The Use of Agricultural Chemicals and Mosquito Response to Pyrethroids in Bungoma East, Sub-County, Western Kenya

by

Rebeccah Nanjala Wafula

Department of Global Health
Duke University

Date:_____

Approved:

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Wendy O'Meara, Supervisor

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Randy Kramer

_____
Joe Egger

Thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science in
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ABSTRACT

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Abstract

Insecticide treated bed nets and indoor residual spraying are the most widely used vector control methods in Africa. The World Health Organization now recommends four classes of insecticides for use against adult mosquitoes in public health programs. Of these four classes of insecticides, pyrethroids have become the insecticides of choice in treating mosquito bed nets and in the use of indoor spraying to prevent malaria transmission. Pyrethroids are not only used in malaria control but also in agriculture to protect against pest insects. This concurrent use of pyrethroids in vector control and protection of crops from pests in agriculture may exert selection pressure on mosquito larval population and induce resistance to this class of insecticides. The main objective of our study was to explore the use of agricultural chemicals and the response of mosquitoes to pyrethroids in an area of high malaria transmission.

We used a cross-sectional study design in a two-step study involving both mosquitoes and human subjects. In this study, we collected larvae growing in breeding sites affected by different agricultural practices. We used purposive sampling to identify active mosquito breeding sites and then interviewed households adjacent to those breeding sites to learn about their agricultural practices that might influence the response of mosquitoes to pyrethroids.

Results from the insecticide susceptibility assays showed noticeable differences in the proportion of mosquitoes, which were knocked down between the breeding sites .The overall mortality for individual breeding sites was less than the 90% threshold set by the WHO defining mosquito resistance to an insecticide. We observed very low mortality of mosquitoes after 24 hours post exposure to the cone bioassays with only one site recording a mortality of 16.67%. The use of fertilizers (84.56%) and non-crop pesticides (70.31%) was more common than the use of pesticides in our household results.

This study confirmed that local mosquitoes reared from agricultural areas in four villages in Western Kenya were resistant to permethrin and deltamethrin insecticides. In our study, cone bioassay tests results indicated that resistance in mosquitoes in Bungoma East sub-county might be undermining the efficacy of insecticide treated nets in the area. Our findings highlight the need for local malaria stakeholders to

Dedication

To my future self, may you look back and realize that the process was worth it.

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2.0 INTRODUCTION

Malaria remains a disease of global importance. The World Health Organization (WHO) estimates that 3.2 billion people are at risk of malaria, of whom 1.2 billion are at high risk [1].In 2015,an estimated 248 million cases of malaria occurred worldwide with 438 000 cases resulting in deaths. Concerted efforts by the global community have resulted in expansion of malaria interventions, which have helped reduce malaria incidence by 37% globally, and by 34% in Africa [1]. Current malaria control strategies include increased diagnostic testing, of all suspected malaria cases and prompt treatment of confirmed cases with effective Artemisinin combination therapies (ACT), chemoprevention of malaria in pregnant women (IPTp), infants and children where applicable and application of appropriate vector control interventions. Vector control interventions include the use of bed nets indoor residual spraying, larviciding, use of larvivorous fish, space spraying and using mosquito repellents among others.

Insecticide treated bed nets and indoor residual spraying are the most widely used vector control methods in Africa. Insecticide treated nets have been proven to be highly effective and cost effective offering up to 50-70% protection against malaria[2-5]. Historically, several insecticides have been used in the vector control of malaria with varying success. Dichlorodiphenyltrichloroethane (DDT) was extensively used in indoor spraying in the 1940's and late 1970's[6].DDT has the longest residual efficacy of any insecticide. When sprayed on walls and ceilings, it can last approximately 6-12 months

dependent on the dosage and nature of substrate[7].In addition, its spatial repellency and irritant effect on malaria vectors strongly limits human-vector contact. However the observed decrease in malaria infection following the introduction of DDT for malaria control was short lived due to the emergence of resistance in malaria vectors.

The WHO now recommends four classes of insecticides for use against adult mosquitoes in public health programs: pyrethroids, organochlorines, organophosphates and carbamates.[8] Of these four classes of insecticides, pyrethroids have become the insecticides of choice in treating mosquito bed nets to prevent malaria transmission. In 2009,pyrethroids accounted for 75% of indoor residual spraying worldwide and the treatment of long lasting insecticide bed nets (LLINs) while carbamates and organophosphates represented a small percentage of global usage[9]. Pyrethroids are considered safer, cheaper and have a longer residual action than other insecticides[10].In addition, pyrethroids are used in indoor residual spraying as an alternative to DDT and malathion where mosquito vectors have become resistant to both insecticides[10]. Examples of pyrethroids include alpha-cypermethrin, cyfluthrin, bifentrin, cypermethrin, cyphenothrin, deltamethrin, etonfenprox, lambda-cyhalothrin and permethrin [11].

In Kenya, malaria is one of the leading causes of morbidity and mortality. Malaria accounts for between 30-50% of all outpatient cases and 20% of hospital admissions to health facilities [12]. Vector control of malaria has been highly prioritized

by the National Malaria Control Unit in Kenya. Currently, malaria prevention strategies are largely concentrated on mass distribution of long lasting insecticide nets, routine distribution of long lasting insecticide treated nets (LLINs) to pregnant women and children under 1 year and indoor residual spraying in some malaria endemic areas[13]. As of 2010,household ownership of long lasting insecticide nets stood at 70% and that of insecticide treated nets at 60% in malaria endemic areas[14].Although household ownership of bed nets and bed net use has significantly reduced malaria infection, this reduction has not been uniform in all settings where bed net coverage is high[15, 16].

There are several reasons as to why the increase in coverage of bed nets may not correspond to a uniform decrease of malaria infection in all areas. One possible reason is that, relying on one class of insecticide in the treatment of bed nets may have accelerated the development of resistance observed in mosquito vector populations. According to the 2014 World Malaria Report, insecticide resistance in malaria vectors has been reported in 53 of the 65 reporting countries around the world since 2010[1]. Of these countries 49 have reported resistance to two or more insecticide classes. In West and Central Africa, countries such as Benin, Cote Divoire, Burkina Faso and Ghana have reported widespread resistance to pyrethroids and organophosphates. Ethiopia has reported resistance to all four classes of insecticides, including widespread resistance to DDT and increasing resistance to pyrethroids. In Southern Africa, a high frequency of metabolic resistance to pyrethroids has now been reported in Zambia and Malawi in

addition to earlier reports in Mozambique and South Africa[9]. Resistance to pyrethroids has also been reported in Kenya[17].

African malaria vectors have been able to develop resistance to pyrethroids primarily through 2 mechanisms. The first is resistance at the target site, in which only 1 point mutation at 1014L in the voltage gated sodium channel causes insensitivity to pyrethroids, resulting in knockdown resistance (*kd*r). The second is metabolic resistance that relates to the elevated activity of 1 or more detoxification enzymes[18]. The point mutations in the voltage-gated sodium channel are commonly reported in *Anopheles gambiae* Giles*[19-21]*. In contrast, such *kdr* mutations do not seem to be common in *Anopheles arabiensis* Patton, instead metabolic resistance seems to be more common in this species [22-24].*Kdr* mutations can also be selected by and do confer cross resistance to the organochlorine DDT, which also targets the insect voltage gated sodium channels [25].

Pyrethroids are not only used in malaria control but also in agriculture to protect against pest insects. The selection of *kdr* mutations may be highly dependent on the use of insecticides targeting the sodium channel which could be affected by agricultural practices and domestic usage based on these insecticides [25].This concurrent use of pyrethroids in vector control and protection of crops from pests in agriculture [26] may exert selection pressure on mosquito larval population and induce resistance to this class of insecticides.

Several studies have shown that agricultural practices can lead to resistance in mosquito populations and may undermine the effectiveness of current vector control programs. In Northern Benin, low mortality rates of *Anopheles gambiae* to permethrin was observed in areas using pesticides in growing cotton.[27] Another study conducted in urban areas of Benin investigated the use of pesticides in vegetable farming. Evidence showed a clear pattern of resistance with some sites recording as low as 17% mortality of Anopheles mosquitoes exposed to permethrin[28]. Resistance to lambda-cyhalothrin (pyrethroid) has been observed in populations of *Anopheles gambiae* in rubber cultivating areas of Cameroon[29]. It is possible that the use of pesticides in agriculture may contaminate nearby breeding sites by diffusing into the water and applying selection pressure on mosquitoes.

In Kenya, Obala *et al[16]* conducted a case control study looking at factors associated with the prevention failure of insecticide treated bed nets using the efficacy decay model in Bungoma East Sub-County. In their results, using pesticides for agricultural purposes increased the odds of infection (Adj1.72, p=0.078) while growing grains like maize, millet or sorghum reduced the odds of infection(Adj OR=0.45).Despite these important relationships between agriculture and malaria infection, pyrethroids represented a small percent of the total pesticide used by households in the study. In addition to the use of pesticides, other chemicals used in agriculture include fertilizers, herbicides, fungicides and their presence in breeding sites has been shown to affect

mosquito tolerance to insecticides .A study conducted by Riaz et al showed that exposing *Ae.egypti* larvae to the herbicide glyphosate, the active molecule of Roundup, led to a significant increase of their tolerance to permethrin together with the induction of multiple detoxification enzymes.[30]

The mechanisms linking resistance and agricultural practices are certainly related to breeding sites but the exact relationships are unknown and require further investigation. It is a possibility that those mechanisms require pathways to phenotypic resistance not specifically related to pyrethroid metabolism pathways. Activation of these phenotypes could be due to exposure to a variety of agricultural chemicals. Bungoma East is an example of an area with high coverage of insecticide treated nets and high malaria infection. Coverage of insecticide treated nets increased from 25% to 67% and in some villages as high as 95%[16]. Most households practice subsistence farming by growing different types of crops. These crops are sometimes sprayed with pesticides to protect them from pest insects. An understanding of the association between agricultural practices and mosquito response to pyrethroids will strengthen current vector control programs in targeting areas with specific insecticides depending on the mosquito susceptibility.

2.1 Rationale

There are several reasons for the continued occurrence of malaria infection in areas with high coverage of insecticide treated nets. These include the reduced efficacy

of insecticide treated nets, improper use of insecticide treated nets, stock-outs of anti-malarial drugs and poor dosing regimen of policy recommended drugs by private outlets. However, the role of agricultural practices such as using pesticides or growing different types of crops in the development of mosquito resistance is yet to be fully understood. In addition, few resistance studies that have been carried out in Kenya as shown in Figure 1 [20].The aim of this study was to explore this association by carrying out susceptibility tests on mosquitoes collected from various breeding sites in agricultural areas and through household surveys of nearby households. In addition, we included data from a case-control study to understand the relationship between growing different types of crops and larval characteristics. We performed secondary data analysis of larval data from Obala et al [16]to examine the relationship between crops, pesticides and whether any of the larvae sites had larvae, and for larvae sites near a home whether the larvae had KDR resistance mutations.

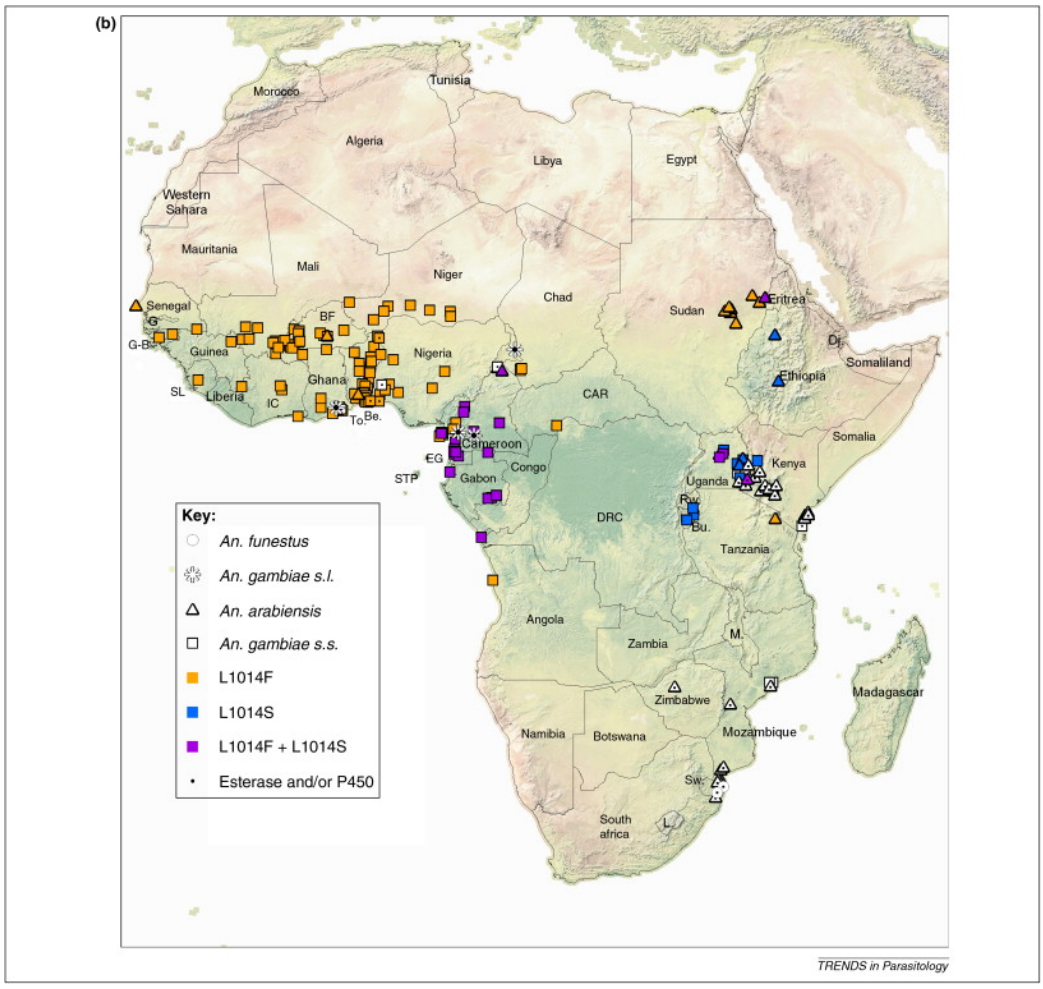

Figure 1: Pyrethroid resistance in Africa

1.1.1 Study objectives

In this study, we identified breeding sites with larvae in target villages. We then interviewed households adjacent to breeding sites whose activities, particularly agricultural activities, may influence those breeding sites. Our outcome measure was phenotypic resistance to permethrin and deltamethrin of adult mosquitoes reared from these larval sites. The study objectives were

1a) To determine mosquito susceptibility to insecticides used in vector control

1b) To identify agricultural practices that influence resistance in the mosquitoes

2) To understand relationships between agricultural practices and larval abundance

3.0 METHODS

3.1 Setting

The study was carried out in Kinesamu, Lukhuna, Matulo and Muji villages in Bungoma East sub –county. Bungoma East sub-county is located 50 km east of the border with Uganda. The sub- county gets its water supply from river Nzoia. Residents of Bungoma East sub-county grow sugar cane as their main cash crop but also practice subsistence farming by cultivating onions, tomatoes, legumes and vegetables. Malaria transmission is perennial with a seasonal peak following the rains in May-June. Prior to scale-up of malaria control efforts, entomological inoculation rate (EIR) was estimated to be 29 infectious bites per person per year[31].

3.2 Mosquito sampling

3.2.1 insecticide susceptibility bioassays

We used purposive sampling in which our first objective was to identify active breeding sites based on the local knowledge of the area. Once we identified active larval sites, we characterized each larval site as to whether there was presence or absence of crops. Larvae and pupae were sampled from breeding sites in study villages through dipping and transferred to falcon tubes for transportation to an insectary. Both culicine and anopheline mosquitoes were collected, as it was difficult to distinguish them at the pupae stage during collection. Mosquito larvae collected from the same breeding site over two or more days were assigned the same identification but were reared

differently. Mosquito larvae were then transferred from falcon tubes to basins and raised in their own breeding water and fed with yeast. Every two days, the water would be replaced with water from the same breeding site and those larvae that had developed into pupae would be transferred into adult cages. Upon emergence culicine and male anopheline mosquitoes were discarded. Adult female anopheline mosquitoes were maintained on 10% sucrose solution until they were used for the insecticide bioassay.

Three to five days old female adult mosquitoes morphologically identified as belonging to the genus anopheles were exposed to discriminating doses of 0.05% deltamethrin and 0.75% permethrin according to the standard WHO test procedures[8].Impregnated papers were obtained from the vector control research unit of University Sains Malaysia, Penang, Malaysia which is the WHO approved source for insecticide assays. Four exposure tubes were lined with a sheet of insecticide-impregnated papers while two control holding tubes were lined with oil-impregnated papers. Batches of 20-25 mosquitoes were transferred into the four exposure tubes and control tubes per insecticide. The mosquitoes were observed for 1-hour, during which the number of mosquitoes knocked down was recorded every 10 minutes. At the end of the 1-hour exposure, the mosquitoes were transferred to the holding tubes and a pad of cotton wool soaked in sugar water was placed on the mesh-screen end of the holding tubes.

Mosquitoes were maintained in holding tubes for 24 hours post-exposure (recovery period). At the end of the recovery period, we counted and recorded the number of dead mosquitoes. We considered an adult mosquito to be alive if it was able to fly, regardless of the number of legs remaining. Any knocked-down mosquitoes, whether or not they had lost legs or wings, were considered moribund and counted as dead. Results of mortality rates were interpreted according to WHO criteria [8]. On completion of the susceptibility test, mosquitoes were transferred to individual clearly labeled Epperndorf tubes. We separated dead and live mosquitoes and stored them separately. Mosquito species were identified morphologically the following day.

3.2.2 WHO Cone bioassays

We also performed standardized WHO cone bioassays because a large sample size (100-150 mosquitoes) was required for the WHO insecticide susceptibility test. We could not always collect this number of mosquitoes from individual larval sites of interest. The WHO cone assays were conducted on four 25 cm x 25 cm pieces cut from a new Olyset permethrin treated LLIN . Two standard WHO cones were fixed with a plastic manifold onto each of the four netting pieces. Five susceptible, non-blood-fed, 3–day-old female Anopheles were exposed for 3 min in each cone and then held for 24 hours with access to sugar solution. Knock-down time was measured 60 min after exposure, and mortality measured after 24 hours. A negative control, from an untreated net, was included in each round of cone bioassay testing.[32]

3.3 Household surveys

For each larvae site, we noted if there was presence or absence of crops. We then proceeded to check if there were households near the breeding sites. Nearness was defined as households that were within 300 meters of the larvae site. We then went to the households near the breeding site and found out which household(s) was responsible with the crops that were near the breeding site. We approached household representatives older than 18 years and sought verbal informed consent for participation in the study. We then proceeded to administer a household survey after obtaining ethical approval from both Duke and Moi Institutional Review Boards. Two research assistants collected data on types of pesticides used in the house and on the farm, type of crops grown, use of fertilizers in farming or any other form of chemicals and bed net use. Some questions on the household survey were adapted from the Vietnam Pesticide Use Questionnaire[33] .We then linked this information to the mosquito response to pyrethroids from each larvae site near the households.

3.4 Secondary Data

We performed a secondary analysis of data from a previous case control study conducted by Obala et al in May 2013 and July 2014 on factors threatening the effectiveness of bed nets in Bungoma East. Cases were recruited from the pediatric ward of Webuye-Sub County Hospital. Each case was visited at home after they were discharged. Households within the same village, but outside of the search radius for

neighbors and larval sites (250 meters) were canvassed to identify an age- and gender matched healthy control. Household information was recorded including the number of households members, education and occupation of the household head, housing construction and agricultural practices including the types of crops grown around the house and the names of the chemical products used on the crops as reported by the family. All potential breeding sites within a quarter–kilometer radius were also mapped and photographed. Each breeding site was dipped to determine the presence of any mosquito larvae. A sample of larvae were collected from active sites and sent for molecular analysis of species and presence of kdr mutations. A detailed description of the methods and the main findings of this study have been published elsewhere [16]and we are only presenting the results of the larvae data analysis in this study

3.5 Data Management

Data were entered into an MS Excel spreadsheet and stored on a password-protected laptop. We did not collect personal identifying data in the household surveys. Study IDs were generated through assigning a larval site a number, two letters of the village and the year. No data were transferred electronically without deidentification.

3.6 Measures

Our study had two primary outcome measures for measuring the response of mosquitoes to pyrethroids .Our first measure was the knock down rate, which was defined as the number of mosquitoes that were unable to stand or fly in a coordinated

way after being exposed to an insecticide for an hour. Our second measure was the 24-hour mortality rate, which was obtained by summing the number of dead mosquitoes across all four-exposure replicates and expressing this as a percentage of the total number of exposed mosquitoes. A similar calculation was made in order to obtain a value for the control mortality. A mosquito was defined as dead if it was immobile, unable to stand or fly in a coordinated way and alive it was still able to fly, irrespective of the number of legs remaining after 24 hours of exposure respectively.

3.7 Data analysis

Data was entered into an MS Excel spreadsheet, cleaned for duplicate entries and imported into STATA version 13[34].We measured resistance using WHO defined criteria [8]. We used a Fishers exact test to compare mortality rates across the four breeding sites .We evaluated statistical significance at p-values of <0.05.Statistical significant results are bolded in all subsequent tables. For our secondary data, we performed logistic regression models to determine the relationship between crops and 1) whether a household had a larval site 2) whether the site had larvae 3) whether the larvae were *Anopheles gambiae* or *Anopheles funestus* and 4) whether there was presence of KDR mutations. We also performed logistic regression models to determine the relationship between pesticides and 1) whether a household had a larval site 2) whether the site had larvae 3) whether the larvae were *Anopheles gambiae* or *Anopheles funestus* and 4) whether there was presence of KDR mutations. We then combined the types of

crops and pesticides into one model and assessed the relationship to whether any sites near a home had a larval site. We reported 95% confidence intervals for the crude and adjusted odds ratio. For household surveys, we performed descriptive analysis.

3.8 Ethics approval

Ethical approval was sought and obtained from both Duke University and Moi Teaching and Referral Hospital Institution Review Boards. Permission was also sought from the Bungoma County Director of Health. We also obtained permission from the local village administration and verbal consent from representatives of households near breeding sites for the household surveys since this was a minimal risk study

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4.RESULTS

4.1 Larval data

In this section, we investigated the relationship between variables correlated with a household having any potential mosquito breeding site within 300 meters, variables correlated with larvae being in the site, variables correlated with confirmed malaria vectors I n the site (i.e. anopheles larvae as determined by molecular genetic assays), and variables correlated with vectors with mutations that are known to confer resistance to pyrethroid-based insecticides and might make them very successful at feeding even when individuals are protected with insecticide impregnated bed nets.

We performed logistic regression models to determine the relationship between crops and 1) whether a household had a larval site 2) whether the site had larvae 3) whether the larvae were *Anopheles gambiae* or *Anopheles funestus* and 4) whether there was presence of KDR mutations. We also performed simple logistic regression models to determine the relationship between pesticides and 1) whether a household had a larval site 2) whether the site had larvae 3) whether the larvae were *Anopheles gambiae* or *Anopheles funestus* and 4) whether there was presence of KDR mutations.

The larval data presented in this thesis was part of a larger case-control study in which 442 case-control pairs were enrolled. The mean age of both cases and controls was 3.5 years. Fifty percent of enrolled children were female. The remaining characteristics are shown in Table 1

Table 1:Summary characteristics of case/control and their households

Variable	Mean	95% CI	Mean(Controls)	95% CI
Children and Households				
Age (Years)	3.58	(3.31-3.85)	3.49	(3.24-3.75)
Male	0.5		0.5	
Proportion testing positive*	0.2		0.08	
Total person in household	0.2	(5.98-6.40)	6.17	(5.95-6.38)
Total nets per household	6.19	(1.75-1.97)	1.91	(1.81-2.02)
Sleeping spaces per person	1.86	(0.44-0.48)	0.48	(0.45-0.47)
HH* finished secondary school	0.3		0.28	
Pesticides used				
Organophosphates	0.13		0.1	
Pyrethroids	0.05		0.06	
Carbamates	0.01		0	
Any pesticide	0.16		0.15	
Types of crops				
Grains	0.7		0.74	
Sugarcane	0.67		0.64	
Napier grass	0.31		0.29	
Banana	0.73		0.75	
Vegetables	0.56		0.56	
Legumes	0.48		0.45	
Tubers	0.44		0.42	
Larval sites*				
Total larval sites	67.31	(311)	51.44	(232)
Total sites with larvae	25.87	(119)	14.19	(64)
Total sites with Anopheles gambi	21.67	(26)	10.67	(7)
Total sites with kdr mutations	14.29	(17)	9.09	(6)

The relationship between households having a larval site was dependent on the type of crop and not associated with the use of pesticides after adjusting for crop type. In a multivariable model that included major crop types and use of pesticides, households that grew vegetables compared to households, which did not grow vegetables, was significantly associated with reduced odds of having a household near a home having a

larval site. (Adjusted Odds Ratio (AOR) 0.53, (95% CI:0.403-0.696). The results are shown in Table 2.

Table 2: Relationship between crops, pesticides and whether a household had a larval site

Variable	%(n)	OR	95% CI	*p*value	AOR	95% CI	pvalue
Maize	72.56(394)	1.11	0.83-1.491	0.474	1.29	0.902-1.845	0.163
Banana	73.3(398)	0.85	0.625-1.154	0.298	1.09	0.757-1.560	0.652
Sugarcane	63.72(346)	0.78	0.594-1.044	0.098	0.89	0.639-1.236	0.484
Millet	11.97(65)	0.97	0.651-1.464	0.907	1.17	0.753-1.803	0.491
Napier grass	28.36(154)	0.77	0.578-1.023	0.072	0.96	0.697-1.318	0.794
Beans	43.28(235)	0.94	0.719-1.225	0.642	1.06	0.769-1.468	0.713
Vegetables	50.46(274)	0.53	0.403-0.696	**0.001**	0.59	0.430-0.808	**0.001**
Groundnuts	9.39(51)	0.73	0.475-1.123	**0.071**	0.92	0.576-1.454	0.709
Sweet potatoes	53.61(268)	0.65	0.496-0.847	**0.002**	0.78	0.569-1.058	0.109
Pesticide	48.98(72)	0.61	0.419-0.851	**0.004**	0.75	0.513-1.105	0.148

The relationship between households having a larval site with larvae was dependent on the type of crop and unrelated to the use of pesticides. In a multivariable model, the odds of having a larval site with larvae were nearly twice in households that grew sugarcane (AOR 1.68:1.104-2.546) and beans (AOR 1.74:1.209-2.508). The odds of having a larval site with larvae were decreased if the households grew bananas and vegetables. The odds of having larval site were decreased if a household grew sweet potatoes although this relationship did not reach statistical significance. The results are shown in Table 3

Table 3: Relationship between crops, pesticide and whether any household had a larval site with larvae

Variable	%(n)	OR	95% CI	*p*value	AOR	95% CI	*p*value
Maize	77.05(141)	1.42	0.969-2.070	**0.071**	1.24	0.777-1.975	0.368
Banana	68.85(146)	0.7	0.490-0.999	**0.048**	0.58	0.380-0.896	**0.014**
Sugarcane	70.49(129)	1.3	0.917-1.854	0.14	1.68	1.104- 2.546	**0.015**
Millet	14.21(26)	1.27	0.791-2.038	0.322	1.32	0.789- 2.202	0.29
Napier grass	32.79 (69)	1.13	0.801-1.604	0.478	1.37	0.919- 2.040	0.123
Beans	51.91(95)	1.5	1.082-2.073	**0.015**	1.63	1.094-2.422	**0.016**
Vegetables	45.9(84)	0.58	0.420-0.806	**0.001**	0.53	0.360-0.784	**0.001**
Groundnuts	10.38(19)	1.02	0.601-1.743	0.931	1.01	0.564-1.817	0.966
Sweet potatoes	36.07(66)	0.71	0.509-0.995	0.939	0.7	0.474-1.041	**0.079**
Pesticide	14.97(22)	0.66	0.163-0.406	0.092	0.73	0.430-1.239	0.245

We compared homes with anopheles larvae nearby to all other homes. In our study the relationship between crops, pesticide use and whether a household had a site near their home with larvae of the *Anopheles gambiae* species was associated with only growing groundnuts and not with using pesticides as shown in Table 4. However the number of households growing groundnuts and having a site with larvae of the *Anopheles gambiae* species was relatively small.

Table 4:Relationship between crops, pesticide and whether any sites had *Anopheles gambiae*

Variable	%(n)	OR	95% CI	*p*value	AOR	95% CI	*p*value
Maize	81.82(27)	1.44	0.55-3.744	0.458	1.33	0.383- 4.620	0.653
Banana	72.73(24)	1.26	0.543-2.904	0.593	0.64	.223-1.830	0.405
Sugar cane	84.85(28)	2.8	1.02-7.681	**0.039**	2.08	.654- 6.627	0.214
Millet	9.09 (3)	0.54	0.152-1.903	0.329	0.32	.082-1.291	0.11
Nappier grass	39.39(13)	1.47	0.673-3.192	0.334	1.08	0.356-2.430	0.866
Beans	57.58(19)	1.3	0.601-2.780	0.492	0.93	0.444- 2.621	0.884
Vegetables	60.61(20)	2.03	0.94-4.371	**0.068**	1.28	0 .476- 3.481	0.617
Sweet potatoes	60.61(20)	3.27	1.502-1.098	**0.002**	1.32	0 .377-4.614	0.665
Groundnuts	15.15(5)	1.64	0.552-4.889	0.368	3.14	1.246-7.917	**0.015**
Pesticide	22.73(5)	1.43	0.487-4.193	0.516	0.91	0.277-2.996	0.881

We compared homes with anopheles larvae, which expressed kdr mutations nearby to homes with anopheles larvae to all other homes. The relationship between crops, pesticide use and whether any sites near a household with *kdr* mutations was not associated with the type of crop grown. We were unable to evaluate the relationship between pesticide use and whether any site near a home had *kdr* mutations due to very few observations.

Table 5:The relationship between crops and whether any site had kdr mutations

Variable	%(n)	OR	95% CI	*pvalue*	AOR	95% CI	*pvalue*
Maize	76.92(20)	0.83	0.308-2.280	0.99	0.75	0.192-2.893	0.672
Banana	69.23(18)	1.26	0.543-2.904	0.96	0.49	0.152-1.551	0.223
Sugarcane	84.62(22)	2.25	0.727-6.934	0.09	2.15	0.59-7.763	0.245
Millet	11.54 (3)	1.27	0.396-4.079	0.67	0.94	0.243-3.614	0.926
Nappier grass	38.46(10)	1.4	0.568-3.443	0.51	1.16	0.407-3.289	0.785
Beans	53.85(14)	1.24	0.513-2.983	0.83	1.18	0.364-0.364	0.788
Vegetables	53.85(14)	1.625	0.673-3.920	0.38	1.30	0.428-3.972	0.641
Sweet potatoes	56.52(13)	2.53	1.042-6.135	0.04	1.39	0.341-5.654	0.646
Groundnuts	11.54 (3)	1.92	0.581-6.348	0.84	2.52	0.855-7.424	0.094

4.2 Insecticide susceptibility tests

4.2.1 Knock down effects of insecticides

Knock down referred to the inability of a mosquito to stand or fly in a coordinated way. We calculated the proportion of mosquitoes that were knocked down during a one-hour exposure period in regular intervals of 10,20,30,40,50 and 60 minutes. Figure 1 and 2 show the relationship between knockdown times for the test populations of mosquitoes during one hour of exposure to permethrin and deltamethrin respectively. There were noticeable differences in the proportion of mosquitoes, which were knocked down between the breeding sites. For permethrin, the highest number of mosquitoes, which were knocked down, was in Kinesamu (91%) and the site with the lowest number of mosquitoes, which were knocked down, was in Muji (60%).

For deltamethrin, the highest number of mosquitoes that were knocked down was in Matulo (82%) and the lowest number of mosquitoes that were knocked down was in Lukhuna (51%).

Table 6:Proportion of mosquitoes knocked down after exposure to deltamethrin

	Exposure(minutes)					
Breeding site	10	20	30	40	50	60
Kinesamu	8 (3.92-16.77)	19 (13.22-30.99)	45 (39.27-60.73)	53 (48.02-69.16)	72 (70.25-87.69)	74 (72.74-89.48)
Muji	16 (11.89-30.44)	29 (25.79-47.76)	42 (41.02-63.79)	48 (48.44-70.80)	51 (52.24-74.21)	48 (48.44-70.80)
Matulo	11 (5.51-18.48)	42 (31.52-51.36)	71 (59.71-78.32)	85 (74.66-89.98)	86 (75.78-90.76)	92 (80.35-93.77)

Table 7:Proportion of mosquitoes knocked after exposure to permethrin

	Exposure(minutes)					
Breeding site	10	20	30	40	50	60
Muji	38 (36.71-59.63)	52 (54.29-76.13)	60 (65.02-84.86)	58 (62.28-82.73)	64 (70.62-88.97)	72 (82.59-96.36)
Matulo	4 (1.13-10.22)	23 (15.66-33.42)	42 (33.27-53.75)	52 (43.19-63.80)	62 (53.54-73.42)	70 (62.14-80.79)
Lukhuna	4 (1.22-10.99)	8 (3.92-16.77)	26 (19.82-39.40)	40 (33.96-55.30)	42 (36.07-57.49)	46 (40.35-61.80)

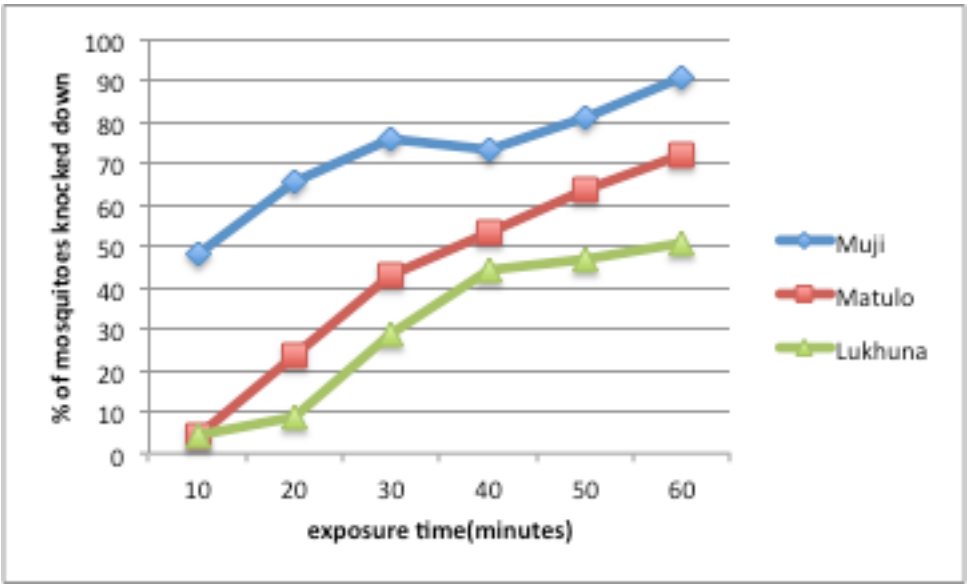

Figure 1:Percentage of mosquitoes knocked down after exposure to permethrin

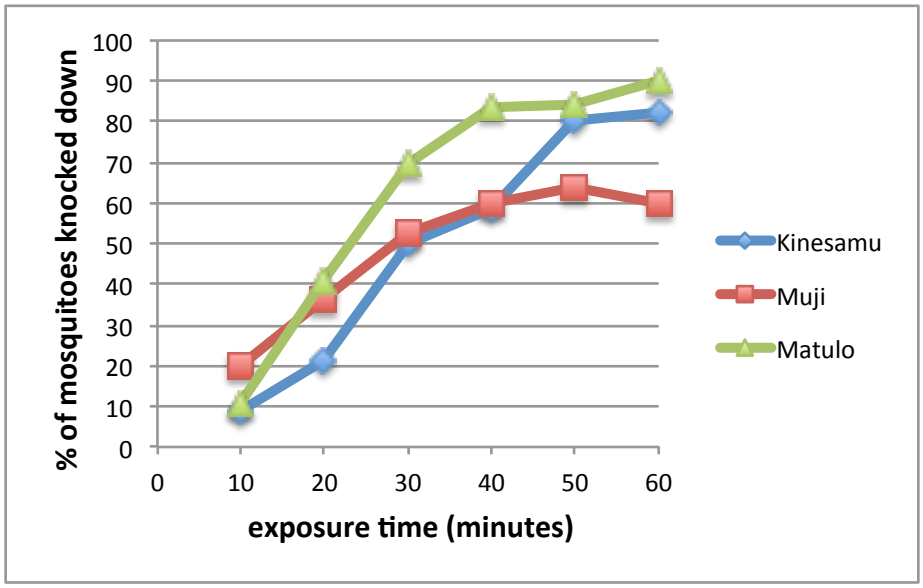

Figure 2: Percentage of mosquitoes knocked down after exposure to deltamethrin

4.2.2 Mortality rates

Mortality was defined as a count of the number of dead mosquitoes in both the exposure and the control tubes. We calculated mortality rates 24 hours post exposure.

$$\text{Observed mortality} = \frac{\text{Total number of dead mosquitoes}}{\text{Total sample size}} \times 100$$

Mortality in the range of 98-100% indicated susceptibility to the insecticide while mortality of less than 98% was suggestive of the existence of resistance. A mortality rate below 90% was considered resistant.

Table 6:Mortality of mosquitoes exposed to permethrin[1]

Breeding site	Status	%Mortality (n)	95%CI
Kinesamu	Exposure**	66.03[a](90)	60.79-80.79
	Control	13.99(40)	3.99-23.99
Muji	Exposure	84.42[b](80)	76.42-92.42
	Control	5.26(40)	0-20.26
Matulo	Exposure	88.56[b](102)	80.56-96.56
	Control	0(52)	*

[1] Numbers in the same column with the same superscript do not differ significantly by Fisher test (p>0.05)

* No confidence intervals

**Mortality corrected using Abbotts formula

Table 7:Mortality of mosquitoes to deltamethrin[2]

Breeding site	Status	%Mortality (n)	95% CI
Muji	Exposure	88.1(92)	81.10-95.1
	Control	5.26(50)	0.74-11.26
Matulo	Exposure	85.11(79)	78.11-92.11
	Control	0(52)	*
Lukhuna	Exposure	36.36(90)	28.36- 44.36
	Control	4.76(42)	10.24-19.76

Numbers in the same column with the same superscript do not differ significantly by Fisher test ($p>0.05$)
* No confidence intervals

4.2.3 WHO Cone bioassays

We tested a total of 80 mosquitoes using the cone bioassay tests. We observed zero mosquitoes knocked down after the standard three-minute exposure to the new Olyset permethrin net and after an hour post exposure in Matulo, Lukhuna and Muji. We also observed very low mortality of mosquitoes after 24 hours post exposure to the cone bioassays with only one site recording a mortality of 16.67%. In addition, none of the mosquitoes in the control cones died.

4.2.4 Species identification

A total of 814 Anopheles mosquitoes were identified morphologically. Most of the mosquitoes belonged to the *Anopheles gambiae* species (93.1%) *Anopheles coustani* (3.9%) and 3.0 percent of the mosquitoes species could not be determined morphologically and required further analysis.

4.3 Household surveys

4.3.1 Demographic characteristics of household respondents

The mean age of household representatives who responded to our surveys was 38.7 years.75.36% of our respondents were female while (24.64%) were male. Most respondents had attained a primary education (65.22%) and some had secondary education (28.99%). Majority of the respondents were self-employed (56.06%) with the

remaining respondents being unemployed (37.88%) or private employees (6.06%). All the households owned land and household representatives were responsible for working on their own farms. The mean number of years that each household had stayed in the village was 16.16 years. Table 8 shows a summary of the types of crops grown , fertilizers and self reported malaria by households living near the breeding sites.

Table 8:Differences in agricultural practices and malaria perception between breeding sites

Variable	Kinesamo %(n)	Lukhuna %(n)	Matulo %(n)	Mujl %(n)
Number of households	9	19	31	10
Crops				
Maize	77.78(7)	78.90(15)	74.19(23)	100(10)
Beans	44.44(4)	78.90(15)	29.03(9)	80(8)
Sweet potatoes	0	5.26(1)	3.23(1)	10.00(1)
Vegetables	0	21.01(4)	16.12(5)	40(4)
Nappier grass	0	5.26(1)	3.23(1)	30(3)
Ground nuts	22.2(2)	10.52(2)	3.23(1)	30(3)
Sugarcane	0	15.70(3)	16.12(5)	20(2)
Yams	100(1)	0	0	0
Use of fertilizer in 2015	100(9)	89.40(17)	74.19(23)	70(7)
Use of fertilizer in 2014	100(9)	100(19)	80.64(25)	100(10)
Fertilizers				
DAP	100(9)	100(19)	58.06(18)	80(8)
CAN	77.78(7)	94.70(18)	48.39(15)	70(7)
UREA	22.2(2)	10.52(2)	9.67(3)	50(5)
MANURE	0	21.01(4)	9.67(3)	70(7)
Last fertilizer used in 2015				
DAP	22.22(2)	78.90(15)	16.12(5)	0
CAN	77.78(7)	10(2)	54.83(17)	90(9)
UREA	0	0	6.45(2)	0
MANURE	0	0	6.45(2)	10(1)
Use of pesticides	22.22(2)	21.10(4)	19.35(6)	10(1)
Use of non crop pesticides	55.55(5)	78.90(15)	54.83(17)	70(7)
Other pesticides				
Triaticks	33.3(3)	57.9(11)	35.48(11)	0
Cattledip	0	5.26(1)	3.23(1)	30(3)
Grenade	22.2(2)	21.01(4)	3.23(1)	40(4)
Pesticide mixing	0	10.52(2)	3.23(1)	0
Bednet use and self-reported malaria				
Any bed nets	100(9)	27.87(17)	87.10(27)	80(8)
Bed net for each sleeping spa	55.55(5)	21.62(8)	61.29(19)	50(5)
Pretreated	100(9)	24.07(13)	83.87(26)	10(1)
Malaria common	100(9)	26.87(18)	100(31)	90(9)
Malaria concern	100(9)	27.27(18)	93.55(29)	100(10)
Infection within last 4 weeks	100(9)	28.81(17)	83.87(26)	70(7)

We linked the information from the household surveys to the results we obtained from the insecticide susceptibility assays of mosquitoes. The majority of the households in our survey used a form of chemical fertilizer, which may or may not be linked to the observed resistance by mosquitoes observed in our study. The remaining results are shown in Table 9.

Table 9: Relationship between mosquito assay results and household surveys. Reported as percent (n)

	Lukhuna	Kinesamo	Muji	Matulo
Mortality (deltamethrin)	NA	66.03(90)	84.42(80)	88.56(90)
Mortality(permethrin)	36.36(90)	NA	88.1(79)	85.11(102)
Mortality Olysset	0	0	16.67(2)	0
Knockdown (deltamethrin)	NA	82.22(74)	60(42)	90.19(42)
Knockdown (permethrin)	51.11(90)	NA	72.16(72)	78.26(92)
Any chemical fertilizer	89.4(17)	100(9)	70(7)	74.19(23)
Any pesticide	21.10(2)	22.22(4)	10(1)	19.35(6)
Any non-crop pesticide	78.90(15)	55.55(5)	70(7)	54.83(17)

5. Discussion

Our results from the insecticide susceptibility bioassay suggest that mosquitoes from the study sites met the WHO criteria for resistance. The WHO indicates that the mortality of mosquitoes needs to meet a susceptibility threshold of between 98%-100 to be considered susceptible to an insecticide and mortality less than 90% indicates resistance. The highest mortality recorded in our study was 88.1% in Muji, which still fell below the 90% threshold set by the WHO guidelines. In Lukhuna, we recorded the lowest mortality of mosquitoes at 36.36%. Similar results have been reported in previous studies conducted in areas with agricultural activities. In Northern Benin, low mortality rates of *Anopheles gambiae* to permethrin was observed in areas growing cotton.[27] Another study conducted in urban areas of Benin showed a clear pattern of resistance in areas growing vegetables with some sites recording as low as 17% mortality of Anopheles mosquitoes exposed to permethrin[28]. In rubber cultivating areas of Cameroon[29], a mortality rate of 41% was observed in populations of *Anopheles gambiae* suggesting resistance to lambda-cyhalothrin (pyrethroid) insecticide

5.1 Relationship between knockdown rate and mortality

We also evaluated the proportion of mosquitoes that were knocked down when exposed to deltamethrin and permethrin after every ten minutes up to an hour respectively.

There were substantial differences in the proportion of mosquitoes that were knocked down after an hour of exposure to the insecticide and the proportion of mosquitoes that eventually died after 24 hours post exposure. For permethrin, the 24-hour mortality was substantially higher than the proportion of mosquitoes knocked down after an hour in Muji when compared to Kinesamu. The proportion of mosquitoes that were knocked down and the proportion of mosquitoes that eventually died after an hour were about equal in Matulo. For deltamethrin, we observed that the proportion of mosquitoes that were knocked down after an hour exposure was substantially lower than the proportion of mosquitoes that eventually died after 24 hours in Muji and Matulo when compared to Lukhuna. This may provide evidence that the mechanisms governing sensitivity to these two outcomes, knockdown versus mortality, are different.

It is possible that in mosquito populations where the major contributing mechanism of resistance is via knockdown, the knock down rate might be expected to be lower than mortality, as the target site mutation enables mosquitoes to temporarily withstand pyrethroid exposure [25]. This phenomenon may be also be observed in areas where the main mechanism of resistance is through metabolic pathways, where the knock down rate may be lower than the mortality rate because the mosquitoes are able to detoxify the insecticide faster enabling them to recover when they are removed from exposure [25] Owusu et al [35]compared the relationship between time to knock down between the Mosquito Contamination Device(MCD) and the WHO insecticide bioassay

and found that the time to knockdown was a poor predictor of 24 hour mortality. However, the MCD bioassay is a relatively new technique and does not have a 24- hour holding period for mosquitoes, which may limit the application of these findings to cone bioassays, as they differ from WHO bioassays.

5.2 Cone bioassays versus insecticide susceptibility assays

The results of mosquito mortality from the WHO cone-bioassays differed substantially from the results of mosquito mortality obtained from the insecticide susceptibility bioassays. None of the mosquitoes were knocked down after the standard three-minute exposure to the new Olyset Permethrin net and after an hour post exposure in Matulo, Lukhuna and Muji. We noted very low mortality of mosquitoes after 24 hours post exposure to the cone bioassays with only one site recording a mortality of 16.67%. Bagi et al[36] also measured the intensity of insecticide resistance by comparing the mortality of mosquitoes obtained from different bioassays. In their study, they observed mortality rates of less than 50 % for all strains of mosquitoes, including the Kisumu susceptible strain when exposed to a new Olyset Net LLIN, which had permethrin incorporated into the polyethylene fibers. Although some studies have reported that Olyset nets perform poorly in cone bioassays [37], these results might be an indication that resistance is also having an impact on the efficacy of this type of insecticide treated bed net.

5.3 Pesticide use and larval characteristics

In our analysis of the larval data from the case-control study, the relationship linking agriculture and larval characteristics was not dependent on the use of pesticides but on the type of crop. This analysis showed that the presence of potential larval sites was correlated with growing vegetables while the presence of active larval sites was correlated with growing sugarcane and bananas. Although 16.39% of larval sites with larvae had anopheles gambiae present, the overall numbers were too few to measure robust correlations .Our study mainly consisted of subsistence crops, which may not require a lot of pesticide application. We did see low reported pesticide use in these areas, but there is another interpretation here. If pesticides were not related to having larvae present, then it could either mean larvae were not exposed to much pesticide or it could mean that they are not sensitive to pesticide. Unfortunately, we had too few kdr results to determine the relationship between pesticides and kdr prevalence.

These results differed from previous studies that have linked agricultural use of pesticides to observed mosquito resistance to insecticides[27-29]. It is also possible that the type of crop grown determines the intensity of pesticide application. This observation may be true in some of the areas, which have reported resistance of mosquitoes in agricultural areas. Some of these agricultural areas grow cash crops such as cotton, rubber or vegetables for commercial purposes. Cotton is noted to be one of the

cash crops that require intense application of insecticides [38]. A study by Diabate et al noted increased resistance of mosquitoes to insecticides in areas that grew cotton [38].

Although vegetable cultivation may require frequent application of pesticide compared to other types of crops, in our study growing vegetables was associated with decreased odds of having any larval site or having a site with larvae present and not with a site having KDR mutations. This is in contrast to several studies[28, 36, 38] , which have linked the cultivation of vegetable farming to the development of insecticide resistant mosquito populations but not in relation to the presence or absence of larval sites.

5.4 Fertilizers and mosquito response to pyrethroids

The results from our household surveys showed that most households used at least one type of fertilizer for growing crops. Fertilizers used by households included Diammonium phosphate (DAP), Calcium ammonium nitrate (CAN), and Urea. The main components of these fertilizers are nitrogen, phosphorus and potassium. Very few studies have explored the relationship between the use of fertilizers in crop cultivation and mosquito resistance to insecticides. One study conducted by Darriel et al [39] in a laboratory study attempted to identify some of the physico- chemical factors favoring the larval development of pyrethroid-resistant *An. gambiae* VKPR. They evaluated various breeding sites and whether the breeding sites contained plant matter alone or was associated with NPK fertilizer. Although mosquitoes are not able to directly

assimilate nitrogen, phosphorus and potassium, these three minerals facilitate the development of algae, fungi, and bacteria, which increases the food biomass of the breeding sites.[39]. The authors conclude that NPK fertilizers "could impact the mosquito environment and even generate new ecological systems beneficial to the proliferation of mosquitoes. They cause a growing eutrophication of the natural breeding sites and select for resistance mechanisms to insecticides"[39]. However, it is possible that exposure to general chemicals in the larval environment may cause the larvae to increase expression of detoxification enzymes that might enhance resistance to all types of toxins.

5.6 Strengths and limitations of the study

One of the main strengths of our study is that we were able to rear larvae in a modified insectary, which enabled us to test mosquitoes that were of the same age in both the insecticide susceptibility and the WHO cone bioassays. Age of the mosquitoes is an important factor in carrying out these two mosquito bioassays. If we had used wild caught mosquitoes it would have been impossible to tell whether the mosquitoes were dying of physiological immaturity or old age. Secondly, the use of the impregnated insecticides papers in the WHO bioassay allowed our study to expose the mosquitoes to a predetermined discriminating dose of insecticide to ensure that mosquitoes had similar exposure. Thirdly, we attempted to identify household and agricultural

practices, which may be relevant in explaining the observed resistance in mosquitoes in future studies.

Our study has several limitations. Due to the transient nature of larval sites, we ended up having very few numbers of breeding sites over the ten-week period than we anticipated. Another reason for the few number of breeding sites was because we ran into a dry spell of mosquito larvae collection in the month of July. We were able to find breeding sites but there were no larvae in them. In some breeding sites, we found very few larvae, which would take us 3-6 weeks to get a sufficient sample size of 100-150 per test given the constraints of carrying out the project over the summer. Secondly, we were only able to administer household surveys after successful rearing mosquito larvae from the breeding sites. As a result of the few number of breeding sites, we did not have a sufficient number of households to be able to carry out a formal statistical analysis of differences in mortality related to household factors. Thirdly, we were unable to perform molecular analysis on mosquitoes in our insecticide susceptibility assay study leaving the possibility that the mechanism of resistance in our sample may or may not be related to the major mechanisms of kdr and metabolic resistance already observed in malaria vectors in Africa. Lastly, this was a cross-sectional study carried out in a period of 10 weeks and our results may not reflect the response of mosquitoes to pyrethroids throughout the year.

5.7 Recommendation for policy makers

5.7.1 Monitoring and evaluation of pyrethroid resistance in agricultural areas

Previous reports have suggested that pyrethroid-resistant populations of *Anopheles gambiae* are prevalent in western and central Africa but not as common in southern and eastern countries of Africa. Our study proposes that the Division of Malaria Control in Kenya, carry out longitudinal monitoring in sentinel sites located in agricultural areas to assist in detecting temporal changes in the prevalence of resistance of malaria vectors. The results from our study only provided a snap shot of the resistance problem in four villages. The results obtained from the sentinel areas will enable the Division of Malaria Control to determine whether current pyrethroid-based vector control programs are still suited for killing malaria vectors in agricultural areas.

5.8 Implications for further research

Our study showed a substantial difference in the results obtained from the cone bioassays and insecticide susceptibility bioassays. Future research is needed to evaluate whether results from the cone bioassay are of more relevance locally, in decision-making compared to the standardized WHO method of exposing malaria vectors to a predetermined discriminating dose of insecticide and recording the percentage mortality of the mosquito population. This is because cone bioassays measure the response of mosquitoes to locally implemented vector control tools such as bed nets, which are

readily available, compared to the bureaucratic process of obtaining insecticide impregnated papers from the only WHO approved Vector Control Unit in Malaysia.

We also observed substantial differences in the rate of knockdown of mosquitoes and eventual mortality after 24 hours post exposure to the insecticides in the WHO susceptibility bioassays. Further research is needed to evaluate the feasibility of using the knockdown rate of mosquitoes as an indicator to detect early resistance in resource constrained areas.

In our study, we were unable to formally evaluate mortality related to household factors due to the small number of households in our sample. We believe this is an important area of research that should be explored in future in a larger study. It would be of interest to note, which household factors play a major role in influencing resistance in this context where the use of pesticides was very low. In addition, further research may include testing of water and soils of breeding sites for pesticide residues to supplement information given by households on agricultural practices.

6. Conclusion

This study confirmed that local mosquitoes reared from agricultural areas in four villages in Western Kenya were resistant to permethrin and deltamethrin insecticides according to the criteria set by the WHO. Our results the from cone bioassay tests suggest that resistance in mosquitoes in Bungoma East sub-county might be undermining the efficacy of insecticide treated nets in the area .Our findings highlight the need for local and national policy makers to develop tools to track resistance of malaria vectors in agricultural areas which if not monitored could undermine the effectiveness of current vector control strategies. Future research should be directed towards determining which bioassay would be more relevant to local malaria stakeholders in determining the resistance of malaria vectors.

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