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MAINLAND TANZANIA INSECTICIDE RESISTANCE MONITORING

2017 FINAL REPORT

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MAINLAND TANZANIA ENTOMOLOGICAL MONITORING REPORT

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II. ABBREVIATIONS/ACRONYMS

ACE	ACETYLCHOLINESTERASE
CDC	CENTERS FOR DISEASE CONTROL AND PREVENTION
DDT	DICHLORODIPHENYLTRICHLOROETHANE
DED	DISTRICT EXECUTIVE DIRECTOR
DMO	DISTRICT MEDICAL OFFICER
GPIRM	GLOBAL PROGRAMME FOR INSECTICIDE RESISTANCE MONITORING
GST	GLUTATHIONE-S TRANSFERASE
IRS	INDOOR RESIDUAL SPRAYING
KCMUCO	KILIMANJARO CHRISTIAN MEDICAL UNIVERSITY COLLEGE
LLIN	LONG-LASTING INSECTICIDAL NET
MFOS	MIXED FUNCTION OXIDASE
MOCDGEC	MINISTRY OF HEALTH, COMMUNITY DEVELOPMENT, GENDER, ELDERLY AND CHILDREN
NIMR	NATIONAL INSTITUTE FOR MEDICAL RESEARCH
NMCP	NATIONAL MALARIA CONTROL PROGRAMME
NSE	NON-SPECIFIC ESTERASES
PCR	POLYMERASE CHAIN REACTION
PMI	PRESIDENT'S MALARIA INITIATIVE
SSA	SUB-SAHARAN AFRICA
TPRI	TROPICAL PESTICIDES RESEARCH INSTITUTE
URT	UNITED REPUBLIC OF TANZANIA
USAID	UNITED STATES AGENCY FOR INTERNATIONAL DEVELOPMENT
VCO	VECTOR CONTROL OFFICER
WHO	WORLD HEALTH ORGANIZATION

III. ACKNOWLEDGEMENTS

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DISCLAIMER

IV. EXECUTIVE SUMMARY

Background: Malaria vector control in Tanzania is based predominantly on the use of residual insecticides deployed through indoor residual spray (IRS) and long lasting insecticide treated nets (LLINs). However, the development of insecticide resistance has become a serious threat to the continued effectiveness of these control tools. To ensure the effectiveness of these vector control interventions is not compromised, and therefore maintain the gains made in malaria control, strategies for preventing and managing insecticide resistance are imperative. The current survey was carried out as a routine monitoring of susceptibility status of major malaria vectors to insecticides in 22 sentinel districts of Mainland Tanzania.

Methods: The WHO standard methods were used to detect knockdown and mortality rates in the wild female *Anopheles* mosquitoes reared from larvae collected in the sentinel districts. The WHO diagnostic doses of 0.75% permethrin; 0.05% deltamethrin; 0.1% bendiocarb, and 0.25% pirimiphos-methyl were used. In addition, synergy tests using PBO were conducted. Molecular diagnostic assays were used to identify the mosquito vectors and detect the prevailing mechanisms of insecticide resistance.

Results: In 50% of the surveyed sites, *Anopheles gambiae* s.l. were found to be resistant to permethrin; namely in Bagamoyo, Kilosa, Kilombero, Manyoni, Muleba, Musoma rural, Nzega, Ruangwa, Mpanda and Uvinza. Likewise, deltamethrin resistant mosquito populations were prevalent in 60% of the sites which included Bagamoyo, Kilosa, Kilombero, Manyoni, Mpanda, Muleba, Musoma rural, Nzega, Ruangwa, Mpanda, Mtwara, Kasulu and Uvinza. Thirty percent (30%) of sentinel districts were found to have suspected permethrin resistant *Anopheles gambiae* s.l. populations (mortality rate of 90% to 97%) in Geita, Kasulu, Magu, Mtwara, Nyasa and Sengerema. Suspected resistance to deltamethrin was only observed in Nyasa district. Malaria vectors were fully susceptible to bendiocarb and pirimiphos-methyl in all sites except Manyoni where mosquitoes exhibited resistance to pirimiphos-methly exposure. Likewise, reduced susceptibility to pirimiphos-methly was observed (mortality rate of 95%) in Musoma Rural. PBO-synergist assays were conducted to evaluate the potential role of cytochrome P_{450} genes in the observed phenotypic resistance. The results showed an increased mean recovery of susceptibility to permethrin 0.75% and deltamenthrin 0.05% of 10.3% (1.2-1.5 fold reduction) and 10.7% (1.0-1.7 fold reduction) respectively when the insecticides were combined with the P_{450} inhibitor. This suggests a likely significant role of cytochrome P_{450} in the pyrethroid resistance.

A total of 9,246 mosquitoes were collected, of these, 2,225 mosquitoes were subjected to PCR analysis for the *An. gambiae* s.l. sibling species identification of which 31.3% and 68.7% were identified as *An. gambiae* s.s and *An. arabiensis*, respectively. The *kdr* east mutation was detected in surviving *An. gambiae s.s* from 5 sentinel sites (Kinondoni, Kyela, Mtwara, Muleba and Ruangwa) with allelic frequencies ranging from 43% to 92%. Only 2 sites (Kinondoni and Muleba) had *kdr* east mutations in surviving *An. arabiensis* populations with allelic frequencies of 10% and 50%. The *kdr* west mutation was detected in *An. gambiae s.s* from only 2 sentinel sites (Muleba & Kyela) equivalent to allelic frequencies of 30% and 100%. We could not find any association between *kdr* east mutation and presence of phenotypic resistance of permethrin in surviving mosquitoes (χ^2 = 0.18, p = 0.66). Conversely, presence of *kdr* east (L1014S) mutation in survivors from bioassay was associated with occurrence of resistance phenotypes in deltamethrin (χ^2 = 5.56, p = 0.0184).

Conclusion: High frequency of pyrethroid resistance was documented in many sites across Tanzania, in some cases coupled with *kdr* east and west mutations. Malaria vectors in the Lake Zone area where IRS operations have been conducted continue to be susceptible to pirimiphos-methyl. Continued monitoring of changing dynamics of insecticide resistance in *Anopheles* mosquitoes is essential for timely management and safeguarding the effectiveness of the current vector control tools.

I. INTRODUCTION

Most countries in sub-Saharan Africa (SSA) depend heavily on two vector control interventions in the battle against malaria: Long-Lasting Insecticidal Nets (LLINs) and Indoor Residual Spraying (IRS). These tools use insecticides belonging to the four main chemical classes: organochlorines, pyrethroids, carbamates and organophosphates. Whereas 14 formulations belonging to these classes are approved by the World Health Organization (WHO) for use in IRS, only pyrethroids are approved for use in LLINs because of their low mammalian toxicity, excito-repellent properties and rapid knock-down and killing effect. It has been estimated that since 2000, the use of these insecticide-based tools in combination with case management and community education have averted more than 670 million cases of malaria (WHO, 2015). However, the occurrence and exponential spread of insecticide resistance among malaria vectors poses a major threat to the sustainability of gains made in malaria vector control. There have been reports of dramatic increases of resistance in all four major classes of insecticides throughout SSA, including Tanzania (Kabula et al., 2013, Nkya et al., 2014, Protopopoff et al., 2014). Thus, insecticide resistance poses major challenges on the immediate and long-term implementation of malaria vector control interventions in several parts of the world, especially in SSA.

In 2012, the WHO reported that insecticide resistance in malaria vectors had already been found in more than 64 malaria endemic countries worldwide, with the majority reporting resistance to pyrethroids. This spread is alarming as it poses serious threats to the efficacy of vector control interventions and the gains made in malaria control over the last 15 years. Following this trend of insecticide resistance, in 2012 WHO developed the Global Plan for Insecticide Resistance Management in malaria vectors (GPIRM), encouraging a broad commitment to act before insecticide resistance compromises current vector control strategies. Consequently, WHO called for all countries to develop and implement insecticide resistance management strategies in their malaria control programmes in order to curb the spread of resistance as well as preserve the effectiveness of vector control tools. As a response to this call, in 2016 Tanzania developed a national insecticide resistance monitoring and management plan (MoHCDGEC, 2012).

The National Institute for Medical Research (NIMR) in collaboration with the National Malaria Control Program (NMCP) undertakes the detection and monitoring of insecticide resistance annually. This surveillance and/or insecticide resistance monitoring started back in 1999 under Government support through the MoHCDGEC. Eleven rounds of insecticide resistance monitoring in the country have so far been carried out in Tanzania mainland (i.e., 1999, 2004, 2008-2017). The continuous national insecticide resistance monitoring has contributed to a better understanding of the levels of vector susceptibility and the current exponential increase and spread of insecticide resistance across the country for developing appropriate and feasible insecticide resistance management strategies.

Malaria vector susceptibility to pyrethroids (permethrin, lambdacyhalothrin, deltamethrin) in several parts of the country has been progressively decreasing compared to the initial status recorded in 1999 (Kisinza *et al*., 2011; Kabula *et al*., 2013). The susceptibility responses across the country indicate the highest frequencies of pyrethroid resistance (Kabula et al., 2013), presence of bendiocarb resistance and emergence of pirimiphos-methyl resistance. Vector genetic and biochemical analyses have indicated presence of both target-site (*kdr* west L1014F and *kdr* east L1014S) mutations and metabolic mechanisms

which involves lower penetration or sequestration, or an increased biodegradation of the insecticide due to enhanced detoxification activities, conferring pyrethroid resistance in *An. gambiae ss* and *An. arabiensis* in various sentinel sites across the country (Kulkarni et al., 2006, Matowo et al., 2010, Protopopoff et al., 2013).

The current report encompasses the findings from the insecticide resistance surveillance in 22 sentinel districts conducted between April and September 2017.

2. STUDY OBJECTIVES

2.1 MAIN OBJECTIVE

The main objective of the study was to detect and monitor the susceptibility status of malaria vectors to insecticides, establish species composition of local malaria vectors and determine the underlying mechanisms of resistance.

2.2 SPECIFIC OBJECTIVES

Specifically, the study sought to:

2.1.1 Determine the resistance status of *Anopheles gambiae* s.l. to permethrin (0.75%), deltamethrin (0.05%), bendiocarb (0.1%) and pirimiphos-methyl (0.25%).

2.1.2 Establish malaria vector species composition and their distribution in the sentinel districts.

2.1.3 Characterize the insecticide resistance mechanisms and their distribution across the country.

2.1.4 Determine the impact of PBO-pyrethroid synergist on killing effect to malaria vectors

2.1.5 Provide recommendations on the best feasible options and appropriate strategies of resistance management in the country

3. METHODS

3.1 STUDY DESIGN

This was a cross-sectional countrywide survey, which was conducted between May and July 2017 across the 22 national sentinel sites (districts) for the insecticide resistance monitoring in mainland Tanzania. The purpose of the survey was to detect and monitor the susceptibility status of malaria vectors to insecticides, establish species composition of local malaria vectors and determine the underlying mechanisms of resistance. The surveillance was preceded by a one-week training workshop to equip the data collectors (field workers and district malaria focal persons) with basic knowledge on malaria entomology, mosquito collection techniques, and field susceptibility tests of malaria vectors including PBO-Pyrethroid synergy testing, as well as rearing, preservation and transportation of mosquito samples for laboratory analyses and molecular characterizations.

3.2 CONTEXT AND STUDY SITES SELECTION

The present national survey for insecticide resistance monitoring was conducted in 22 sentinel districts. These districts are shown in Table 1. Additionally, testing of insecticide resistance intensity was planned to be conducted in 10 districts with a history of pyrethroid resistance. The ten districts were Bagamoyo, Geita, Kilombero, Magu, Muleba, Musoma Rural, Ngara, Uvinza and Ruangwa. Unfortunately, testing of insecticide resistance intensity could not be executed due to delay in delivery of CDC test kit and insecticides for this purpose.

Selection of sentinel sites and/or districts for the insecticide resistance monitoring was based on the WHO recommended selection criteria, with emphasis on the following principles:

a) Evidence of presence or absence of the insecticide resistance detected by the previous surveys

b) The use of insecticides for IRS (include most IRS sites in the Lake Victoria zone with unknown resistance status)

c) Districts bordering other countries with resistance e.g. Ngara, Kyela, Mpanda

d) Malaria endemicity in the area (priority was given to the districts with high malaria prevalence e.g. Nyasa, Muleba, Ngara, Mpanda, Kasulu, Uvinza, Geita, Songea)

The selection of the ten districts for insecticide resistance intensity testing was solely based on previous history of high level of permethrin and deltamethrin resistance.

Table 1: Sentinel sites/districts used for Insecticide Resistance Monitoring in 2017

S/N	Region	District	Geo-coordinates	#**Prevalence of malaria in children aged 6-59 months	#*LLIN Coverage (%)
1	Dar es Salaam	Kinondoni	6° 47'S;39°16'E	1.1	65.9
2	Geita	Geita DC	2°54'S;32°15'E	38.2	96.4
3	Kagera	Muleba	1°45'S; 31°40'E	41.0	89.5
4	Kagera	Ngara	2°30′S; 30°39′E	41.0	89.5
5	Katavi	Mpanda	6° 20′ S; 31° 4′ E	13.5	94.7
6	Kigoma	Uvinza	5° 6′ S; 30° 23′E	38.1	93.7
7	Kigoma	Kasulu	8°45'S; 32°45' E	38.1	93.7
8	Lindi	Ruangwa	10°06′S; 8°93′E	17.4	69.9
9	Mara	Musoma rural	1° 30'S; 33°48'E	19.1	91.4
10	Mbeya	Kyela	9°35'S; 33°55'E	0.7	50.4
11	Morogoro	Kilombero	8°31'S; 37°22'E	22.5	55.2
12	Morogoro	Kilosa	06°49′S 036°59′E	22.5	55.2
13	Mtwara	Mtwara urban	10°43′S; 38°48′E	20.0	61.3
14	Mwanza	Magu	2°30'S; 33°30'E	15.1	90.3
15	Mwanza	Sengerema	02°20′S; 032°30′E	15.1	90.3
16	Pwani	Bagamoyo	6°26′S; 38°54′E	15.4	64.6
17	Ruvuma	Nyasa	11° 17′ S; 34° 46′ E	22.6	66.1
18	Ruvuma	Songea rural	10°41'S; 35°39'E	22.6	66.1
19	Shinyanga	Kahama	03°50'S; 32°36'E	16.5	78.7
20	Simiyu	Bariadi	02°48′S; 33°59′E	13.4	97.6
21	Singida	Manyoni	5°45′S; 34°50'E	5.5	43.9
22	Tabora	Nzega	4° 13' S; 33°10' E	19.5	90.8

Source: TDHS-MIS, 2015-16

*Percentage of households with at least one insecticide-treated net (ITN)

** Malaria prevalence according to RDT

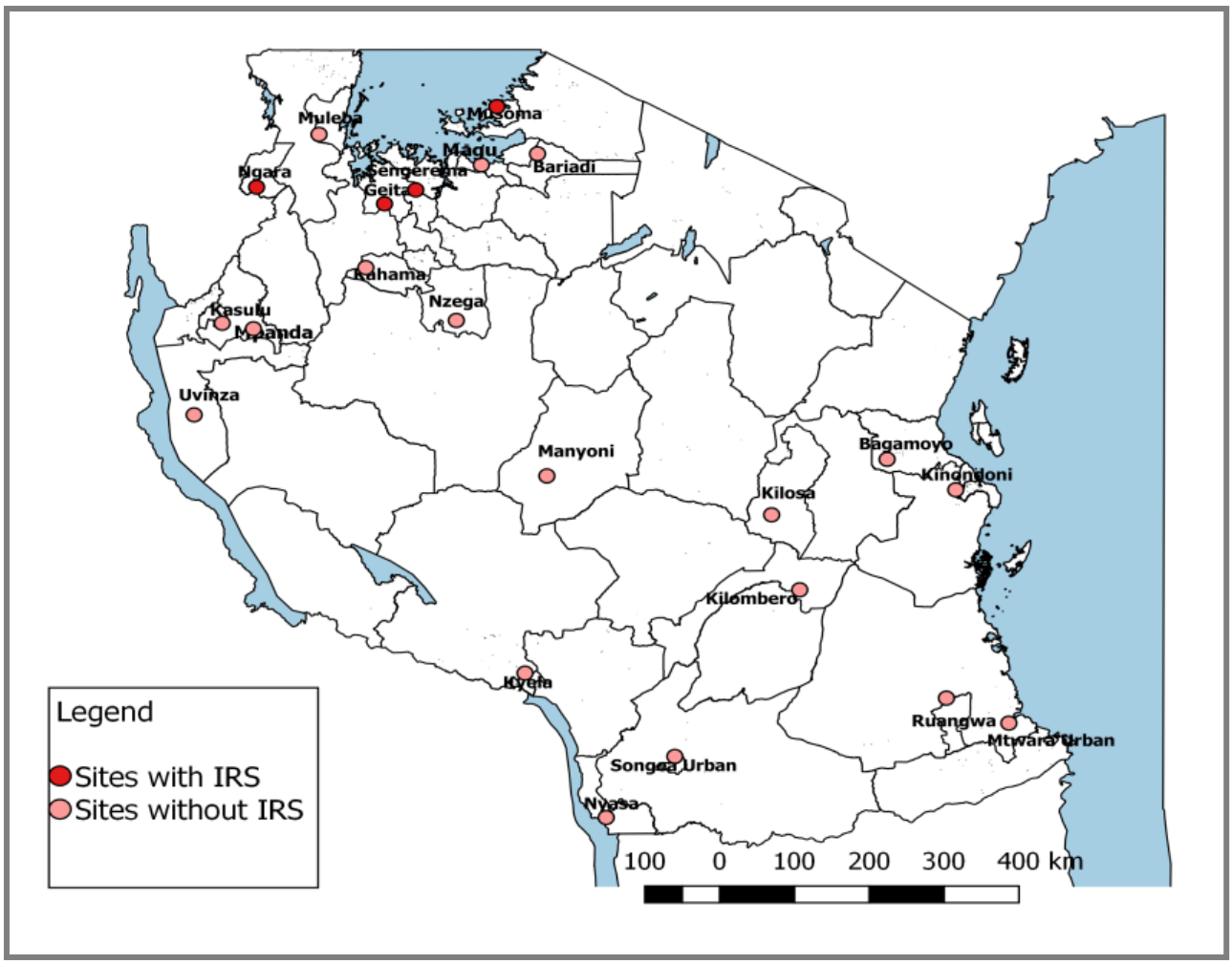


Figure 1: Map showing surveyed sentinel sites for insecticide resistance monitoring with and without IRS, 2017. All sentinel sites received LLINs universal coverage.

3.3 MOSQUITO COLLECTIONS IN THE FIELD

Mosquito larval searches were done and *Anopheles* larvae were carefully collected with a 350 ml dipper and transferred into plastic containers, which were then loosely capped to allow aeration. These were transported to the field laboratory where they were reared at 27-30°C. Larvae collected from several breeding sites in the same village were pooled together for rearing and testing. The larvae were fed with Tetramin® fish food. The development of the larvae was monitored regularly and all those that pupated were transferred into shallow plastic cups/small beakers using Pasteur pipettes, and then placed in appropriately labeled cages for adult emergence. Female adult mosquitoes aged 2-5 days were used for WHO susceptibility tests and PBO synergy testing. Adult mosquitoes for biochemical assay were not exposed to insecticides but were freshly frozen when they were four (4) days old. They were kept under -80°C until ready for the assays. The Cryo Express (CX) dry shippers were used to transport these frozen mosquito samples from the field to the laboratory. Storage temperature inside the shipping cavity remained at approximately -190°C until the liquid nitrogen evaporated from the absorbent material. From each sentinel district, a minimum of 100, freshly frozen adult female mosquitoes were preserved for subsequent biochemical assays. Geographical coordinates of each sampling site were recorded using calibrated smart phones.

Figure 2: Mosquito larval sampling sites with geographical coordinates displayed.

3.4 WHO INSECTICIDE SUSCEPTIBILITY TESTS

Susceptibility tests were carried out in the field using the World Health Organization insecticide susceptibility test kits for adult mosquitoes (WHO, 2013). The kit is basically comprised of insecticide impregnated test papers and non-impregnated papers for control and plastic tubes that are marked red for exposure and green for holding. Prior to conducting susceptibility testing in the field, the insecticide susceptibility test kits (i.e., insecticide impregnated test papers and non-impregnated papers) were first tested using laboratory reared susceptible mosquito strains in the laboratory as a quality check for the treated papers.

Two to five-day old F1 generation mosquitoes were tested using standard WHO insecticide susceptibility procedures with four replicates of 15–25 wild adult female mosquitoes per tube. Mosquitoes were exposed to papers impregnated with the WHO-recommended discriminating concentrations of deltamethrin (0.05%), bendiocarb (0.1%), permethrin (0.75%) and pirimiphos-methyl (0.25%) prepared at Universiti Sains Malaysia (WHO, 2013). Knock-down (KD) rates were recorded 10, 15, 20, 30, 40, 50 and 60 minutes after the start of exposure (for pyrethroids). The time for mosquito knockdown was recorded at pre-determined intervals during exposures to the pyrethroid insecticides only. A mosquito was considered knocked down if it lay on its side on the floor of the exposure tube and unable to fly.

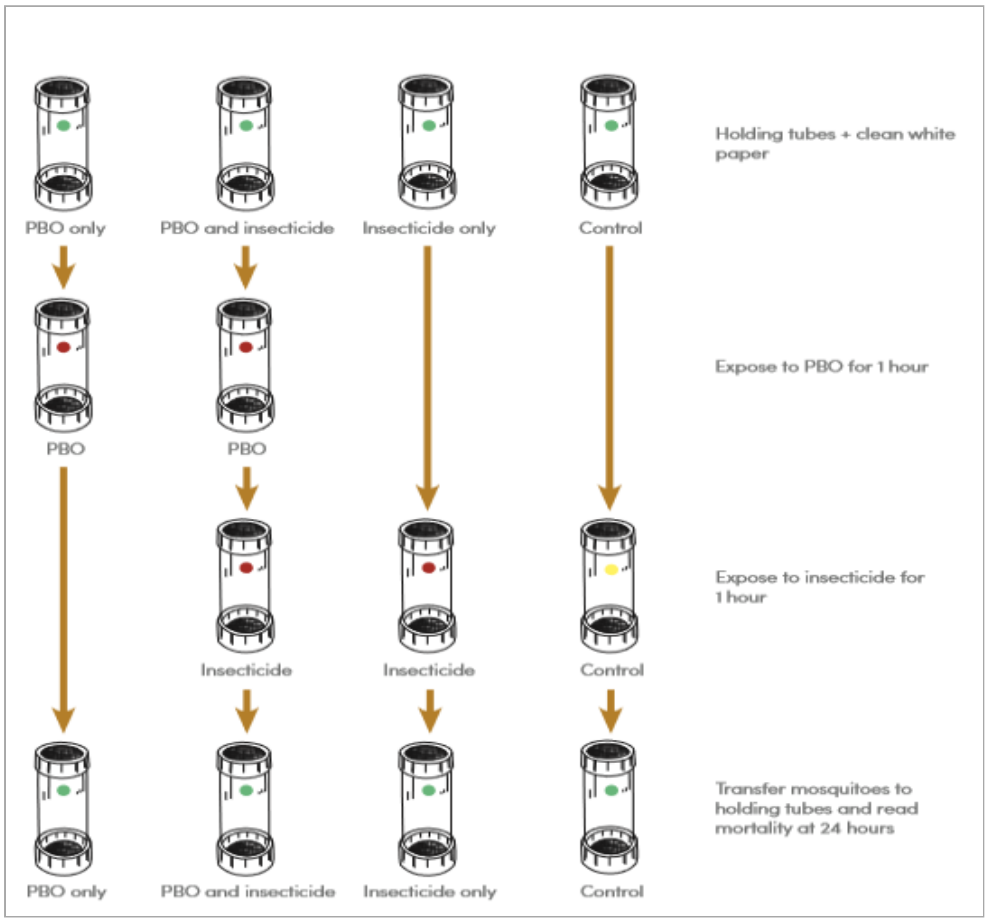
At the end of exposure period, mosquitoes were then transferred into holding tubes (lined with untreated papers) by gently blowing them through the open space between the exposure and the holding tubes. Cotton soaked in 10% sugar was placed on top of the holding tube. This provided nourishment so as to avoid death by starvation. The mortality was scored 24 hours post-exposure. The susceptibility status was evaluated based on the WHO criteria i.e. 98-100% mortality indicate susceptibility; 90-97% mortality required confirmation and less than 90% mortality indicate resistance (WHO, 2013). When the control mortality was scored between 5% and 20%, the mean observed mortality was corrected using Abbott's formula (Abbott, 1925). Tests with control mortality scores above 20% were discarded. Time taken for 50% knockdown of mosquitoes (KT_{50}) and its corresponding 95% confidence intervals were determined by probit analysis using the computer program PoloPlus (Version 1.0, LeOra Software) (Finney 1971).

All tested mosquitoes were preserved dry over *silica gel* in 1.5 ml eppendorf tubes and transported to Amani Medical Research Centre for further laboratory analysis (molecular species identification and detection of genetic mechanisms of insecticide resistance).

3.5 PBO – SYNERGIST BIOASSAYS

Piperonyl butoxide (PBO) synergist tests were carried out in sentinel sites where mosquitoes were found to be resistant to permethrin and/or deltamethrin. The aim of this test was to ascertain the involvement of mixed function oxidases in the observed phenotypic resistance. In this test, 2–5 days old F1 adult mosquitoes were pre-exposed to 4% piperonyl butoxide (PBO) paper for 1 h and immediately exposed to 0.75% permethrin, or 0.05% deltamethrin 4% for 1 h. Two controls were used during this experiment: control 1 constituted mosquitoes exposed to clean papers neither with insecticides nor with PBO, while control 2 constituted mosquitoes exposed to papers treated with PBO only (Fig. 3). The number of mosquitoes tested for each insecticide varied between 40 to 80 (table 3). Mortalities were later assessed after exposure; the PBO synergized group was compared to the un-synergized group after 24 h post-exposure. This comparison was used to evaluate the potential role of cytochrome P_{450} genes in the observed resistance.

Figure 3: Diagrammatic presentation showing the steps to perform the synergist-insecticide bioassay test using WHO testing procedures (Source: WHO, 2016)



3.6 LABORATORY ANALYSES

3.6.1 IDENTIFICATION OF *AN. GAMBIAE* S.L. AND DETECTION OF TARGET SITE RESISTANCE MECHANISMS

For each sentinel site, genomic DNA (gDNA) from 40-130 female adult mosquitoes were individually extracted using the method described by Collins et al. (1987). Sibling species of the *An. gambiae* s.l. were identified using standard PCR method of Scott et al. (1993). The target site mutations were screened using Taqman assay genes (Bass et al, 2010)

3.6.2 DETECTION OF METABOLIC RESISTANCE MECHANISMS

Mosquito samples, which were freshly frozen and kept at -80˚C, were transported in liquid nitrogen at approximately -190˚C to the respective laboratory for analysis of biochemical resistance mechanisms. Biochemical assays were carried out to quantify levels of cytochrome P_{450} mono-oxygenases/mixed function oxidase (MFO), non-specific esterase (NSE), acetyl-cholinesterase and glutathione-S-transferase (GST) in individual mosquitoes. Adult female individuals of 3–4 days-old, not previously exposed to insecticide and reared under insecticide-free environment were used in the assay, following the CDC plate bioassay method (CDC, 2000).

4. RESULTS

4.1 SUSCEPTIBILITY STATUS OF *AN. GAMBIAE* S. L. TO INSECTICIDES

Table 2 and figure 3 below presents detailed susceptibility status from 20[a] out of 22 (90.9%) surveyed sentinel districts, which indicate that in 50% of the sites, *Anopheles gambiae* s.l. were resistant to permethrin (i.e. <90% mortality rate). Thirty percent (30%) of sentinel districts were found to have *Anopheles gambiae* s.l. populations suspected to be resistant to permethrin (mortality rate of between 90% to 97%). Likewise, deltamethrin resistant mosquito populations were present in 60% of the sites. Suspected resistance to deltamethrin was only observed in Nyasa district. Unlike permethrin and deltamethrin insecticides, malaria vectors were fully susceptible (mortality rate of ≥98%) to bendiocarb and pirimiphos-methyl in all sites, except Manyoni where mosquitoes exhibited resistance to pirimiphos-methly exposure. Likewise, reduced susceptibility (suspected resistance) to pirimiphos-methyl was observed (mortality rate of 95%) in Musoma rural (see table 2).

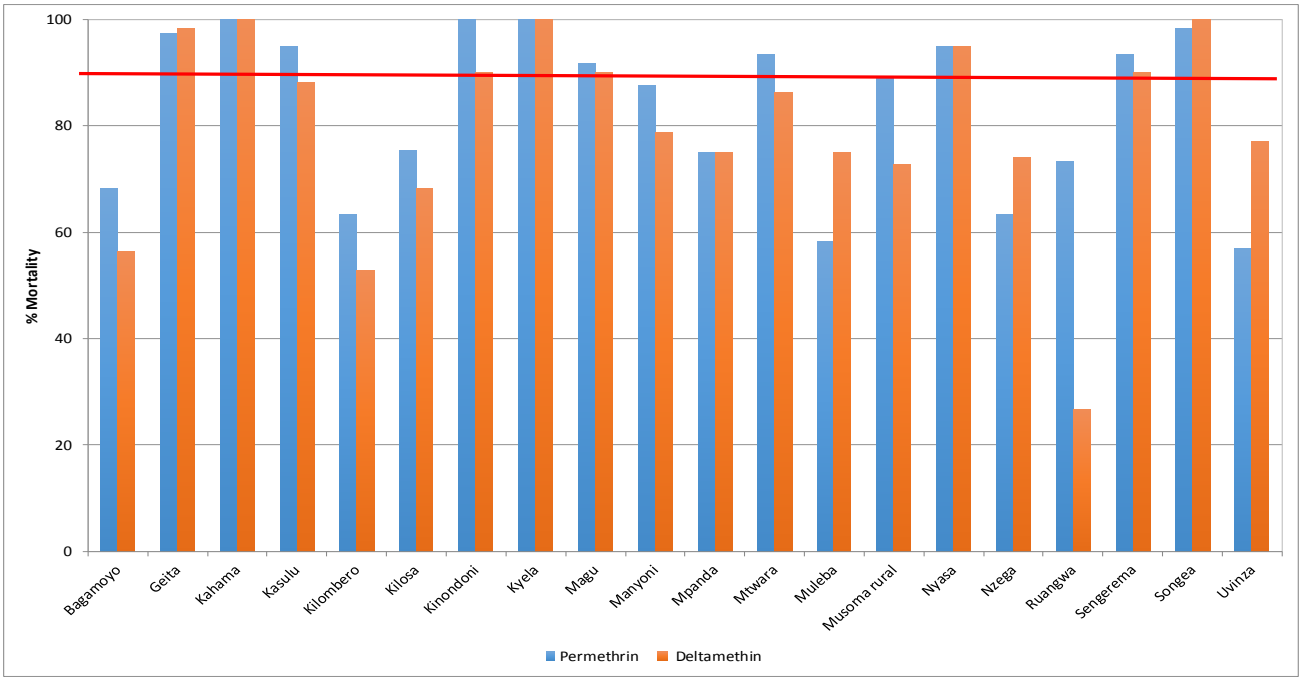


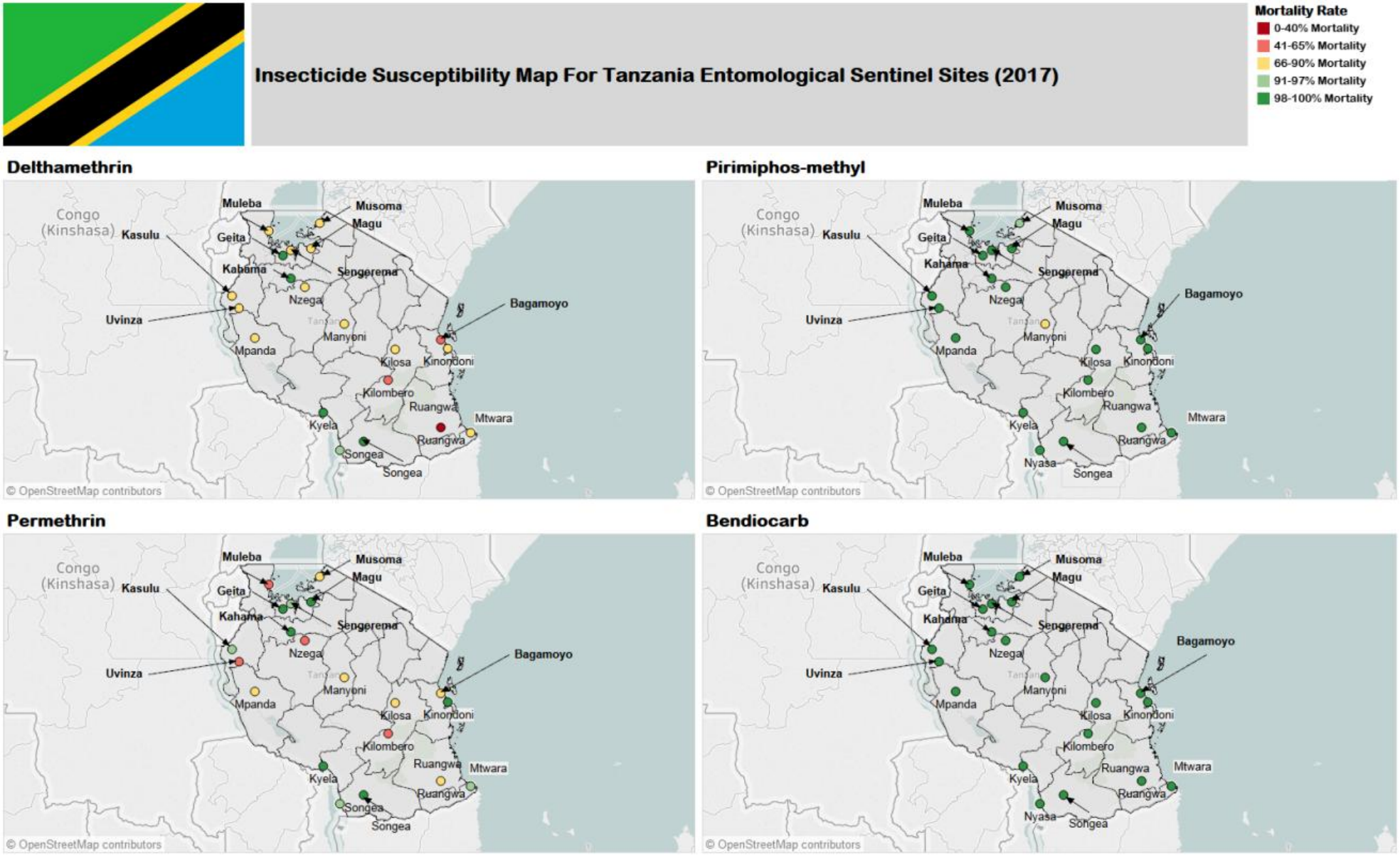
Figure 4: Mortality response of wild female *Anopheles gambiae s.l.* local populations to discriminatory dosages of permethrin and deltamethrin. ***The red bold line indicates the cut off point of susceptibility level. Mortalities below the line indicate resistance to an insecticide***

[a] **Mosquito collection was carried out in all 22 sites. However, the results from 2 sites are not included in this report. This is because the number of adult mosquitoes raised from two sites were not adequate to conduct a meaningful test. This was due to lack of larval sampling sites as a result of drought in those 2 sites and/or high mortality of immatures in the rearing process.**

Table 2: Susceptibility status (mortality rates[2]) of *An. gambiae* s.l to the WHO- discriminating concentrations of four different insecticides

S/N	Sites	Permethrin				Deltamethin				Bendiocarb			Actellic 300CS		
		N	Mortality (%)	SE	KD50	95%CI	N	Mortality (%)	SE	KD50	95%CI	N	Mortality (%)	SE	N	Mortality (%)	SE
1	Bagamoyo	79	68.3	3.3	51.34	(48.30-55.46)	82	56.3	6.3	54.47	(51.52-58.67)	80	100	0	80	100	0
2	Geita	80	97.5	1.7	41.52	(39.04-44.37)	80	98.3	1.6	35.18	(33.25-37.16)	80	100	0	80	100	0
3	Kahama	60	100	0	32.4	(30.08-34.92)	80	100	0	24.38	(22.59-26.12)	60	100	0	60	100	0
4	Kasulu	78	94.9	2.89	15.71	(13.05-17.88)	80	88.3	7.3	51.34	(37.91-103.39)	80	100	0	80	100	0
5	Kilombero	80	63.3	12	18.57	(16.61-20.52)	80	52.7	21.1	47.71	(38.57-67.69)	80	100	0	80	100	0
6	Kilosa	80	75.4	2.9	41.98	(39.58-44.71)	80	68.3	14.5	36.99	(35.12-37.23)	80	100	0	80	100	0
7	Kinondoni	80	100	0	29.1	(25.94-32.66)	80	90	0	23.66	(21.06-26.38)	80	100	0	80	100	0
8	Kyela	60	100	0	30.65	(26.95-35.07)	80	100	0	21.44	(18.89-24.02)	80	100	0	80	100	0
9	Magu	80	91.7	4.4	26.03	(23.67-28.57)	80	90	5.7	33.85	(30.67-37.37)	80	100	0	80	100	0
10	Manyoni	79	87.6	2.1	22.16	(19.48-24.92)	80	78.7	2.7	22.43	(21.27-23.62)	100	100	0	95	86.2	2.6
11	Mpanda	80	75	0	38.52	(34.77-43.13)	80	75	2.9	34.26	(32.44-36.09)	80	100	0	80	100	0
12	Mtwara	82	93.3	4.4	14.48	(11.92-16.77)	80	86.4	2.6	31.16	(29.15-33.31)	88	100	0	88	100	0
13	Muleba	80	58.3	6.7	51.24	(41.76-77.90)	80	75	5	43.11	(40.88-45.52)	80	98.3	1.6	80	100	0
14	Musoma rural	88	89.4	1.5	19.9	(17.32-22.58)	88	72.7	2.62	21.11	(19.20-23.06)	88	100	0	88	95.45	2.62
15	Nyasa	80	95	2.8	18.8	(17.75-19.90)	80	95	2.9	21.21	(19.79-22.69)	80	100	0	80	100	0
16	Nzega	102	63.3	8.8	54.02	(50.32-59.24)	97	74.1	8.8	52.62	(49.01-57.60)	97	100	0	99	100	0
17	Ruangwa	80	73.3	12	45.15	(42.96-47.62)	80	26.7	8.3	45.73	(41.05-52.24)	80	100	0	80	100	0
18	Sengerema	80	93.3	4.4	45.18	(42.04-49.04)	80	90	2.9	51.34	(37.91-103.39)	80	100	0	80	100	0
19	Songea	84	98.3	1.7	27.01	(25.47-28.60)	80	100	0	28.25	(26.29-30.29)	80	100	0	80	100	0
20	Uvinza	84	57.1	11.9	50.04	(46.96-54.06)	88	77.2	4.6	44.57	(41.43-48.38)	80	100	0	80	100	0

[2]Based on WHO criteria for insecticide susceptibility levels [i.e., Mortality-rate based criteria was used to determine the levels of mosquito susceptibilities: Susceptible (≥ 98%); resistant to be confirmed (97 – 90%) and resistant (≤ 90%).


Insecticide Susceptibility Map For Tanzania Entomological Sentinel Sites (2017)
Mortality Rate
0-40% Mortality
41-65% Mortality
66-90% Mortality
91-97% Mortality
98-100% Mortality
Delthamethrin
Pirimiphos-methyl
Permethrin
Bendiocarb
Congo (Kinshasa)
Kasulu
Muleba
Musoma
Magu
Geita
Kahama
Sengerema
Nzega
Uvinza
Bagamoyo
Manyoni
Mpanda
Kilosa
Kinondoni
Kilombero
Ruangwa
Mtwara
Kyela
Songea
Nyasa
© OpenStreetMap contributors

4.2 IDENTIFICATION OF MALARIA VECTORS

A total of 9,246 mosquitoes were collected, morphologically identified as *An. gambiae* s.l. and tested for insecticide resistance across sentinel sites. Of these, 2,225 mosquitoes were subjected to PCR analysis for the *An. gambiae* s.l. sibling species identification of which 31.3% and 68.7% were identified as *An. gambiae* s.s and *An. arabiensis*, respectively. The distribution of these two sibling species at each of the sentinel districts is shown in figure 5. Only mosquito samples which amplified in PCR are included in this analysis.

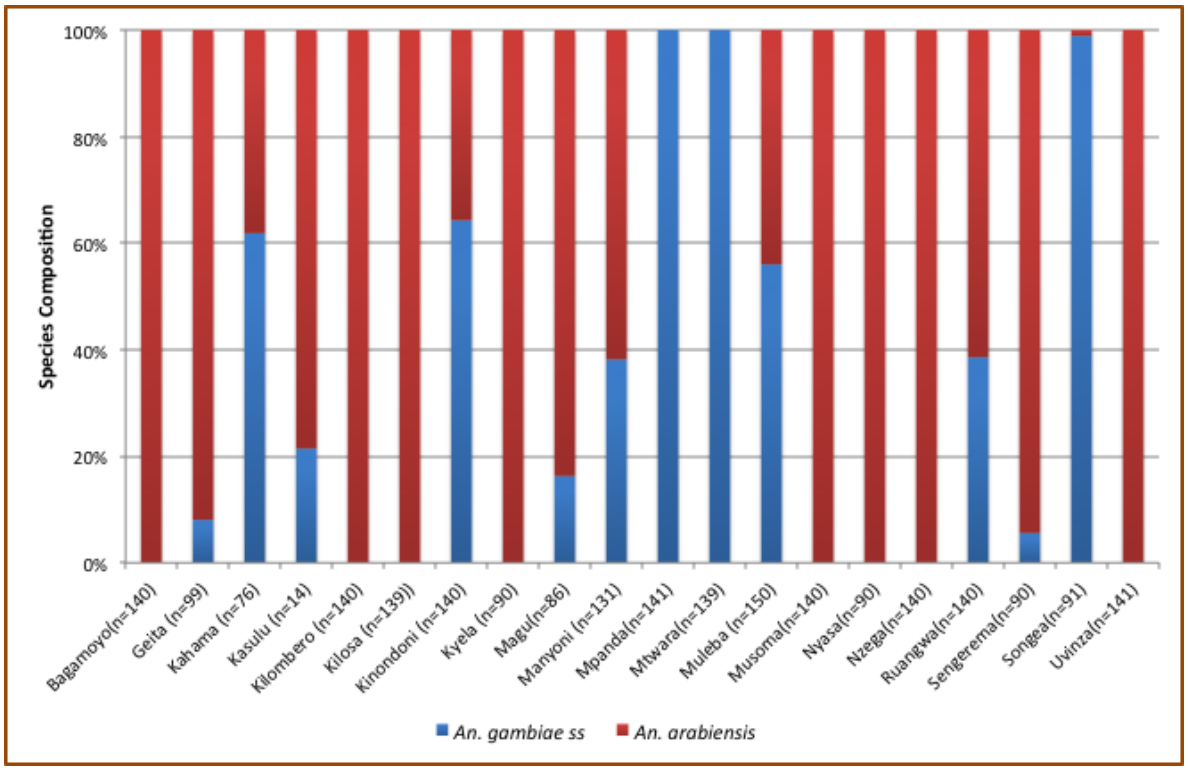


Figure 5: Species composition of *Anopheles gambiae* s.l. collected from 20 sentinel sites.

4.3 SYNERGIST TESTS WITH PBO

No mortality was observed in the control mosquitoes exposed to control papers with only PBO in all assays. There was a statistically significant increase in mortality to permethrin in 4 sites following pre-exposure to PBO. This suggests a likely significant role of cytochrome P_{450} in the observed pyrethroid resistance phenotype.

Table 3: Comparison of mortality rates of *Anopheles gambiae s.l.* exposed to permethrin 0.75% and deltamethrin 0.05% alone and piperonyl butoxide (PBO) in combination with permethrin 0.75% and deltamethrin 0.05% respectively per site in 2017

Site	Permethrin only		95% Confidence Interval	PBO + Permethrin		95% Confidence Interval
	N	% Mortality		N	% Mortality	
Bagamoyo	50	70	56.3-80.9	50	86	73.8-93.1
Geita	40	85	70.1-92.9	40	100	91.0-100
Kilombero	40	42.5	28.5-57.8	40	100	91.2-100*
Magu	40	40	26.4-55.4	40	100	91.2-100*
Muleba	40	72.5	57.2-83.9	40	97.5	87.1-99.6*
Musoma	40	85	70.9-92.9	40	100	91-100
Ruangwa	40	62.5	47.0-75.8	40	82.5	68.0-91.3
Sengerema	40	85	70.9-92.9	40	100	91.0-100
Uvinza	83	70.7	60.6-79.7	83	95.2	82.2-98.1*

* Significant resistance reduction when PBO was included in bioassay

Site	Deltamethrin only		95% Confidence Interval	PBO + Deltamethrin		95% Confidence Interval
	N	% Mortality		N	% Mortality	
Bagamoyo	50	66	52.2-77.6	50	94	83.8-97.9*
Geita	40	90	76.9-96.0	40	100	91.2-100
Kilombero	40	42.5	28.5-57.8	40	100	91.2-100*
Muleba	40	62.5	47.0-75.8	40	100	91.2-100*
Musoma	40	80	65.2-89.5	40	95	91.2-100
Ruangwa	40	75	59.8-85.8	40	100	91.2-100*
Sengerema	40	80	65.2-89.5	40	100	91.2-100*
Uvinza	83	71.7	59.2-81.5	83	100	93.9-100*

* Significant resistance reduction when PBO was included in bioassay

4.4 KNOCKDOWN RESISTANCE (*KDR*) AND *ACE-1* MUTATIONS

After being exposed to the various discriminatory insecticide dosages using WHO test kits, all surviving mosquitoes and twenty percent of the dead mosquitoes (from all 20 sentinel sites) were analyzed using the Taqman assay (Bass et al., 2008) for presence of *kdr* east and *kdr* west mutations. The *kdr* east (L1014S) mutation was detected in surviving *An. gambiae* s.s from 5 sentinel sites (Kinondoni, Kyela, Mtwara, Muleba and Ruangwa) with allelic frequencies ranging from 43% to 92% (Table 4). Only 2 sites (Kinondoni and Muleba) had *kdr* east mutations in surviving *An. arabiensis* population with allelic frequencies of 10% and 50%.

Likewise, the *kdr* west (L1014F) mutation was detected in *An. gambiae* s.s from only 2 sentinel sites (Muleba & Kyela) equivalent to allelic frequencies of 30% and 100% (Table 5). All surviving *An. arabiensis* from most sentinel sites (with the exception of Kahama, Mtwara & Songea) were found to have *kdr* west mutations with 100% allelic frequency.

Analysis of *kdr* genotype frequency indicated no association of *kdr* east (L1014S) mutation with the presence of phenotypic resistance of permethrin in surviving mosquitoes (χ^2 = 0.18, p = 0.66). Conversely, presence of *kdr* east (L1014S) mutation in surviving mosquitoes was associated with occurrence of resistance phenotypes for deltamethrin (χ^2 = 5.56, p = 0.0184). The detailed analysis is presented in table 6. Additionally, *Ace-1* mutation was not detected in any mosquito collected from all sites.

Table 4: Distribution of *kdr*-East (L1014S) mutation among wild *An. gambiae s.s* and *An. arabiensis* mosquitoes from 19 districts

Site	*An. gambiae* s.s.						*An. arabiensis*					
	N	Genotype count			Allelic frequency		N	Genotype count			Allelic frequency	
		RR	RS	SS	R	S		RR	RS	SS	R	S
Bagamoyo	0	0	0	0	0	0.0	46	0	0	46	0	1.0
Geita	0	0	0	0	0	0.0	5	0	0	5	0.0	1.0
Kahama	5	0	0	5	0	1.0	0	0	0	0	0.0	0.0
Kilombero	0	0	0	0	0	0.0	59	0	0	59	0.0	1.0
Kilosa	0	0	0	0	0	0.0	53	0	0	53	0.0	1.0
Kinondoni	9	8	0	1	0.89	0.1	2	1	0	1	0.5	0.5
Kyela	1	1	0	0	1	0.0	21	1	0	20	0.0	1.0
Magu	3	0	0	3	0	1.0	8	0	0	8	0.0	1.0
Manyoni	9	0	0	9	0	1.0	28	0	0	28	0.0	1.0
Mpanda	0	0	0	0	0	0.0	39	0	0	39	0.0	1.0
Mtwara	20	7	3	10	0.43	0.6	0	0	0	0	0.0	0.0
Muleba	12	11	0	1	0.92	0.1	12	1	0	11	0.1	0.9
Musoma rural	0	0	0	0	0	0.0	32	0	0	32	0.0	1.0
Nyasa	0	0	0	0	0	0.0	12	0	0	12	0.0	1.0
Nzega	0	0	0	0	0	0.0	53	0	0	53	0.0	1.0
Ruangwa	35	12	17	6	0.59	0.4	41	0	2	39	0.0	1.0
Sengerema	1	0	0	1	0	1.0	20	0	0	20	0.0	1.0
Songea	9	0	0	9	0	1.0	0	0	0	0	0.0	0.0
Uvinza	0	0	0	0	0	0.0	128	1	1	126	0.0	1.0

N=No. Identified as *An. gambiae* s.s or *An arabiensis*; SS=Homozygous susceptible; RS=Heterozygous resistant; RR=Homozygous resistant; R=Resistance allele; S= Susceptible allele

Table 5: Distribution of *kdr*-West (L1014F) mutation among wild *An. gambiae* s.s and *An. arabiensis* mosquitoes from 19 districts

Site	*An. gambiae* s.s.						*An. arabiensis*					
	N	Genotype count			Allelic frequency		N	Genotype count			Allelic frequency	
		RR	RS	SS	R	S		RR	RS	SS	R	S
Bagamoyo	0	0	0	0	0.0	0.0	32	0	0	32	0	1
Geita	0	0	0	0	0.0	0.0	5	0	0	5	0	1
Kahama	5	0	0	5	0.0	1.0	0	0	0	0	0	0
Kilombero	0	0	0	0	0.0	0.0	26	0	0	26	0	1
Kilosa	0	0	0	0	0.0	0.0	47	0	0	47	0	1
Kinondoni	0	0	0	0	0.0	0.0	1	0	0	1	0	1
Kyela	1	1	0	0	1.0	0.0	20	0	0	20	0	1
Magu	3	0	0	3	0.0	1.0	8	0	0	8	0	1
Manyoni	9	0	0	9	0.0	1.0	27	0	0	27	0	1
Mpanda	0	0	0	0	0.0	0.0	26	0	0	26	0	1
Mtwara	12	0	0	12	0.0	1.0	0	0	0	0	0	0
Muleba	4	1	0	3	0.3	0.8	10	0	0	10	0	1
Musoma rural	0	0	0	0	0.0	0.0	32	0	0	32	0	1
Nyasa	0	0	0	0	0.0	0.0	11	0	0	11	0	1
Nzega	0	0	0	0	0.0	0.0	53	0	0	53	0	1
Ruangwa	30	1	0	29	0.0	1.0	36	0	0	36	0	1
Sengerema	2	0	0	2	0.0	1.0	14	0	0	14	0	1
Songea	9	0	0	9	0.0	1.0	0	0	0	0	0	0
Uvinza	0	0	0	0	0.0	0.0	113	0	0	113	0	1

N=No. Identified as *An. gambiae* s.s or *An arabiensis* ; SS=Homozygous susceptible; RS=Heterozygous resistant; RR=Homozygous resistant; R=Resistance allele; S= Susceptible allele

Table 6: Association between permethrin and deltamethrin phenotypic resistance and presence of resistance genotypes for *An. gambiae* s.l.

PERMETHRIN	N	***kdr*** East genotype count			Allelic frequency		Statistics
		RR	RS	SS	R	S	
Survivors (Resistant)	162	6	11	145	0.07	0.93	$\chi^2 = 0.18$ $p = 0.6692$
Deads (Susceptible)	132	15	0	117	0.11	0.89	

DELTAMETHRIN	N	***kdr*** East genotype count			Allelic frequency		Statistics
		RR	RS	SS	R	S	
Survivors (Resistant)	207	7	9	191	0.06	0.94	$\chi^2 = 5.56$ $p = 0.0184$
Deads (Susceptible)	162	15	3	144	0.10	0.90	

N=No. *An. gambiae* s.l. genotyped; SS=Homozygous susceptible; RS=Heterozygous resistant; RR=Homozygous resistant; R=Resistance allele; S= Susceptible allele

4.5 METABOLIC RESISTANCE MECHANISMS

Laboratory analysis for metabolic resistance mechanisms were successfully performed on samples from 15 sites. Field collected *Anopheles gambiae* s.l. from all sites did not show overexpression of mixed function oxidases (MFOs) activity when compared to the susceptible Kisumu reference strain (**Figure 6**). This was similar for non-specific esterases (NSE) activity in which did not show any increased expression when compared to the susceptible Kisumu reference strain **(Figure 8).** The level of acetyl-cholinesterases (ACE) activity in these populations was higher than that of the Kisumu strain in Uvinza, Ruangwa, Muleba, Kasulu, Geita, Kilombero and Nzega **(Figure 7).**

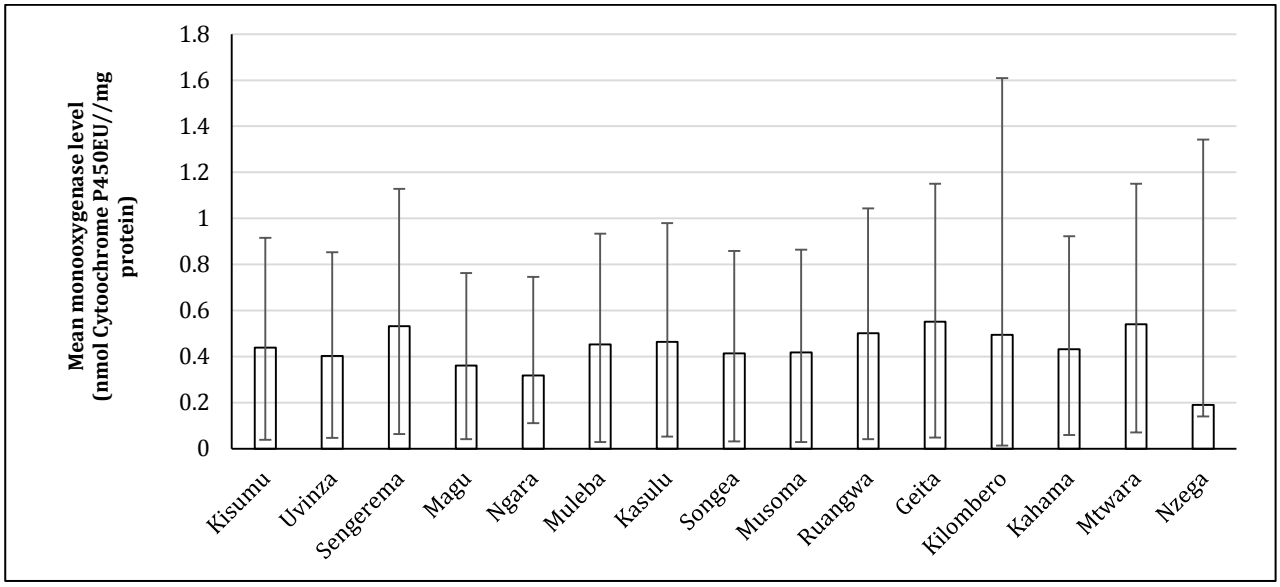


Figure 6: Mean level of mixed function oxidases activity in field-collected *Anopheles gambiae* s.l. from different sentinel sites. Kisumu refers to the reference susceptible strain of *An. gambiae s.s* used as control

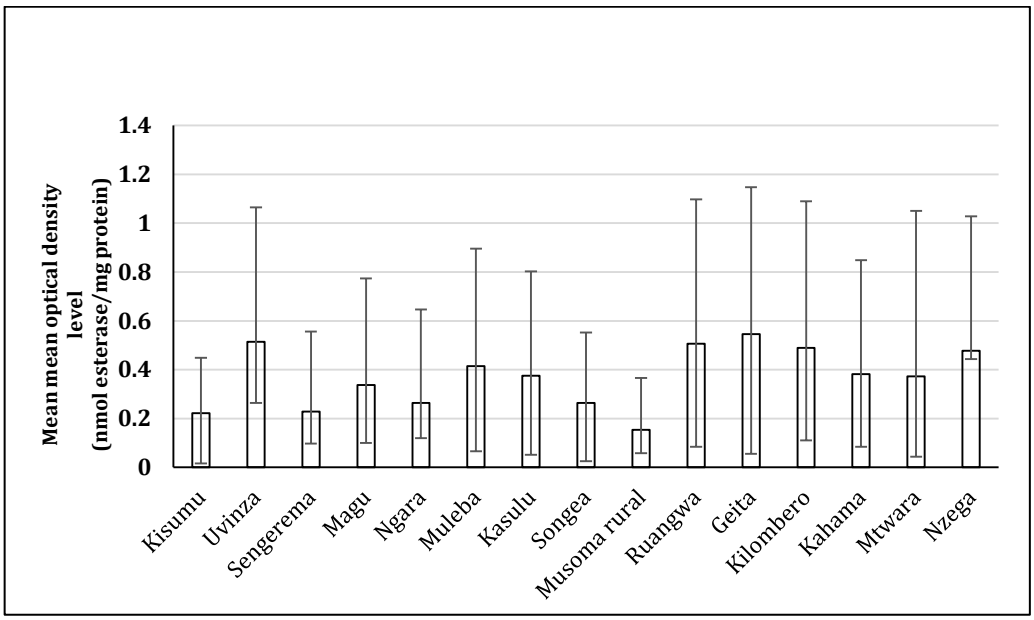


Figure 7: Mean level of acetyl-cholinesterases activity in field-collected *Anopheles gambiae s.l.* from different sentinel sites. Kisumu refers to the reference susceptible strain of *An. gambiae s.s* used as control.

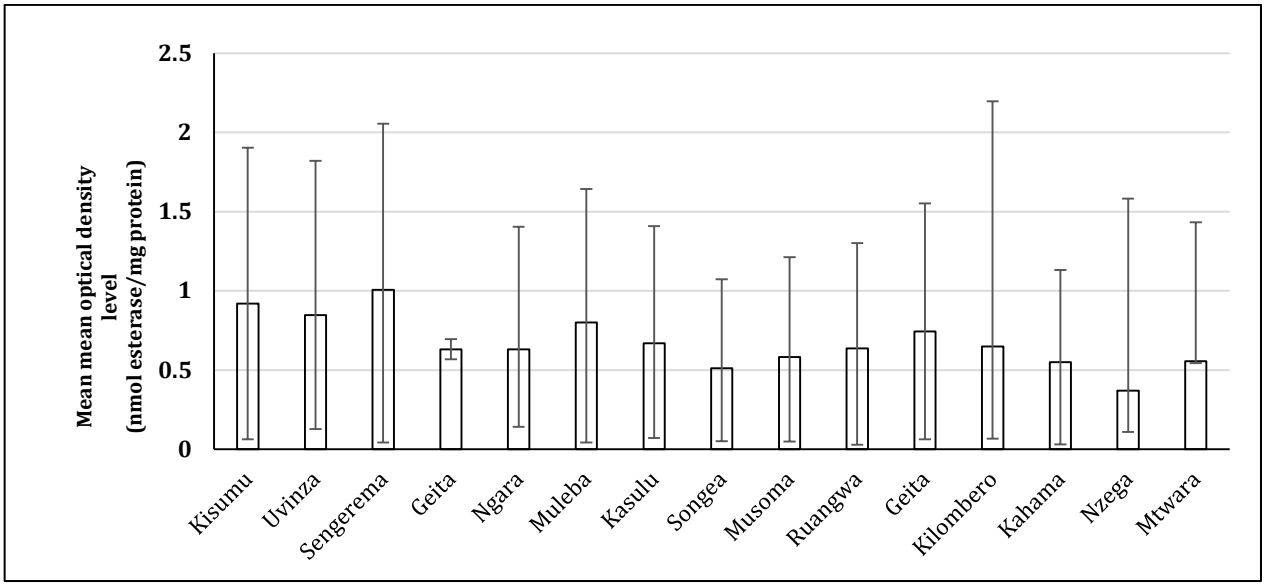


Figure 8: Mean level of non-specific esterases activity in field-collected *Anopheles gambiae s.l.* from different sentinel sites. Kisumu refers to the reference susceptible strain of *An. gambiae s.s* used as control

5. DISCUSSION

The present survey demonstrated presence of wide distribution and high frequency resistance to type I and II pyrethroids (i.e. permethrin & deltamethrin), while there was widespread susceptibility to carbamate and organophosphate insecticides across the surveyed sentinel districts.

Such high resistance to permethrin and deltamethrin has been associated with repeated exposure of mosquitoes to permethrin on bed nets following rolling out of LLINs nationwide (IRM report 2016, Maxwell et al., 2003). Equally, resistance to deltamethrin is progressively mounting and increasingly exacerbated by the rolling out of deltamethrin LLINs (Permanet 2.0) to pregnant women attending antenatal clinics in the country (NBS report, 2016). Formerly, deltamethrin was extensively used in the re-treatments for conventional bed-nets (ITNs) since early 2000s (Maxwell et al., 2003). Currently LLINs coverage ranges between 44% and 98% in the surveyed sites (NBS report, 2016).

Genotyping and allelic distribution of *kdr* mutation indicated that *kdr* east (L1014S) is predominant among *An. gambiae* s.s. (4 sites) compared with 2 sites among *An. arabiensis* populations with allelic frequencies of 6% to 11% for mosquitoes that survived exposure to permethrin and deltamethrin. However, occurrence of insecticide resistance to deltamethrin in the surveyed sites is associated with a high frequency of *kdr*-east (L1014S) in mosquitoes (χ^2 = 5.56, p = 0.0184). Cross-resistance between pyrethroids (permethrin and deltamethrin) and DDT has been reported in Sudan due to mutations in the *kdr* allele 1014F (Abdalla et al. 2014,). Interestingly, one *An. gambiae s.s* mosquito in Kyela was found to carry both *kdr* allele (east & west) mutations.

An. arabiensis was found to be the predominant vector in nearly 70% of the sites. This observation may be a strong indication that residual malaria transmission in some of the districts might potentially be associated with the predominance of *An. arabiensis*; a known exophagic vector (Mahande et al., 2007) that might be contributing to relatively high malaria prevalence rates in the surveyed sites as exhibited in table 1. The importance of *An. arabiensis* as an outdoor biting vector, particularly early in the evening hours (before bedtime) may result in residual malaria transmission as described in other parts of Tanzania (Govella et al., 2012, Milali et al. 2017).

The absence of resistance to bendiocarb in all sites and presence of pirimiphos-methyl resistance in one new site (Manyoni) among populations of *An. gambiae* s.l may be partially attributed to resistance mitigation action implemented by removing bendiocarb from IRS operations since 2014 in Lake Victoria regions (PMI report, 2015). Likewise, pirimiphos-methyl is a relatively new insecticide used for IRS to replace bendiocarb since 2015, therefore it is not yet widely selected for resistance. However, it is understood that a different formulation of the same active ingredient has been in use as an agrochemical in post harvest pest control for grains, cereals and legumes; potentially creating insecticide pressure in *Anopheles* populations in Manyoni district.

Analysis for resistance mechanisms using synergist tests with piperonyl butoxide (PBO) revealed varied responses in tested mosquitoes across surveyed sites. Full restoration of susceptibility after pre-exposure to PBO was observed against permethrin and deltamethrin exposure in all sites except for permethrin in Bagamoyo, Muleba and Ruangwa, and deltamethrin in Bagamoyo sites. These responses suggest that insecticide resistance in *An. gambiae* population is mainly mediated by oxidase (cytochrome P_{450}) based metabolic resistance with minor contribution from other mechanisms including *kdr*. This is

the first finding that supports observations reported previously using biochemical tests in Tanzania (Kisinza et al. 2016).

6. RECOMMENDATIONS

1) Continue with insecticide resistance monitoring for proper management of any emerging/expanding resistance in malaria vectors.
2) Expand the intensity of insecticide resistance testing in our local malaria vectors. This will help link the observed phenotypic resistance and the performance of the vector control tools in the field. This information can then be used to inform operational decisions such as a change of insecticide for IRS or the introduction of a non-pyrethroid for IRS in areas with LLINs as the main intervention.
3) Include the synergist assay in the insecticide resistance monitoring. This will help to easily assess the involvement of metabolic resistance mechanisms in the production of resistance phenotype.
4) Determine the resistance status of *Anopheles funestus* in the country. Despite adequate information on the susceptibility status of *Anopheles gambiae* vectors to insecticides, little is known on the susceptibility status of another major malaria vector, *Anopheles funestus* to insecticides.
5) In the presence of insecticide resistance, Integrated Vector Management approach (i.e., Environmental management, biological control and larviciding) should be encouraged depending on specific local settings, particularly targeting those areas with resistance and/or reduced susceptibility to some insecticides of public health importance.

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