	43.6%
	An. funestus	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	An. mascarensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

July* baseline for South Eastern sites

August* baseline for East coast sites