ABUNDANCE AND PYRETHROID RESISTANCE OF AEDES AEGYPTI MOSQUITOES COLLECTED IN SELECTED WARDS OF MUHEZA DISTRICT, TANZANIA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ONE HEALTH MOLECULAR BIOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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EXTENDED ABSTRACT

Aedes aegypti mosquitoes are primary vectors that carry mosquito-borne diseases such as dengue fever, Zika and Yellow fever. Despite mosquito control measures employed in Tanzania such as indoor residual spraying and larvae source management systems, several studies have reported the presence of insecticide resistance. The present study aimed at investigating the abundance of *Ae. aegypti* and their susceptibility to pyrethroids in Muheza district in Tanga region. A total of 7200 mosquito larvae were collected from selected wards in Muheza district using standard dipping method and reared into adults. Some of the reared larvae died and others escaped during the rearing process leaving 2572 of the collected larvae that emerged into adults. Adult mosquitoes were identified using standard taxonomic keys. Female *Ae. aegypti* mosquitoes aged three to five days old were tested for susceptibility to pyrethroids using WHO guidelines and the insecticides used were permethrin (0.75%), alphacypermethrin (0.05%) and deltamethrin (0.05%). Mosquito DNA was then extracted and voltage-gated sodium channel genes were amplified targeting Domain II and Domain III yielding expected amplicons size of 640 and 740 bp, respectively. Abundant *Ae. Aegypti* species were from Mbaramo ward representing 21% (n=267), followed by Zeneti representing 19% (n=240), Kwafungo 19% (n=236), Genge 13% (n=161), Ngomeni 12% (n=153), Misozwe 10% (n=131) and Magila 6% (n=78). Tested *Ae. aegypti* mosquitoes were susceptible to alphacypermethrin and permethrin with a percentage mortality of 100 and 98.75%, respectively, and resisted to deltamethrin with a percentage mortality of 68%. S989P and V1016I point mutations were identified. Increase in *Ae. aegypti* resistance to deltamethrin is attributed to prolonged use of insecticides as residual sprays and on pyrethroids impregnated bed nets. *Ae. aegypti* resistance to deltamethrin and high abundance of this specie in some wards pose a high risk for mosquito-borne diseases and this calls for rational vector control measures.

DECLARATION

I, Neema Ally Bendera, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

Neema Ally Bendera — Date

(Candidate: MSc. One Health Molecular Biology)

The declaration is hereby confirmed by;

Prof. Gerald Misinzo — Date

(Supervisor)

Dr. Elisa Mwega — Date

(Supervisor)

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DEDICATION

I dedicate this piece of work to God Almighty for His love and provisions. I also dedicate this work to my parents Dr. Ally Bendera and Anna Martin Hizza, my sibling Dr. Rehema Ally Bendera and my good friends Neema Ngoti and Rosemary Peter Nshama for their prayers, guidance, love, words of encouragement and support.

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ABBREVIATIONS AND ACRONYMS

µL	Microliter
bi	Breteau index
Bp	base pairs
CDC	Centres for Disease Control and Prevention
CI	confidence interval
CTAB	cetyltrimethylammonium bromide
ddNTPs	dideoxynucleotides triphosphates
DDT	Dichlorodiphenyltrichloroethane
DEET	N,N-Diethyl-meta-toluamide
Df	dengue fever
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
GPS	global positioning system
IRS	indoor residual spray
ITN	Insecticide treated nets
kdt	knockdown time
Ld	Ladder
LD_{50}	lethal dose 50
LD_{90}	lethal dose 90
LLINS	Long-lasting insecticidal nets
LSM	larval source management system
Mbv	Mosquito-borne viruses
$MgCl_2$	magnesium chloride
NIMR	National Institute for Medical Research

PCR	polymerase chain reaction
RVFV	Rift Valley fever virus
SUA	Sokoine University of Agriculture
TDS	total dissolved solids
ULV	ultra-low volume
URT	United Republic of Tanzania
UV	Ultraviolet
WHO	World Health Organization
YFV	yellow fever virus

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background Information

There are 41 recognized genera incorporating about 3500 species of mosquitoes known so far (Goodwin *et al*., 2021). Mosquitoes from two families of Anophelines (all *Anopheles* mosquitoes) and Culicines (*Aedes*, *Mansonia*, *Culex*) are potential vectors that transmit diseases to humans and domestic animals (Tandina *et al.,* 2018)**.**

Aedes mosquitoes breed in different habitats including those with fresh water, brackish water, cans, rooftop gutters, tree logs and shrubs (Ferede *et al*., 2018). Urbanization is known to be the major cause for increased mosquito breeding habitats due to poor house planning that increases potential breeding sites for mosquitoes (Weaver and Reisen, 2010). In general mosquito habitat productivity can be estimated using mosquito indices such as container index (CI), that shows the percentage of water-holding containers infested with larvae or pupae (Gutu *et al*., 2021). Breteau index (BI) that shows number of positive containers per 100 houses inspected (Abílio *et al.*, 2018).

Human diseases transmitted by *Ae. aegypti* mosquitoes include Zika, Chikungunya, Rift Valley fever, yellow fever, dengue and many other of medical and veterinary importance (Lee *et al*., 2018). In addition to human diseases they carry, mosquitoes cause allergic reactions and blood loss when they are in large number (Díaz-Quiñonez, 2020). Nearly 700 million people contract mosquito-borne diseases each year leading to about a million deaths (Huang *et al*., 2019). The disease burden is highest in tropical and subtropical areas and highly affects the poorest populations (Wu *et al*., 2017). Major outbreaks of mosquito-borne diseases such as dengue, chikungunya, Zika and yellow fever have afflicted many

populations by increasing mortality rates and burdening health systems in various countries (Huang *et al*., 2019).

Several mosquito-borne diseases outbreaks have been reported in Tanzania. These include Rift Valley fever which has been reported on average of every 8 to 10 years since the first outbreak in 1931. In 2006 and 2007 Rift Valley fever was reported in different regions of Tanzania namely Arusha, Dar es Salaam, Dodoma, Iringa, Manyara, Mwanza, Morogoro, Pwani, Singida and Tanga (Sindato *et al*., 2011). Recurring dengue outbreaks have been reported in the year 2010, 2012, 2013, 2014 and the recent one has been reported in the year 2018 and 2019 in different regions such as Morogoro, Tanga and Dar es Salaam (Chipwaza *et al.,* 2021). Chikungunya was first reported in Tanzania in 1952 and recently in 2019 it has been reported in different regions and districts such as Hai, Moshi, Tanga, Kilosa, Kisarawe, Kilombero, Kyela and Sengerema (Mwanyika *et al*., 2021).

In addition to the developed vaccines for a number of the mosquito-borne diseases such as malaria, yellow fever and dengue, measures such as indoor residual spray, use of repellents, larval source management, improvement of infrastructure and house modifications are still used in mosquito vector control (Huang *et al*., 2021; O'Leary, 2021). Some common vector control strategies observed in Tanzania include use of Long-lasting insecticide-treated Nets (LLINs) such as Permanet®3.0 and Olyset® plus and indoor residual sprays (IRS). Insecticides used in indoor residual sprays include insecticides such as organophosphate, these have been known to be very effective by increasing mortality, knockdown and exophily of female mosquitoes (Yukich *et al.,* 2020).

The National Institute for Medical Research (NIMR), Amani Research Center in collaboration with the National malaria control programs and different higher learning

research institutions have been conducting annual insecticide resistance surveillance since 1999 aiming at early detection and containment of resistance to insecticides. However, the intensive use of insecticides in households and different agricultural settings have led to an increase in mosquito insecticide resistance (Lorenz *et al*., 2014).

There are four different ways in which mosquitoes become resistant to insecticides, these include resisting to insecticides through hardening of their cuticle preventing the penetration of insecticides into their bodies, behavioral changes where they tend to feed indoors and rest outdoors, mutation of the insecticide target site proteins, and by secreting enzymes that detoxifies insecticides before reaching the target site (Vontas and Mavridis, 2019). Several mutations such as I1011M/V, V1016/I, S989P in domain II and F1534C occurring in domain III of the voltage gated-sodium channel play a key role in pyrethroid resistance of *Ae. aegypti* mosquitoes (Aponte *et al.,* 2019).

This study aimed at determining the abundance and pyrethroid resistance of *Ae. aegypti* mosquitoes collected in selected wards of Muheza District, Tanzania. The information gained from this research will show the effectiveness of pyrethroids in *Aedes* mosquitoes interventions and will also contribute to planning future vector control efforts and strategies.

1.2 Problem Statement and Study Justification

Tanzania is facing an increased prevalence of mosquito-borne diseases. Mosquitoes play a crucial role in transmitting diseases from human to human and from animals to human and vice versa making them critical players for vector-borne diseases transmission across the country (Failloux, 2018). This increase in mosquito-borne diseases is associated with a number of factors that favour the survival of vectors including increased mosquito

resistance to insecticides, increased mosquito productivity, increased global travel and trade, unplanned urbanization leading to an increase in mosquito breeding sites, climatic change and mosquitoes behavioural changes (Marques-Toledo *et al.,* 2017 ; Trumbetta *et al*., 2020).

Essential strategies commonly used in Tanzania to control mosquitoes include indoor residual sprays, larval source management, use of long-lasting insecticidal nets, use of slow burning coils and house modifications (Schmidt *et al*., 2021). Despite control efforts taken by the country against these vectors, there has been an increase in mosquito-borne diseases leading to an increase in morbidity and mortality rates, socio-economic burdens, cost of interventions, and vulnerability to possible outbreaks in many parts of the country (Chipwaza *et al*., 2021).

This study aimed at determining the abundance and pyrethroid resistance of *Ae. aegypti* mosquitoes collected in selected wards of Muheza District in Tanzania. Knowledge gained from the study will help set an early warning for mosquito-borne disease outbreaks, understand the exposure risk in selected areas of Muheza district in Tanga region also in knowing the current status of pyrethroids interventions and help inform on how it should be improved.

1.3 Research Objectives

1.3.1 Overall objective

The main objective of this study was to determine the abundance and susceptibility status of *Aedes aegypti* mosquitoes to pyrethroids in Muheza district, Tanga region.

1.3.2 Specific objectives

The specific objectives of this study were:

i. To determine the abundance of *Ae. aegypti* and its association with the physicochemical characteristics of the breeding sites,

ii. To assess the susceptibility status of *Ae. aegypti* to pyrethroids,

iii. To detect knockdown resistance genes in *Ae. aegypti*.

CHAPTER TWO

MANUSCRIPT ONE

Abundance of *Aedes aegypti* mosquitoes and the physicochemical characteristics of the breeding sites in the selected wards of Muheza district in Tanga, Tanzania.

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Abstract

Mosquito-borne diseases remain to be the major problem causing increased morbidity and mortality rates in tropical and subtropical regions. *Ae aegypti* mosquitoes are the most important vectors associated with the outbreaks breeding on water storage containers, tins, car tires and rice fields. The present study aimed at investigating the abundance of *Ae. aegypti* mosquitoes and the physicochemical characteristics of the breeding sites in the selected wards of Muheza district. A total of 7 200 mosquito larvae were collected from selected wards in Muheza district and reared into adults. A total of 2 572 collected larvae successfully emerged into female adult mosquitoes. Three genera namely *Aedes* 46%, *Culex* 30%, and *Anopheles* 18% were identified. *Aedes aegypti* was the abundant mosquito specie accounting for 49.2% of the total female adult mosquitoes. *Culex quinquefasciatus* accounted for 32%, *Anopheles gambiae* accounted for 17.3% and *Anopheles funestus* accounted for 1.5%. Majority of the *Ae. aegypti* were collected from Mbaramo ward. The

Poisson regression model was used to determine the rate of association between the expected number of *Ae. aegypti* and Physicochemical characteristics of the breeding sites. TDS refers to total dissolved solid which was measured by using a handheld Hanna TDS meter. Expected number of *Ae. aegypti* decreased for every unit increase in algae cover, temperature, pH, and vegetation (with correlation coefficient of -2.073, -3.424, -12.502 and -1.85 respectively). While for temporary habitats and total dissolved solids the expected number of *Ae. aegypti* increased (with correlation coefficient of = 9.537 and 30.003 respectively).

Key words: Abundance, *Aedes aegypti*, physicochemical characteristics, breeding sites, Muheza, Tanga.

2.1 Introduction

Emergence and re-emergence of mosquito-borne diseases in tropical and subtropical regions in recent times is alarming (Cheang *et al*., 2021). *Aedes aegypti* and *Aedes albopictus* are known to be major vectors for mosquito borne diseases such as zika, yellow fever, Rift valley fever and dengue (Kraemer *et al*., 2015). The occurrence of mosquito-borne disease outbreaks is highly dependent on the vector abundance, availability of the breeding habitats and circulating virus (Fang *et al*., 2021).

Primary *Aedes* mosquito developmental stages are entirely aquatic and require water for their survival. Breeding sites such as pots, concrete wells, tires, plastic containers, rice fields are known to harbour the development of *Aedes* mosquitoes (Dalpadado *et al*., 2022a). Also habitat physicochemical characteristics such as PH, salinity, total dissolved solids (TDS) and factors such as climate, environment, ecology and socioeconomic are

known to affect the productivity, distribution and abundance of *Aedes* mosquitoes hence affecting mosquito-borne diseases transmission (Amini *et al*., 2020).

The ability of physicochemical characteristics of mosquito breeding habitats to directly affect the immature stages in mosquito development such as life span, body size, biting behaviour have an important contribution on the intensity of diseases transmission and mosquito control (Khater *et al.,* 2013). Breeding habitat PH values between 4 and 10 and high temperature is known to reduce immature developmental period of *Aedes* mosquitoes increasing mosquito production (Medeiros-Sousa *et al*., 2020).

The knowledge on breeding habitats physicochemical characteristics can be used as a guide in developing mosquito control tools. Not much data is available on the breeding sites physichochemical characteristics of *Aedes* mosquitoes in Muheza district in Tanga region, Tanzania. Hence this study is aimed at assessing the abundance of *Aedes aegypti* mosquitoes and the physicochemical characteristics of the breeding sites in the selected wards of Muheza district.

2.2 Materials and Methods

2.2.1 Description of the study area

Data collection for this study was conducted at Muheza district in Tanga region. Muheza district lies along the North-eastern coast of Tanzania. Muheza district occupies a total of 4 922 square kilometres with an elevation of 1050m above the sea level. According to the census conducted in 2012, the district had a total of 279 423 people. Muheza district is comprised of 33wards and 175 villages (URT, 2013).

The sites were selected based on some ecological and demographic characteristics such as the presence of mosquito habitats, topography, vegetation, areas of high human habitation and the presence of artificial containers. Sampling was conducted from different wards which were Mbaramo, Misozwe, Genge, Ngomeni, Magila, Kwafungo and Zeneti.

The selected wards are also characterized by high population, unplanned settlements, presence of garages, poor waste disposal, high vegetation cover, mining activities and agricultural activities. All these are factors that favour the breeding of *Ae. aegypti* mosquitoes hence they were selected.

2.2.2 Study design

A cross-sectional study design was adopted in this study.

2.2.3 Sampling procedures and data collection

Sampling was successfully done at Muheza district in the Tanga region. Mosquito larvae and pupae collection was done between April and July 2021. Mosquito sampling forms were used to record the ecological data and each sampling site was georeferenced using a handheld Global Positioning System (GPS) device (Appendix 3).

2.3 Mosquito Larvae Collection and Rearing

A standard of ten dips were done to collect mosquito larvae from the breeding sites using a 350mls handheld dipper. At every site, stagnant water, water holding containers, discarded tyres, tins, buckets, ponds and streams were searched for larvae. Not all mosquito species were sampled from the sampling areas due to time and other limitations but equal efforts were applied in sampling all breeding sites selected. The collected larvae were then kept in plastic paper cups and transported to the insectary where they were reared at a high

temperature of about 32ºC and were fed cat food (Whiskas®). Emerged pupae were collected and transferred into containers kept in cages for adult mosquitoes to emerge at a temperature of 26±1ºC and relative humidity of 45%.

Emerging adult mosquitoes were fed 30% sucrose prepared in the laboratory and separated based on sex using standard method (Kittayapong *et al.*, 2018). The number of female emerging were recorded together with the habitat type, GPS coordinates, water pH, and water temperature, site sampled. The collected mosquitoes were counted, separated according to species and mosquito specie abundance was recorded. Male mosquitoes were excluded because they are not disease vectors.

2.3 Data Analysis

The data were entered, coded, validated and stored into the spread sheet of Microsoft Excel Window 2007 and analysed using Statistical Package for Social Sciences (SPSS) version 16.0. The Poisson regression model was used to determine the rate of association between the expected number of *Ae. aegypti* and Physicochemical characteristics of the breeding sites. The model is taken due to its ability in making valid predictions when the outcome variable is discrete and counted. The Poisson regression model is a non-parametric technique which does not consider the distribution of the outcome variable, over dispersion and homoscedasticity. The Pearson chi-square test had shown a high level of statistical significance ($p<0.0001$) on the association between mosquito species abundance and the sampled wards.

2.4 Ethical Consideration

This study clearance and ethical protocols were approved by the Medical Research Coordinating Committee of the National Institute for Medical Research in Tanzania (Ref No. NIMR/HQ/R.8a/Vol.IX/3278).

2.5 Results

Abundance of *Aedes aegypti* and its association with the physicochemical characteristics of the breeding sites

A total of 7 800 mosquito larvae were collected in Muheza district from different breeding sites located in seven wards namely, Magila, Mbaramo, Misozwe, Zeneti, Kwafungo, Genge and Ngomeni. The mosquito larvae were then reared into adults out of which 2572 of the collected larvae emerged into three mosquito genera (*Aedes*, *Culex* and *Anopheles*) identified through standard taxonomic key. More mosquitoes were collected from Mbaramo representing 17.9% (n=462), followed by Zeneti 17.3% (n=444), Kwafungo 16.8% (n=432), Genge 14.1% (n=362), Misozwe 12.5% (n=323), Magila 10.7% (n=275) and Ngomeni 10.7% (n=274) (Table 2.1).

As shown in Table 1 and (Figure 1) below, the *Aedes* genus (46%; n=1266) was the most abundant, followed by *Culex* (30%; n=824) and *Anopheles* (18%; n=482) which was the least abundant.

Table 2.1: Mosquitoes distribution (%) by genus between sampling locations (wards)

Ward	**Geographical coordinates**	***Aedes*** **N (%)**	***Culex*** **N (%)**	***Anopheles*** **N (%)**	**Total N (%)**
Genge	S-5.174421/E38.792200	161 (13)	150 (18.2)	51 (10.5)	362 (14.1)
Mbaramo	S-5.170549/E38.778731	267 (21)	130 (15.8)	65 (13.5)	462 (17.9)
Misozwe	S-5.06458/ E38.77127	131 (10)	120 (14.5)	72 (15)	323 (12.5)
Magila	S-5.162510/E38.760294	78 (6)	89 (10.8)	108 (22.4)	275 (10.7)
Ngomeni	S-5.120646/E38.857403	153 (12)	52 (6.2)	69 (14.3)	274 (10.7)
Kwafungo	S-5.24893/ E38.66999	236 (19)	120 (14.6)	76 (15.8)	432 (16.8)
Zeneti	S-5.225010/ E38.660581	240 (19)	163 (19.8)	41 (8.5)	444 (17.3)
Total		**1 266 (49)**	**824 (32)**	**482 (19)**	**2 572**

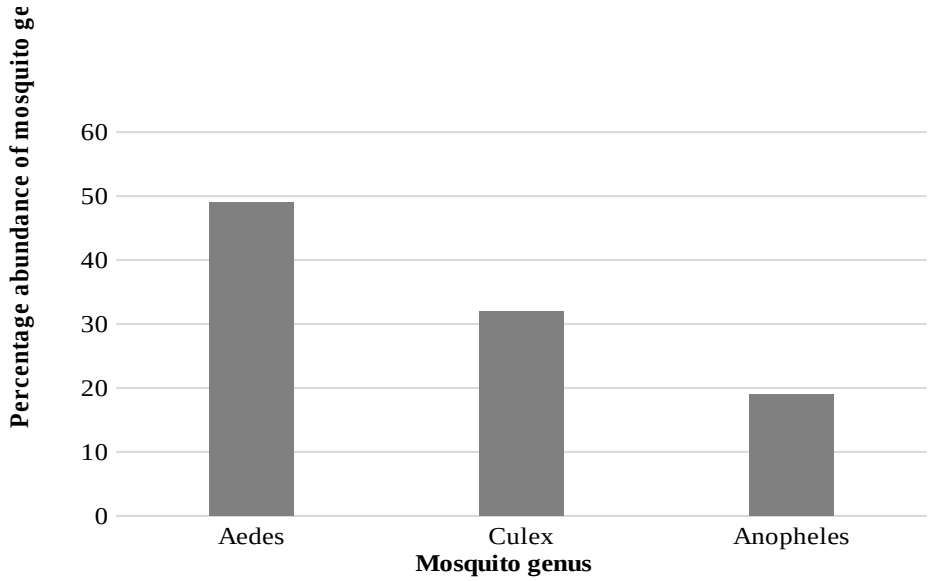

Figure 2.1: A graph showing proportion of mosquito genus sampled from the wards in Muheza district. X- axis shows mosquito genus sampled from Muheza district and y-axis shows the percentage abundance of mosquito genus sampled from Muheza district.

From the seven wards, *Ae. aegypti* were more abundant in Mbaramo representing 21% (n = 267), followed by Kwafungo (19% n = 236), Zeneti (19% n = 240), Genge (13% n=161), Ngomeni (12% n=153), Misozwe (10% n = 131) and Magila (6% n= 78) as shown on Table 2 and Figure 2.

Table 2.2: Abundance of mosquito species from selected wards in Muheza district

		Wards								
		Genge	**Mbaramo**	**Misozwe**	**Magila**	**Ngomeni**	**Kwafungo**	**Zeneti**	**Total**	
		n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**p-value**
Mosquito species	*Ae. Aegypti*	161(13.0)	267(21.0)	131(10.0)	78(6.0)	153(12)	236(19)	240(19.0)	1 266(100)	
	Cx. quinquefasciatus	150(18.2)	130(15.7)	120(14.6)	89(10.8)	52(6.3)	120(14.6)	163(19.8)	824(100)	
	An. Gambiae	41(9.2)	58(13.0)	67(15.0)	105(23.6)	69(15.5)	70(16)	34(7.7)	444(100)	
	An. Funestus	10(26.3)	7(18.4)	5(13.2)	3(7.9)	0(0.0)	6(15.8)	7(18.4)	38(100)	
Total		**362**	**462**	**323**	**275**	**274**	**432**	**444**	**2 572**	**0.0001**

Source: Computed by researcher using SPSS (2021).

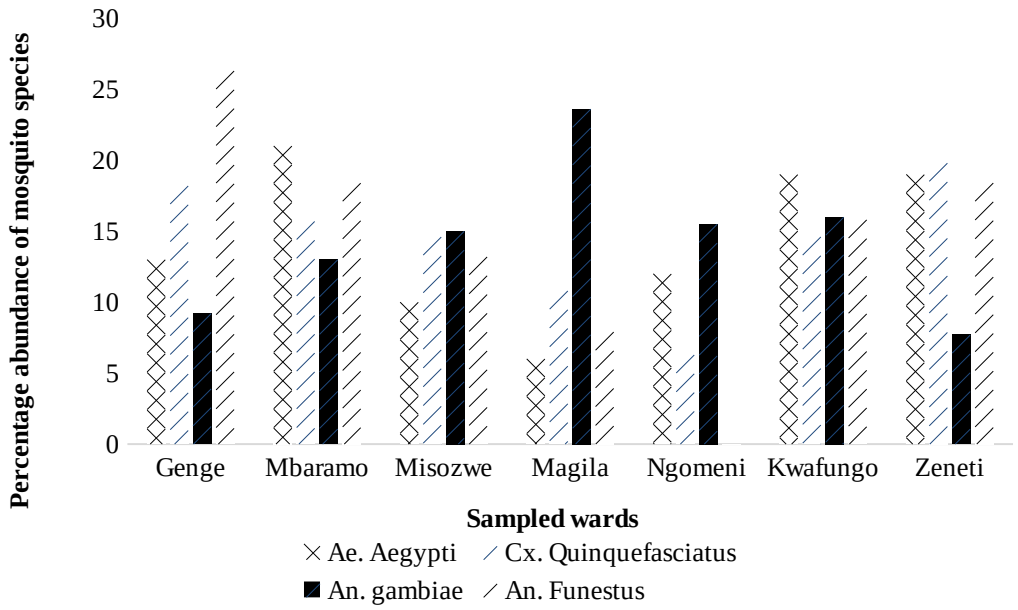

Figure 2.2: A graph showing mosquito species composition in each sampled ward x-axis shows sampled wards and y-axis shows the percentage abundance of mosquito species sampled from each ward.

The Poisson regression model was used to determine the rate of association between the expected number of *Ae. aegypti* and Physicochemical characteristics of the breeding sites. As shown, the model fit best (prob>chi2 = 0.0001), there is about 77.2% of variation explained by the set of independent variables which suggests that the model has a strong determination.

Table 2.3: Association between the expected number of *Aedes aegypti* and physicochemical characteristics of the breeding sites.

Ae. Aegypti	Coef.	St. Err.	t-value	p-value	[95% Conf	Interval]	Sig
Tds	30.003	5.928	5.06	0.0001	41.621	18.384	***
Algae cover	-2.073	.469	-4.42	0.0001	-2.993	-1.153	***
Temperature	-3.424	.641	-5.34	0.0001	-4.679	-2.168	***
pH	-12.502	2.318	-5.39	0.0001	-17.046	-7.958	***
Vegetation	-1.85	.538	-3.44	.001	-2.904	-.796	***
Temporary habitats	9.537	1.886	5.06	0.0001	5.84	13.234	***
Constant	199.421	36.402	5.48	0.0001	128.074	270.768	***

Mean dependent var	180.857	SD dependent var	68.550
Pseudo r-squared	0.772	Number of obs	7
Chi-square	165.105	Prob > chi2	0.0001
Akaike crit. (AIC)	62.748	Bayesian crit. (BIC)	62.370

**** p<.01, ** p<.05, * p<.1*

Coef = coefficient; St. Err = standard error; Conf = confidence; Sig = significance; SD = standard deviation; Var = variance; Obs = observations; Prob = probability.

2.6 Discussion

Emergence and re-emergence of mosquito-borne diseases in tropical and subtropical regions in recent times is alarming (Schwab *et al*., 2018). A number of factors such as transportation of people and animals, increase in population, poor waste disposal, increase in abundance of mosquito-borne diseases vectors and mosquito resistance to insecticides favor worldwide spread of mosquito-borne diseases (El-Sayed and Kamel, 2020; Chang *et al*., 2014).

In this study we assessed the abundance of *Ae. aegypti* mosquitoes in Muheza district which is among dengue outbreak hubs. Mosquito breeding habitats included in the study were those potential for harboring *Ae. aegypti* mosquito larvae. The habitats that were found to have *Ae. aegypti* larvae were old tires, discarded tins, concrete wells, rice fields,

caves and water storage containers. Discarded tires were mostly found in car garages while concrete wells and water storage containers were found around houses and this is because most houses stored water in containers due to water scarcity in some wards of Muheza district.

Our findings were consistent with previous studies that also reported discarded tires and water-holding containers to have high abundance of *Ae. aegypti* larvae (Ferede *et al.*, 2018). Similarly, a study conducted in Viet Nam had shown positive relationship between domestic water storage containers and high abundance of immature *Ae. aegypti* (Tsunoda *et al.,* 2013). The findings obtained on this study suggests that domestic water holding containers are the leading habitats that infest immature *Ae. aegypti.*

TDS coefficient was positive and strongly significant at 5% level. TDS refers to total dissolved solid which was measured by using a handheld Hanna TDS meter. The coefficient means that the expected number of *Ae. aegypti* increased at the rate of 30.003 for every unit increase in total dissolved solid. This observation is valid since salinity and conductivity are considered to be predictor variables for mosquito abundance where the increase in the values of salinity and conductivity results into an increase in mosquito species abundance (Alahmed *et al.,* 2009).

The finding is consistent with other studies that indicated total dissolved salts (TDS) to be a significant contributor in determining mosquito larvae abundance (Sallam *et al.,* 2013). In a similar study conducted in Columbia, infestation of immature *Ae. aegypti* was positively associated with an increase in TDS (Sanchez-Vargas *et al.,* 2021). This study finding suggest that regulation of breeding habitats TDS can be used as a mosquito control measure but this parameter is not reliable since it tends to vary among habitats.

Algae cover had a negative coefficient and strong significance of 5%. The coefficient indicates that the number of mosquitoes on the breeding sites decreased at a rate of 2.073 with an increase in algae cover. This observation is valid despite that algae are nutritious foods for mosquitoes and most of the mosquito breeding sites are infested by algae cover but some algae species such as *Kirchneriella* and *Scenedesmus* are toxic to the larvae when ingested in large quantities (Ranasinghe and Amarasinghe, 2020). *Cyanobacteria* (blue-green algae) are toxic and tend to kill mosquito larvae when ingested hence leading to a decrease in the number of *Ae. aegypti* mosquitoes (Garcia-Sánchez *et al.,* 2017).

These findings are consistent with the study done in Colombia that assessed ecological characteristics of *Aedes* larvae habitats and had reported the negative effect of algae cover on mosquito abundance (Fei *et al.,* 2020). This study has shown that introduction of indigestible algae into the breeding sites can be used as an important mosquito control measure though more research is needed in order to assess the techniques employed so as ensure complete replacement of all nutritious algae on the habitats.

The temperature had a negative coefficient and a strong significance of 5%. The temperature was measured on the breeding sites using a thermometer. The coefficient means that the expected number of *Ae. aegypti* decreased at a rate of 3.424 for every unit increase in temperature of water in the breeding sites. This observation is valid because extreme temperature above the thermal upper limit of 40ºC affects the embryonic development of mosquito eggs and the survival of mosquito larval stages leading to mortality of mosquito larvae and hence reduction of the expected number of *Aedes* mosquitoes (Schrama *et al*., 2018). These findings are consistent with the study conducted to determine the impact of temperature on the bionomics of *Ae. aegypti* where extreme

temperature reduced the number of *Ae. aegypti* on the breeding sites (Eisen *et al*., 2014). Despite that these study findings are inconsistent with other studies which showed that increased temperature had positive impact in mosquito development (Tran *et al.,* 2020).

PH had a negative coefficient and a strong significance of 5%. PH was measured using a benchtop pH meter by collecting water from the breeding sites. The coefficient means that the expected number of *Ae. aegypti* decreased at the rate of 12.502 for every unit increase in pH. This observation is valid because *Ae. aegypti* mosquitoes tend to breed in water with moderate pH, any increase or decrease in pH alters the expected number of *Ae. aegypti* (Pelizza *et al.,* 2007). This finding is consistent with the study done in Brazil that assessed the influence of water's physical and chemical parameters on mosquito assemblage in larval habitats (Medeiros-Sousa *et al.,* 2020). Although some mosquito species such as *Aedes fluviatilis* have adopted extreme pH levels enabling them to survive (Medeiros-Sousa *et al*., 2020).

Vegetation cover had a negative coefficient and strong significance of 5%. The coefficient means that the expected number of *Ae. aegypti* decreased at a rate of 1.82 for every unit increase in vegetation cover. *Ae. aegypti* mosquitoes prefer to breed in urban areas with low vegetation unlike *Ae. albopictus* that prefer to breed on shaded water bodies surrounded by vegetation (Tetreau *et al*., 2013). These results are inconsistent with various studies undertaken that found vegetation cover to have a significant positive association with the mean abundance of *Ae. aegypti* mosquitoes (Sun *et al.*, 2021).

Temporary habitats had positive coefficient and strong significance of 5%. The coefficient means that the expected number of *Ae. aegypti* increased at a rate of 9.537 for every unit increase in temporary habitat. *Ae. aegypti* mosquitoes mostly prefer to breed on temporary

habitats such as plastic tanks, water storage jars, flower vases, old rubber tyres, plastic bottles and concrete streams (Dalpadado *et al.,* 2022b). These results are consistent with various studies that have reported that most containers are positively infested by *Ae. aegypti* mosquitoes (Abílio *et al*., 2018).

2.7 Conclusion

The findings of this study have shown high abundance of *Ae. aegypti* mosquitoes on the sampled wards of Muheza district. *Ae. aegypti* being important vectors for various mosquito-borne diseases this poses a high risk for increased diseases outbreaks in Muheza district. Thus study also identified the potential breeding habitats bionomics reported to have impacts on the mosquito population hence control measures of the respective physicochemical characteristics impacting mosquito population should be employed in primary vector control strategies. These study findings also provide a baseline for modification of the ongoing mosquito control measures employed in Tanzania.

2.8 Recommendations

i. Shortage of water has led to household water storage in plastic containers which pose as potential breeding habitats for *Ae. aegypti* moquitoes. Thus, the government should improve the infrastructure in the corresponding wards so as to reduce the necessity of household water storage. Also storage containers should be well covered and treated in order to prevent mosquitoes from breeding.

ii. Proper waste disposal and regular cleaning of the environment will reduce potential mosquito breeding habitats such as discarded tires, cans, coconut shells and shrubs that pose as potential mosquito breeding habitats.

iii. Indigestible algae should be introduced on mosquito breeding habitats as they are known to be non-toxic mosquito control measures.

iv. More public education should be provided on the potential diseases spread by mosquitoes so as to create awareness and individual efforts in mosquito control. This will reduce the country's burden in the overall vector control strategies.

2.9 References

Abílio, A. P., Abudasse, G., Kampango, A., Candrinho, B., Sitoi, S., Luciano, J., Tembisse, D., Sibindy, S., de Almeida, A. P. G., Garcia, G. A., David, M. R., Maciel-de-Freitas, R. and Gudo, E. S. (2018). Distribution and breeding sites of *Aedes aegypti* and *Aedes albopictus* in 32 urban/peri-urban districts of Mozambique: implication for assessing the risk of arbovirus outbreaks. *PLoS Neglected Tropicao Disease* 12(9): 1 – 30.

Alahmed, A. M., Al Kuriji, M. A., Kheir, S. M., Alahmedi, S. A., Al Hatabbi, M. J., Al Gashmari, M. A. M. (2009). Mosquito fauna (Diptera: Culicidae) and seasonal activity in Makka Al Mukarramah Region, Saudi Arabia. *Journal of Egypt Social Parasitology* 39: 991–1013.

Amini, M., Hanafi-Bojd, A. A., Aghapour, A. A. and Chavshin, A. R. (2020). Larval habitats and species diversity of mosquitoes (Diptera: Culicidae) in West Azerbaijan Province, Northwestern Iran. *BMC Ecol*ogy 20(1): 60-70.

Chang, A. Y., Fuller, D. O., Carrasquillo, O. and Beier, J. C. (2014). Social justice, climate change, and dengue. *Health Human Rights* 16: 93–104.

Cheang, Y. Z. N., Ting, H. R. D., Koh, H. Q. V. and Alonso, S. (2021). In vitro and in vivo efficacy of Metformin against dengue. *Antiviral Research* 195: 1-13.

Dalpadado, R., Amarasinghe, D. and Gunathilaka, N. (2022a). Water quality characteristics of breeding habitats in relation to the density of Aedes aegypti and Aedes albopictus in domestic settings in Gampaha district of Sri Lanka. *Acta Trop* 229: 106-115.

Dalpadado, R., Amarasinghe, D., Gunathilaka, N. and Ariyarathna, N. (2022b). Bionomic aspects of dengue vectors Aedes aegypti and Aedes albopictus at domestic settings in urban, suburban and rural areas in Gampaha District, Western Province of Sri Lanka. *Parasite Vectors* 15: 148-161.

Fang, Y., Li, X. S., Zhang, W., Xue, J. B., Wang, J. Z., Yin, S. Q., Li, S. G., Li, X. H., Zhang, Y. (2021). Molecular epidemiology of mosquito-borne viruses at the China-Myanmar border: discovery of a potential epidemic focus of Japanese encephalitis. *Infections Discovery Poverty* 10: 57-67.

Garcia-Sánchez, D. C., Pinilla, G. A. and Quintero, J. (2017). Ecological characterization of Aedes aegypti larval habitats (Diptera: Culicidae) in artificial water containers in Girardot, Colombia. *Journal of Vector Ecological* 42: 289–297.

Khater, E. I., Sowilem, M. M., Sallam, M. F. and Alahmed, A. M. (2013). Ecology and habitat characterization of mosquitoes in Saudi Arabia. *Tropical Biomedical* 30: 409–427.

Kraemer, M. U. G., Sinka, M. E., Duda, K. A., Mylne, A. Q. N., Shearer, F. M., Barker, C. M., Moore, C. G., Carvalho, R. G., Coelho, G. E., Van Bortel, W., Hendrickx, G., Schaffner, F., Elyazar, I. R. F., Teng, H. J., Brady, O. J., Messina, J. P., Pigott, D. M., Scott, T. W., Smith, D. L., Wint, G. R. W., Golding, N. and Hay, S. I. (2015). The global distribution of the arbovirus vectors Aedes aegypti and Ae. albopictus. *Elife* 4: e08347.

Medeiros-Sousa, A.R., de Oliveira-Christe, R., Camargo, A.A., Scinachi, C.A., Milani, G.M., Urbinatti, P.R., Natal, D., Ceretti-Junior, W., Marrelli, M.T., 2020. Influence of water's physical and chemical parameters on mosquito (Diptera: Culicidae) assemblages in larval habitats in urban parks of São Paulo, Brazil. *Acta Trop* 205: 1-27.

Nnko, E. J., Kihamia, C., Tenu, F., Premji, Z. and Kweka, E. J. (2017). Insecticide use pattern and phenotypic susceptibility of Anopheles gambiae sensu lato to commonly used insecticides in Lower Moshi, northern Tanzania. *BMC Research Notes* 10(1): 1-12.

Pelizza, S. A., López Lastra, C. C., Becnel, J. J., Bisaro, V. and García, J. J. (2007). Effects of temperature, pH and salinity on the infection of Leptolegnia chapmanii Seymour (*Peronosporomycetes*) in mosquito larvae. *Journal of Invertebrate Pathology* 96: 133–137.

Ranasinghe, H. A. K. and Amarasinghe, L. D. (2020). Naturally Occurring Microbiota Associated with Mosquito Breeding Habitats and Their Effects on Mosquito Larvae. *Biomed Research International* 2020: 1-11.

Sanchez-Vargas, I., Williams, A. E., Franz, A. W. E. and Olson, K. E. (2021). Intrathoracic Inoculation of Zika Virus in Aedes aegypti. *Biology Protoc* 11: e4165.

Schrama, M., Gorsich, E. E., Hunting, E. R., Barmentlo, S. H., Beechler, B., van Bodegom, P. M. (2018). Eutrophication and predator presence overrule the effects of temperature on mosquito survival and development. *PLoS Negl Trop Dis* 12: e0006354.

Schwab, S. R., Stone, C. M., Fonseca, D. M. and Fefferman, N. H. (2018). The importance of being urgent: The impact of surveillance target and scale on mosquito-borne disease control. *Epidemics* 23: 55–63.

Tran, B. L., Tseng, W. C., Chen, C. C. and Liao, S. Y. (2020). Estimating the Threshold Effects of Climate on Dengue: A Case Study of Taiwan. *International Journal of Environmental Research and Public Health* 17(4): 1-21.

Tsunoda, T., Kawada, H., Huynh, T. T. T., Luu, L. L., Le, S. H., Tran, H. N., Vu, H. T. Q., Le, H. M., Hasebe, F., Tsuzuki, A. and Takagi, M. (2013). Field trial on a novel control method for the dengue vector, Aedes aegypti by the systematic use of Olyset® Net and pyriproxyfen in Southern Vietnam. *Parasitology Vectors* 6(1): 6-17.

CHAPTER THREE

MANUSCRIPT TWO

Pyrethroid resistance of *Aedes aegypti* mosquitoes collected from Muheza district in Tanga region, Tanzania.

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Abstract

There has been an increase in mosquito-borne diseases, since most of the diseases have no vaccines. Developing countries like Tanzania have employed different mosquito control measures such as indoor residual sprays (IRS), long lasting insecticidal nets (LLIN) and larvae source management systems (LSMS). Recently, there have been reports of mosquito resistance to insecticides making the control measures ineffective. This study aimed at determining the abundance and the susceptibility status of *Aedes aegypti* mosquitoes to pyrethroids in Muheza district, Tanga region. 300 reared female *Ae. aegypti* mosquitoes aged three to five days old were tested for susceptibility to pyrethroids using WHO guidelines and the insecticides used were permethrin (0.75%), alphacypermethrin (0.05%) and deltamethrin (0.05%). Mosquito DNA was then extracted and voltage-gated sodium channel genes were amplified targeting Domain II and Domain III yielding expected amplicons size of 640 and 740 bp, respectively. Tested *Ae. aegypti* mosquitoes were susceptible to alphacypermethrin and permethrin with a percentage mortality of 100

and 98.75%, respectively, and resisted to deltamethrin with a percentage mortality of 68%. S989P and V1016I point mutations were identified. Increase in *Ae. aegypti* resistance to deltamethrin is attributed to prolonged use of insecticides as residual sprays and on pyrethroids impregnated bed nets. *Ae. aegypti* resistance to deltamethrin and high abundance of this specie in some wards pose a high risk for mosquito-borne diseases outbreaks and this calls for rational vector control measures.

3.1 Introduction

In Tanzania the classes of insecticides currently in use for Indoor Residual Spray (IRS) are pyrethroids, carbamates and organophosphates which have proven to be effective (Nnko *et al.,* 2017). Pyrethroids are highly used for they are known to increase mosquitoes exophily and to reduce knockdown time (Choi *et al*., 2019). Different mosquito control strategies have been used such as Long-Lasting Insecticidal Nets (LLIN) which is shown to be more effective when combined with other mosquito control strategies such as IRS and larva source reduction (Finda *et al.,* 2020). Urban areas have employed the method of larvae source management system (LSM) and it has been found to be effective when larvae sources are few and manageable (Ramirez *et al*., 2009).

Other control methods used in Tanzania include the use of repellents such as DEET and other plant-based repellents like lemon grain oil and citronella oil (Warikoo *et al*., 2011). Another intervention used for mosquito control in Tanzania is household modification in different regions and this has been successful due to public health education given to the community (Phiri *et al.,* 2021). House improvements done to control mosquitoes are screening doors and windows, sealing caves which have reduced house entry of mosquitoes (Furnival-Adams *et al*., 2021).

Pyrethroids are a group of manufactured pesticides similar to pyrethrum produced by *chrysanthemum* flowers. In Tanzania, *pyrethrum* is cultivated at the highlands of Mbeya and Iringa regions of southern Tanzania (Tungu *et al.,* 2021). Pyrethroids are added to commercial products used to control insects such as household insecticides and pest sprays (Matiya *et al*., 2019). In Tanzania, vector control programs are largely dependent on synthetic pyrethroids such as deltamethrin, permethrin, lambdacyhalothrin, cyfluthrin and entofenprox which are recommended by the WHO for insecticide-treated nets (ITNs) such as Permanet® 3.0 and olyset® plus (Lines and Addington, 2001) (Kabula *et al.*, 2012).

Pyrethroids are used for indoor residual sprays (IRS) because of their relatively low mammalian toxicity and rapid knockdown effect that causes paralysis of an insect (Edi *et al*., 2014). Pyrethroids are applied by trained personnel in mosquito control or public health officials. Mosquito control professionals apply pyrethroids as an ultra-low volume (ULV) spray (Zhu *et al.*, 2020). Pyrethroids are mostly mixed with water or oil and sprayed, after spraying they settle onto the ground and flat surfaces, then they are broken down by sunlight or other chemicals in the atmosphere. Sprayed pyrethroids often last for one to two days in the environment (Li *et al.,* 2016).

Mosquitoes have adapted different mechanisms that enable them to resist to insecticides. Among the resistance mechanisms are behavioural changes, some mosquitoes tend to feed and rest indoors but in the escape of the insecticides they tend to feed indoors and rest outdoors (Carrasco *et al*., 2019). Other resistance mechanism is cuticle resistance, mosquitoes tend to harden their outer cuticle and prevent the insecticides from penetrating and reaching their target site (Bass and Jones, 2016). Metabolic-mediated resistance occurs when mosquitoes release enzymes that metabolize the insecticide before reaching

their target site. Cytochrome P450 monooxygenases, glutathione S- transferases (GSTs) and esterase are three major enzyme families involved in breaking down insecticides (Balmert *et al*., 2014).

Pyrethroids and DDT targets the voltage-gated sodium channels in mosquito bodies. Alteration of the proteins targeted by pyrethroids is through point mutation or substitution making sodium gates open, continuously discharged and depolarized leading to mosquito death (Fung *et al*., 2021). Mutation at position 1 016 in domain II segment 6 of voltage-gated sodium channel gene in *Ae. aegypti* leads into valine to glycine substitution that causes resistance to deltamethrin. Mutation at position 1534in domain III leads into phenylalanine to cysteine substitution that confers resistance to permethrin (Ranson *et al*., 2000) (Ranathunge *et al*., 2021).

In Tanzania, there are two methods commonly used to test for mosquito susceptibility to insecticides which are WHO method and CDC bottle bioassay. WHO guidelines require mosquitoes aged 2 to 5 days old collected from different breeding sites. Batches of 20 non-blood fed female mosquitoes are then aspirated into WHO holding tubes lined with untreated papers for about one hour (Vatandoost *et al*., 2019).

They are then exposed to the insecticides and the outcomes are recorded for each insecticide used for 60 minutes as knock down time (kdt) and then transferred into the recovery tubes (Rajatileka *et al*., 2011). Thereafter the mosquitoes in the recovery tubes are provided with 30% sucrose solution soaked on small cotton pieces after being exposed to insecticides before scoring mortality after 24 hours. Recovery is monitored at 26% ± 1% and 80% ± 10% humidity. To assess susceptibility status, if mortality is between 98-100% it indicates that the mosquitoes are susceptible to the insecticide. Mortality less than

98% suggests the existence of resistance that needs to be confirmed and mortality less than 90% suggests resistance to insecticides (Soni *et al*., 2018) (Tomlinson *et al*., 2019).

For CDC bottle assay, the test bottles used are coated with insecticide by acetone or ethanol. Mosquitoes are then aspirated into the insecticide-coated bottles and the number of mosquitoes in each bottle does not necessarily have to be equal. After aspirating mosquitoes into the bottles, a timer is started and mortality is scored from time 0. Mortality is scored after every 15 minutes for two hours (Parker *et al*., 2020). If mortality in the control bottles is 3% and 10% the bioassay results should be repeated. If the mortality is greater than 10% the experiment should be discarded (Al-Amin *et al*., 2020).

Insecticide resistance especially against pyrethroids is a major challenge toward vector-borne diseases control worldwide. To the authour's knowledge there is no study to address the overall pyrethroids susceptibility status of Aedes aegypti mosquitoes in Muheza district in Tanga region.

3.2 Study Area

This cross-sectional study was conducted in Muheza district in Tanga region, Tanzania. Muheza district lies along the North-eastern coast of Tanzania, it occupies a total of 4922 square kilometres and it is located at 5º10'S, 38º46'E with an elevation of 1050m above the sea level. According to census conducted in 2012, Muheza district had a total of 204461 people. The climate of the region can be described as tropical with dense rainforest that covers the Usambara mountains (URT, 2013).

The sites were selected based on ecological and demographic characteristics such as the presence of mosquito habitats, topography, vegetation, highly populated areas and the

presence of artificial containers. Sampling was conducted in Mbaramo, Misozwe, Genge, Ngomeni, Magila, Kwafungo and Zeneti wards.

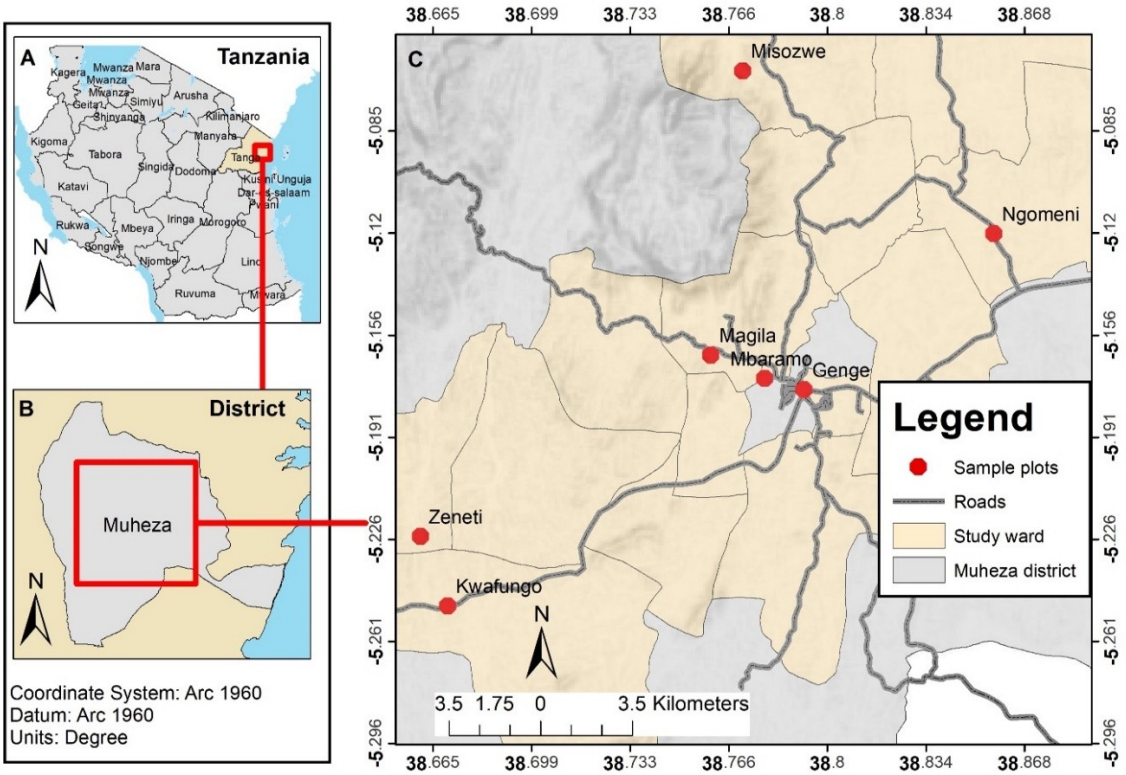

Figure 3.1: Map of Muheza district showing wards where sampling was undertaken. The map was developed using QGIS version 3.16 (https://qgis.org/en/site/forusers/download.html).

3.3 Study Design

3.3.1 Adult bioassays for insecticide susceptibility test

Susceptibility was performed on 300 emerged female *Ae. aegypti* mosquitoes of about two to five days old selected randomly from different wards as stated by WHO guidelines. Female adult mosquitoes were aspirated into WHO exposure tubes lined with paper impregnated by pyrethroid insecticides. For each exposure, four treated tubes with similar insecticide and one control were used. A total of 20 mosquitoes were aspirated into each exposure tube and the procedure was done in four replicates. The experiment was set up

25ºC and a relative humidity of 60%. The number of mosquitoes dead in each insecticide was recorded on a WHO cylinder susceptibility record sheet (Appendix 2).

Control mortality was obtained by dividing the number of dead mosquitoes in the control tube by the total number of mosquitoes applied to each control tube then multiplied by one hundred, while exposure mortality was obtained by dividing the number of dead mosquitoes in the exposure tube by the total number of mosquitoes applied to each exposure tube then multiplied by one hundred.

Exposure mortality rate between 99-100% showed that the mosquitoes were susceptible to the insecticide whereas for mortality of 98% and below indicated mosquito resistance to the insecticide (de la Cruz-Ramos *et al*., 2019).

3.3.2 DNA extraction

Genomic DNA was isolated separately from both live and dead mosquitoes using CTAB solution (Appendix 1). Briefly, 100µl of 2% CTAB solution was added to each tube containing whole-mosquito tissue, then homogenized using plastic applicator stick. Samples were then incubated on a heat block at 65ºC for 30 minutes. 100µl of Chloroform was then added to each tube, then mixed by pulse-vortexing for 5 seconds and centrifuged at 12 000 rpm for 5 minutes to protect DNA during catastrophe. 2-propanol was added to each tube and mixed for 5 seconds by pulse-vortexing then centrifuged at 12 000 rpm for 10 minutes at 4ºC to precipitate DNA molecules from mosquito cells. The procedure was completed by adding 70% ethanol then centrifuged at 1200 rpm for 5 minutes to precipitate DNA and form pellets. The supernatant was discarded and the tubes were uncapped and incubated overnight in a biosafety cabinet. After incubation, the DNA pellets were resuspended by 50µl of ultrapure distilled water. Tubes were then capped and

placed in a 55ºC heat block for 5 minutes to facilitate solubilization. The extracted DNA was lastly stored at -20ºC until PCR.

3.3.3 Molecular analysis

Conventional PCR was used to amplify voltage gated sodium channel gene. The following primers AGA CAA TGT GGA TCG CTT CC, GGA CGC AAT CTG GCT TGT TA, GAG AAC TCG CCG ATG AAC TT and GAC GAC GAA ATC GAA CAG GT were used to amplify domain II and domain III of the voltage gated sodium channel gene respectively (Saavedra-Rodriguez *et al*., 2007). PCR was performed in a 25µL reaction mixture containing 3µL of extracted genomic DNA as a template, 0.2 mm of dNTPs, 1.5 mm $MgCl_2$, 0.4 µM of each primer and 1.5U of Amplitaq polymerase. Initial denaturation was at 94ºC for 15 seconds, annealing at 57 ºC for 30 seconds and extension was done at 72 ºC for 30 seconds. Final elongation was done at 72 ºC for 10 minutes. The quality of the PCR product was assessed by 2% gel electrophoresis stained by SYBR gold DNA stain and visualized using iClear BV20 LED-Transilluminator. PCR products were then sequenced by automated dideoxynucleotide cycle using BigDye Terminator cycle sequencing kit version 3.1, AaSCF3, AaSCR6 and AaSCR8 primers were used.

Table 3.1: Table showing list of primers used for PCR and sequencing of the targeted genes

Primer name	Sequence	Target site	Purpose	Amplicon size (bp)
AaSCF1	AGA CAA TGT GGA TCG CTT CC	S989P, I1011M, I1011V,	PCR	640
AaSCR4	GGA CGC AAT CTG GCT TGT TA	V1016G, V1016I, L1014F		
AaSCF7	GAG AAC TCG CCG ATG AAC TT	F1534C	PCR	740
AaSR7	GAC GAC GAA ATC GAA CAG GT			
AaSCF3	GTG GAA CTT CAC CGA CTT CA	S989P, I1011M, I1011V, L1014F	Sequencing	
AaSCR6	CGA CTT GAT CCA GTT GGA GA	V1016G, V1016I	Sequencing	
AaSCR8	TAG CTT TCA GCG GCT TCT TC	F1534C	Sequencing	

3.4 Data Analysis

Sequence scanner software version 2.0 (Applied Biosystems, Foster City, CA) and Bioedit version 7.2.5 (Ibis Biosciences, Carlsbad, CA) were used to check for the quality of raw sequences data and to obtain consensus nucleotide sequence from both forward and reverse primers for each amplified regions. Multiple consensus sequences were aligned with sequences obtained from GenBank database having accession number MK495870_Aedes.aegypti and MT250048_Aedes.aegypti using Clustal W alignment program in Mega X version 10.1.1.

As shown on Table 2, 0.05% deltamethrin had shown resistance with a percentage mortality of 68% on the collected *Ae. aegypti* mosquitoes. L_{50} was 18.153 with the confidence interval (CI) of 13.367 to 22.547 while LD_{90} was 41.470 with the confidence interval (CI) of 32.215 to 66.103.

Bioassay for 0.75% permethrin had shown susceptibility with a percentage mortality of 98.75% on the collected *Ae. aegypti* mosquitoes. LD_{50} was 13.890 with a confidence interval of 12.199 to 15.431 while LD_{90} was 25.737 with a confidence interval of 22.755 to 30.572.

Bioassay for 0.05% Alphacypermethrin had shown susceptibility with a percentage mortality of 100% on the collected *Ae. aegypti* mosquitoes. LD_{50} was 15.567 with a confidence interval of 11.845 to 19.072 while LD_{90} was 26.929 with a confidence interval of 21.594 to 42.477.

3.5 Ethical Consideration

This study clearance and ethical protocols were approved by the Medical Research Coordinating Committee of the National Institute for Medical Research in Tanzania (Ref No. NIMR/HQ/R.8a/Vol.IX/3278).

Table 3.2: Table showing susceptibility status of *Aedes aegypti* mosquitoes

Insecticide	n	Replicates	% Mortality	LD_{50}	95% CI	LD_{90}	95% CI	Status
Deltamethrin (0.05%)	80	4	68	18.153	13.367-22.547	41.470	32.215- 66.103	R
Permethrin (0.75%)	80	4	98.75	13.890	12.199-15.431	25.737	22.755- 30.572	S
Alphacypermethrin (0.05%)	80	4	100	15.567	11.845-19.072	26.929	21.594- 42.477	S

n = number of samples; CI = confidence interval; SD = standard deviation; KDT = knock-down time; LD_{50} = lethal dose at which 50% of the mosquitoes would be killed; LD_{90} = lethal dose at which 90% of the mosquitoes would be killed; S = Full susceptible (observed mortality 98-100%); R = Resistant (observed mortality ≥ 98%).

3.6 Detection of Knockdown Resistance Genes Mutations in *Aedes aegypti*

The PCR successfully amplified the domain II and domain III genes. The genes amplified for domain III produced PCR products of approximately 740 bp (Figure 13). Whereas the genes amplified for domain II produced PCR products of approximately 6 (Figure 12).

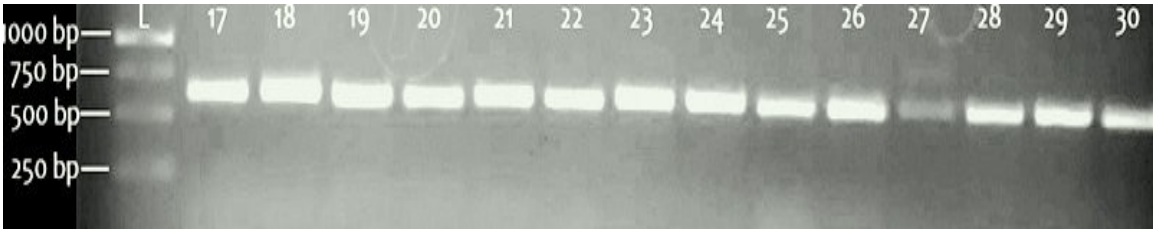

Figure 4. 1: Gel electrophoresis picture showing bright bands of approximately 640bp after kdr gene amplification of Domain II.

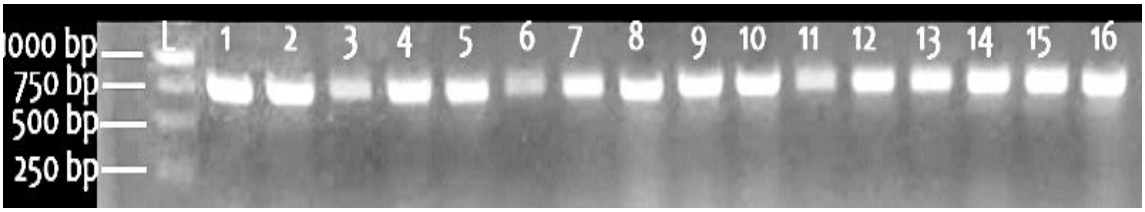

Figure 4.2: Gel electrophoresis picture showing bright bands of approximately 740bp after kdr gene amplification of domain III.

A total of 60 specimens were sequenced out of which 35 sequences were of good quality and were included in detecting mutations on the targeted genes at domain II and domain III of the voltage gated sodium channel gene. The sites analyzed were S989, I1011, L1014, V1016 and F1534 resulting into two point mutations (S989P and V1016I) at domain II and no mutation was detected at domain III of the voltage gated sodium channel gene. On samples 94, 86, 83, 67, 64, 4, 41, 3, 2, 26 the substitution of Serine by Proline was observed. This is the product of transition in the first base of codon 989, the non-mutated codon is TCG and the mutated codon is CCG. On sample number 64 the substitution

Valine by Isoleucine was observed. This is the product of a transition in the first base of codon 1016, the non-mutated codon is ATA and the mutated codon is GTA.

Figure 4.3: Partial section of nucleotide sequence at position 1016 of the VGSC gene. Sample number 94, 86, 83, 67, 64, 4, 41, 3, 2, and 26 are contrasted with reference sequence (MK 495870) from GenBank and V1016 mutation was detected on sample number 64 with the substitution of Valine by Isoleucine as shown on the figure 4.4 below.

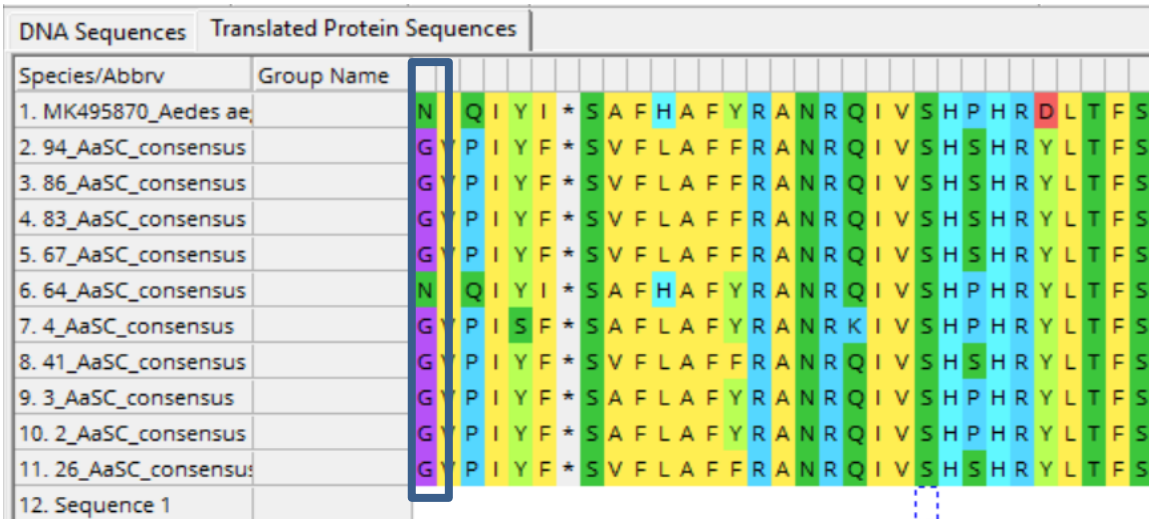

Figure 4.4: Amino acid sequence showing the substitution of Valine by Isoleucine on sample number 64.

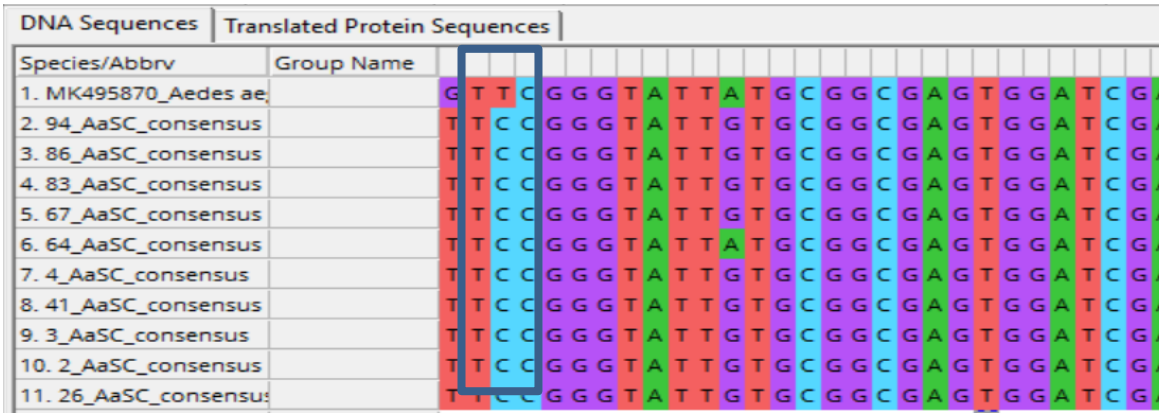

Figure 4.5: Partial section of nucleotide sequence at position 989 of the VGSC gene. Sample number 94, 86, 83, 67, 64, 4, 41, 3, 2, and 26 are contrasted with reference sequence (MK 495870) from GenBank and S989 mutation was detected on all samples with the substitution of Serine by Proline as shown on the figure 4.6 below.

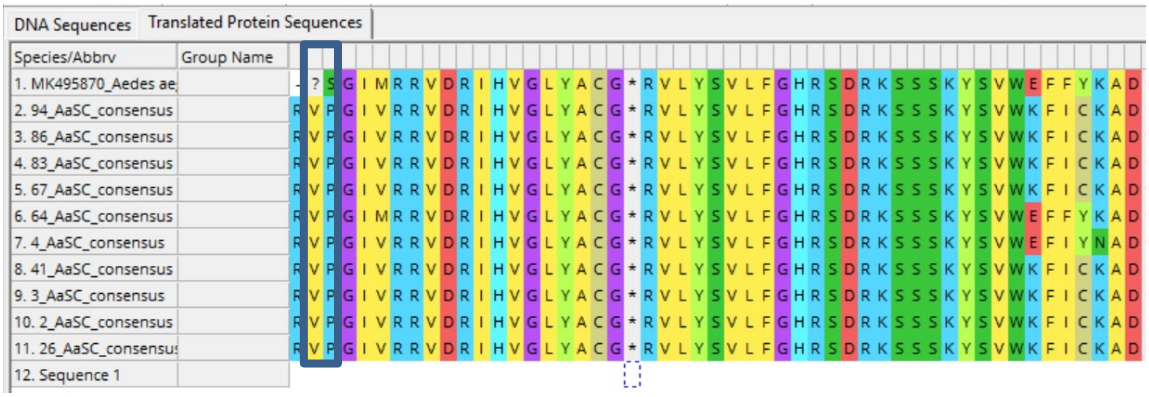

Figure 4.6: Amino acid sequence showing the substitution of Serine by Proline.

3.7 Discussion

The susceptibility test results of *Ae. aegypti* mosquitoes generally demonstrated that *Ae. aegypti* populations from Muheza district subjected to deltamethrin had shown resistance in all study sites selected, with mortality rate of 68%, while when subjected to permethrin and alphacypermethrin *Ae. aegypti* mosquitoes had shown to be susceptible with mortality rates of 98.75 and 100%, respectively. Similar results on deltamethrin resistance were reported on the study done in Papua New Guinea where they found strong deltamethrin

resistance in urban *Ae. aegypti* populations collected from north and south coast of the country (Demok *et al*., 2019).

Deltamethrin had developed higher levels of resistance compared to other insecticides because of its intensive use in manufacturing Long-Lasting Insecticidal Nets (LLINs) along with Indoor Residual Spray (IRS), the main vector control strategies recommended by WHO (Benelli and Beier, 2017). However, cross-resistance with insecticides used in malaria control is also speculated (Brooke, 2008). In other countries, studies have assessed detailed mosquito mechanisms involved in insecticide resistance, which also needs to be done in Tanzania for effective control of *Ae. aegypti* as done in Peru (Pinto *et al.*, 2019).

In general, the increase in pyrethroid resistance among the *Aedes* mosquitoes' population can be attributed to prolonged use of insecticides in controlling mosquitoes domestically such as the use of pyrethroids as residual sprays, slow burning coils and pyrethroids impregnated bed nets. Pyrethroids have also been widely used in agriculture and this has compromised vector control strategies (Dou *et al*., 2020; Medjigbodo *et al*., 2021).

Bioassay results are well supported by the genetic analyses where PCR was done using forward and reverse primers targeting amino acid loci of domain II segment 6 (S989P, I1011M, I1011V, V1016G and V1016I) and F1534C of domain III segment 6 of the voltage-gated sodium channel gene. The two sets of primers successfully amplified 240 samples and domain II had yielded amplicons of approximately 640 bp while domain III had yielded amplicons of approximately 740 bp.

Upon sequencing substitution of Serine by Proline was observed. This is the product of transition in the first base of codon 989, the non-mutated codon is TCG and the mutated

codon is CCG. Also the substitution Valine by Isoleucine was observed. This is the product of a transition in the first base of codon 1016, the non-mutated codon is ATA and the mutated codon is GTA. Similar results have been reported on Indonesian *Ae. aegypti* where co-occurrence of S989P and V1016G point mutations had contributed to the insensitivity of the voltage gated sodium channel leading to pyrethroid resistance (Amelia-Yap *et al*., 2019). Co-occurrence of V1016I and F1534C mutation in Costa Rican *Ae. aegypti had* shown resistance to permethrin and deltamethrin (Zardkoohi *et al*., 2020).

3.8 Conclusion

The findings of this study have confirmed on the presence of vgsc gene mutation on the collected *Ae. aegypti* from selected wards of Muheza district and the detected mutations are attributed to pyrethroids resistance. The mutations may be due improper household application of insecticides and intensive use of one type of insecticide in mosquito control. The data obtained provide additional improvements of the ongoing mosquito control measures in Tanzania.

3.9 Recommendations

i. There is a need to strengthen mosquito control programs undertaken by the country due to an increase in mosquito resistance to insecticides.

ii. It is necessary to ensure the mosquito rearing conditions such as temperature and humidity, food supply and water cleanliness are well met before drawing conclusion on mosquito insecticide susceptibility as these factors may affect mosquito growth making them susceptible to the insecticides they are exposed to.

iii. Future studies should consider other mosquito resistance mechanisms to insecticides such as metabolic resistance and behavioral changes.

iv. More than one class of insecticide should be used to test for mosquito susceptibility to determine if they are resistant to multiple insecticides.

3.10 References

Al-Amin, H. M., Johora, F. T., Irish, S. R., Hossainey, M. R. H., Vizcaino, L., Paul, K. K., Khan, W. A., Haque, R., Alam, M. S. and Lenhart, A. (2020). Insecticide resistance status of Aedes aegypti in Bangladesh. *Parasitology Vectors* 13(622): 1 – 44.

Balmert, N. J., Rund, S. S. C., Ghazi, J. P., Zhou, P. and Duffield, G. E. (2014). Time-of-day specific changes in metabolic detoxification and insecticide resistance in the malaria mosquito Anopheles gambiae. *Journal Insect Physiology* 64: 30–39.

Bass, C. and Jones, C. M. (2016). Mosquitoes boost body armor to resist insecticide attack. *Proceeding National Academic Science* 113: 9145–9147.

Benelli, G. and Beier, J. C. (2017). Current vector control challenges in the fight against malaria. *Acta Tropica* 174: 91–96.

de la Cruz-Ramos, J. M., Hernández-Triana, L. M., García-De la Peña, C., González-Álvarez, V. H., Weger-Lucarelli, J., Siller-Rodríguez, Q. K., Sánchez Rámos, F. J., Rodríguez, A. D., and Ortega-Morales, A. I. (2019). Comparison of two DNA extraction methods from larvae, pupae, and adults of Aedes aegypti. *Heliyon* 5: e02660.

Carrasco, D., Lefèvre, T., Moiroux, N., Pennetier, C., Chandre, F. and Cohuet, A. (2019). Behavioural adaptations of mosquito vectors to insecticide control. *Current Opinion Insect Science* 34: 48–54.

Choi, L., Pryce, J. and Garner, P. (2019). Indoor residual spraying for preventing malaria in communities using insecticide-treated nets. *Cochrane Database System Review* 5: 1 – 80.

de Morais, L. M. O., Jussiani, E. I., Zequi, J. A. C., Dos Reis, P. J. and Andrello, A. C. (2019). Morphological study of Aedes aegypti and Aedes albopictus (Diptera: Culicidae) eggs by X-ray computed. *Microtomography* 126: 102 – 734.

Demok, S., Endersby-Harshman, N., Vinit, R., Timinao, L., Robinson, L. J., Susapu, M., Makita, L., Laman, M., Hoffmann, A. and Karl, S. (2019). Insecticide resistance status of Aedes aegypti and Aedes albopictus mosquitoes in Papua New Guinea. *Parasitology Vectors* 12(1): 333-311.

Dou, R., Sun, J., Deng, F., Wang, P., Zhou, H., Wei, Z., Chen, M., He, Z., Lai, M., Ye, T. and Zhu, L. (2020). Contamination of pyrethroids and atrazine in greenhouse and open-field agricultural soils in China. *Science of Total Environment* 701: 1-37.

Edi, C. A. V., Koudou, B. G., Bellai, L., Adja, A. M., Chouaibou, M., Bonfoh, B., Barry, S. J. E., Johnson, P. C. D., Müller, P., Dongus, S., N'Goran, E. K., Ranson, H. and Weetman, D. (2014). Long-term trends in Anopheles gambiae insecticide resistance in Côte d'Ivoire. *Parasitology Vectors* 7(1): 1-10.

Finda, M. F., Christofides, N., Lezaun, J., Tarimo, B., Chaki, P., Kelly, A. H., Kapologwe, N., Kazyoba, P., Emidi, B. and Okumu, F. O. (2020). Opinions of key stakeholders on alternative interventions for malaria control and elimination in Tanzania. *Malaria Journal* 19(1): 164-177.

Fung, C. Y., Zhu, K. Y., Major, K., Poynton, H. C., Huff Hartz, K. E., Wellborn, G. and Lydy, M. J. (2021). The contribution of detoxification pathways to pyrethroid resistance in Hyalella azteca. *Environmental Pollution* 284: 1-10.

Furnival-Adams, J., Olanga, E. A., Napier, M. and Garner, P. (2021). House *Modifications for Preventing Malaria*. John Wiley and Sons, South Africa. 60pp.

Kabula, B., Tungu, P., Matowo, J., Kitau, J., Mweya, C., Emidi, B., Masue, D., Sindato, C., Malima, R., Minja, J., Msangi, S., Njau, R., Mosha, F., Magesa, S. and Kisinza, W. (2012). Susceptibility status of malaria vectors to insecticides commonly used for malaria control in Tanzania. *Tropical Medicine International Health* 17: 742–750.

Li, H., Lydy, M. J. and You, J. (2016). Pyrethroids in indoor air during application of various mosquito repellents: Occurrence, dissipation and potential exposure risk. *Chemospher*e 144: 2427–2435.

Lines, J. and Addington, W. (2001). Insecticide-treated nets in Tanzania. *Lancet* 358: 0-671.

Matiya, D. J., Philbert, A. B., Kidima, W. and Matowo, J. J. (2019). Dynamics and monitoring of insecticide resistance in malaria vectors across mainland Tanzania from 1997 to 2017: A systematic review. *Malaria Journal* 18(102): 1 – 16.

Medjigbodo, A. A., Djogbenou, L. S., Koumba, A. A., Djossou, L., Badolo, A., Adoha, C. J., Ketoh, G. K. and Mavoungou, J. F. (2021). Phenotypic Insecticide Resistance in Anopheles gambiae (Diptera: Culicidae): Specific Characterization of Underlying Resistance Mechanisms Still Matters. *Journal of Medical Entomology* 58: 730–738.

Nnko, E. J., Kihamia, C., Tenu, F., Premji, Z. and Kweka, E. J. (2017). Insecticide use pattern and phenotypic susceptibility of Anopheles gambiae sensu lato to commonly used insecticides in Lower Moshi, northern Tanzania. *BMC Research Notes* 10: 443-555.

Parker, C., Ramirez, D., Thomas, C. and Connelly, C. R. (2020). Baseline Susceptibility Status of Florida Populations of Aedes aegypti (Diptera: Culicidae) and Aedes albopictus. *Journal Medicine Entomology* 57: 1550–1559.

Phiri, M. D., McCann, R. S., Kabaghe, A. N., van den Berg, H., Malenga, T., Gowelo, S., Tizifa, T., Takken, W., van Vugt, M., Phiri, K. S., Terlouw, D. J. and Worrall, E. (2021). Cost of community-led larval source management and house improvement for malaria control: a cost analysis within a cluster-randomized trial in a rural district in Malawi. *Malaria Journal* 20: 1-17.

Pinto, J., Palomino, M., Mendoza-Uribe, L., Sinti, C., Liebman, K. A. and Lenhart, A. (2019). Susceptibility to insecticides and resistance mechanisms in three populations of Aedes aegypti from Peru. *Parasitology Vectors* 12(494): 1 – 11.

Rajatileka, S., Burhani, J. and Ranson, H. (2011). Mosquito age and susceptibility to insecticides. *Trans Research Society Tropical Medicine Hygiene* 105: 247–253.

Ramirez, J. L., Garver, L. S. and Dimopoulos, G. (2009). Challenges and approaches for mosquito targeted malaria control. *Current Molecular Medicine* 9: 116–130.

Ranathunge, T., Udayanga, L., Sarasija, S., Karunathilaka, S., Nawarathne, S., Rathnarajah, H., Dulficar, F. F., Shafi, F. N., Dassanayake, R. S. and Gunawardene, Y. I. N. S. (2021). Voltage-Gated Sodium Channel (Vgsc) Mutation-Based Pyrethroid Resistance in Aedes aegypti Populations of Three Endemic Dengue Risk Areas of Sri Lanka. *Biomed Research International* 2021: 1-11.

Ranson, H., Jensen, B., Vulule, J. M., Wang, X., Hemingway, J. and Collins, F. H. (2000). Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan Anopheles gambiae associated with resistance to DDT and pyrethroids. Insect *Molecular Biology* 9: 491–497.

Saavedra-Rodriguez, K., Urdaneta-Marquez, L., Rajatileka, S., Moulton, M., Flores, A. E., Fernandez-Salas, I., Bisset, J., Rodriguez, M., McCall, P. J., Donnelly, M. J., Ranson, H., Hemingway, J. and Black, W. C. (2007). A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American Aedes aegypti. *Insect Molecular Biology* 16: 785–798.

Soni, M., Bhattacharya, C., Sharma, J. and Dutta, P. (2018). Bioassay and molecular study for detection of insecticide resistance dengue causing mosquito vectors. *Indian Journal Medicine Microbiology* 36: 435–438.

Tomlinson, S., Carrington Yates, H., Oruni, A., Njoroge, H., Weetman, D., Donnelly, M. J. and Hof, A. E. V. (2019). Open source 3D printable replacement parts for the WHO insecticide susceptibility bioassay system. *Parasit Vectors* 12: 539-546.

Tungu, P. K., Sudi, W., Kisinza, W., Rowland, M. 2021. Effectiveness of a long-lasting insecticide treatment kit (ICON® Maxx) for polyester nets over three years of household use: a WHO phase III trial in Tanzania. *Malaria Journal* 20: 345-352.

Vatandoost, H., Abai, M. R., Akbari, M., Raeisi, A., Yousefi, H., Sheikhi, S. and Bagheri, A. (2019). Comparison of CDC Bottle Bioassay with WHO Standard Method for Assessment Susceptibility Level of Malaria Vector, Anopheles stephensi to Three Imagicides. *Journal of Arthropod Borne Diseases* 13: 17–26.

Warikoo, R., Wahab, N. and Kumar, S. (2011). Oviposition-altering and ovicidal potentials of five essential oils against female adults of the dengue vector, Aedes aegypti L. *Parasitology Research* 109: 1125–1131.

Zhu, Q., Yang, Y., Zhong, Y., Lao, Z., O'Neill, P., Hong, D., Zhang, K. and Zhao, S. (2020). Synthesis, insecticidal activity, resistance, photodegradation and toxicity of pyrethroids (A review). *Chemosphere* 254: 126 – 779.

CHAPTER FOUR

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

4.1 Discussion

This study accessed the abundance and pyrethroid susceptibility status of *Ae. aegypti* mosquitoes in the selected wards of Muheza district. The targeted habitats were the ones known to favour the breeding of *Ae. aegypti* mosquitoes. A total of 2572 collected larvae successfully emerged into female adult mosquitoes.

Three genera namely *Aedes* 46%, *Culex* 30%, and *Anopheles* 18% were identified. *Aedes aegypti* was the abundant mosquito specie accounting for 49.2% of the total female adult mosquitoes. *Culex quinquefasciatus* accounted for 32%, *Anopheles gambiae* accounted for 17.3% and *Anopheles funestus* accounted for 1.5%. Majority of the *Ae. aegypti* were collected from Mbaramo ward. From the seven wards, *Ae. aegypti* were more abundant in Mbaramo representing 21% (n = 267), followed by Kwafungo (19% n = 236), Zeneti (19% n = 240), Genge (13% n=161), Ngomeni (12% n=153), Misozwe (10% n = 131) and Magila (6% n= 78).

The Poisson regression model was used to determine the rate of association between the expected number of *Ae. aegypti* and Physicochemical characteristics of the breeding sites. TDS refers to total dissolved solid which was measured by using a handheld Hanna TDS meter. Expected number of *Ae. aegypti* decreased for every unit increase in algae cover, temperature, pH, and vegetation (with correlation coefficient of -2.073, -3.424, -12.502 and -1.85 respectively). While for temporary habitats and total dissolved solids the expected number of *Ae. aegypti* increased (correlation coefficient = 9.537 and 30.003 respectively).

Similar findings on breeding habitats were reported on other studies conducted in Viet Nam. The results had shown positive relationship between domestic water storage containers and high abundance of immature *Ae. aegypti* (Bitsindou *et al*., 2018). TDS is reported to be a significant contributor in determining mosquito larvae abundance, similar results were reported on a study conducted in columbia where infestation of immature *Ae. aegypti* was positively associated with an increase in TDS (Kache *et al*., 2020).

Algae cover had a negative effect on *Aedes* larvae habitats and similar results were reported on a study done in Colombia (Baharmand *et al*., 2020). The findings obtained on the effects of temperature were inconsistent to other studies (Betanzos-Reyes *et al*., 2018). On this study finding pH had a negative effect on immature stage of *Ae. aegypti*, our results were inconsistent to the study done in Brazil where species such as *Aedes fluviatilis* adopted to extreme pH levels enabling them to survive (Multini *et al*., 2021). Results on vegetation cover and artificial containers were found to have a significant positive association with the mean abundance of *Ae. aegypti*, similar results were reported on other studies (Barrera *et al*., 2006; Sun *et al*., 2021).

4.2 Conclusion

In conclusion, this study has shown that *Ae. aegypti* mosquitoes are abundant in the selected wards in Muheza district which is among dengue outbreak hubs. *Ae. aegypti* have previously been documented as potential vectors for various mosquito-borne diseases such as Zika, yellow fever virus, dengue fever and Japanese encephalitis. In this study, the collected *Ae. aegypti* mosquitoes have shown resistance to deltamethrin and this poses a high risk of disease transmission due to their abundance. Overall the data obtained provide a baseline for systematic planning and proper selection of mosquito control measures before commencement.

4.3 Recommendations

i. Abundance of *Ae. aegypti* in Muheza district calls for public education and campaign on the potential risks brought by these vectors and this could reduce the rate of disease exposure.

ii. There is a need to enhance continuous surveillance for *Ae. aegypti* and other potential vectors for mosquito-borne diseases in Muheza district to reduce the spread of diseases and predict future outbreaks.

iii. There should be proper waste management, house modification and house planning to curtail breeding habitats for *Ae. aegypti* mosquitoes in particular as well as other mosquito species.

iv. There is a need to strengthen mosquito control programs undertaken by the country due to an increase in mosquito resistance to insecticides.

v. It is necessary to ensure the mosquito rearing conditions such as temperature and humidity, food supply and water cleanliness are well met before drawing conclusion on mosquito insecticide susceptibility as these factors may affect mosquito growth making them susceptible to the insecticides they are exposed to.

vi. Future studies should consider other mosquito resistance mechanisms to insecticides such as metabolic resistance and behavioral changes.

vii. More than one class of insecticide should be used to test for mosquito susceptibility before drawing conclusion on insecticide resistance.

viii. Immediate mosquito control measures should include public education on how to deal with key containers such as used car tires and cans that hold stagnant water and enable mosquitoes to breed.

ix. Proper use of insecticides and drug recombinants should also be considered to reduce the rate of insecticide resistance.

x. Breeding grounds could also be targeted by applying larvicides or getting rid of them.

4.4 References

Baharmand, I., Coatsworth, H., Peach, D. A. H., Belton, P. and Lowenberger, C. (2020). Molecular relationships of introduced Aedes japonicus (Diptera: Culicidae) populations in British Columbia, Canada using mitochondrial DNA. *Journal of Vector Ecology* 45(2): 285–296.

Barrera, R., Amador, M. and Clark, G. G. (2006). Ecological factors influencing Aedes aegypti (Diptera: Culicidae) productivity in artificial containers in Salinas, Puerto Rico. *Journal of Medical Entomology* 43: 484–492.

Betanzos-Reyes, Á. F., Rodríguez, M. H., Romero-Martínez, M., Sesma-Medrano, E., Rangel-Flores, H. and Santos-Luna, R. (2018). Association of dengue fever with Aedes spp. abundance and climatological effects. *Salud pública de méxico* 60: 12–20.

Bitsindou, P., Bantsimba-Ndziona, M. J. and Lenga, A. (2018). Current Distribution and Bioecological Characterizations of Aedes aegypti and Aedes albopictus in Brazzaville. *Bulletin de la Societe de Pathologie Exotique* 111: 301–308.

Failloux, A. B. (2018). Mosquitoes as vectors of arboviruses: an endless story. *Biology Aujourdhui* 212: 89–99.

Ferede, G., Tiruneh, M., Abate, E., Kassa, W. J., Wondimeneh, Y., Damtie, D. and Tessema, B. (2018). Distribution and larval breeding habitats of Aedes mosquito species in residential areas of northwest Ethiopia. *Epidemiol Health* 40: e2018015.

Gutu, M. A., Bekele, A., Seid, Y., Mohammed, Y., Gemechu, F., Woyessa, A. B., Tayachew, A., Dugasa, Y., Gizachew, L., Idosa, M., Tokarz, R. E. and Sugerman, D. (2021). Another dengue fever outbreak in Eastern Ethiopia-An emerging public health threat. PLoS Neglected Tropical Diseases 15: e0008992.

Huang, C. H., Tsai, Y. T., Wang, S. F., Wang, W. H. and Chen, Y. H. (2021). Dengue vaccine: an update. *Expert Review of Anti-infective Therapy* 19: 1495–1502.

Huang, Y. J. S., Higgs, S. and Vanlandingham, D. L. (2019). Emergence and re-emergence of mosquito-borne arboviruses. *Current Opinion in Virology* 34: 104–109.

Kache, P. A., Eastwood, G., Collins-Palmer, K., Katz, M., Falco, R. C., Bajwa, W. I., Armstrong, P. M., Andreadis, T. G. and Diuk-Wasser, M. A. (2020). Environmental Determinants of Aedes albopictus Abundance at a Northern Limit of Its Range in the United States. *The American Journal of Tropical Medicine and Hygiene* 102: 436–447.

Lee, H., Halverson, S and Ezinwa, N. (2018). Mosquito-Borne Diseases. *Prim Care* 45: 393–407.

Multini, L. C., Oliveira-Christe, R., Medeiros-Sousa, A. R., Evangelista, E., Barrio-Nuevo, K. M., Mucci, L. F., Ceretti-Junior, W., Camargo, A. A., Wilke, A. B. B. and Marrelli, M. T. (2021). The Influence of the pH and Salinity of Water in Breeding Sites on the Occurrence and Community Composition of Immature Mosquitoes in the Green Belt of the City of São Paulo, Brazil. Insects. 12pp.

O'Leary, K. (2021). A malaria vaccine at last. *Nature Medicine* 27: 1-20.

Schmidt, T. L., Endersby-Harshman, N. M. and Hoffmann, A. A. (2021). Improving mosquito control strategies with population genomics. *Trends Parasitology* 37, 907–921.

Sun, H., Dickens, B. L., Richards, D., Ong, J., Rajarethinam, J., Hassim, M. E. E., Lim, J. T., Carrasco, L. R., Aik, J., Yap, G., Cook, A. R. and Ng, L. C. (2021). Spatio-temporal analysis of the main dengue vector populations in Singapore. *Parasite Vectors* 14: 1-11.

Trumbetta, J. M., Placentia, V. and Connelly, P. H. (2020). Meeting Increased Demand for Mosquito Adulticides Containing the Active Ingredient Naled Following

Hurricanes and Tropical Storms. *Journal of the American Mosquito Control Association* 36: 98–102.

Vontas, J. and Mavridis, K., (2019). Vector population monitoring tools for insecticide resistance management: Myth or fact? *Pesticide Biochemistry and Physiol*ogy 161: 54–60.

Weaver, S. C. and Reisen, W. K. (2010). Present and future arboviral threats. *Antiviral Research* 85: 328–345.

Wu, C., Guo, X., Zhao, J., Lv, Q., Li, H., McNeil, E. B., Chongsuvivatwong, V. and Zhou, H. (2017). Behaviors Related to Mosquito-Borne Diseases among Different Ethnic Minority Groups along the China-Laos Border Areas. *International Journal of Environmental Research and Public Health* 14: 1-11.

APPENDICES

Appendix 1: CTAB DNA extraction protocol

Standard Operating Procedure	CTAB extraction of mosquito genomic DNA
Developed by:	Benard Batengana
Authorizing position:	
Effective date:	October 2019
Edit date :	December 2020

1. **Objective:** This document describes the standard operating procedure for the CTAB extraction of DNA from mosquito tissue
2. **Reference:** Echevery F.D *et al*., 2017
3. **Personal protective equipment required:**
    - Gloves
    - Lab coat
    - Face mask (if available)
4. **CTAB Extraction**

    4.1 **Materials and instructions**
    - CTAB (Cetyltrimethylammonium Bromide, Titr. 99%, Molec. Biology, *ITW Reagents¥: A6284.0100-100gm*
    - Tris Buffer, 1M, PH 8.0 for Molec. Biology, *ITW Reagents: A3145.1000-1lt*
    - EDTA SOLUTION, 0.5m, pH 8.0, *ITW Reagents: 131659.1211-1kg*
    - Chloroform Biochemica, GC 99.5%, *ITW Reagents: A3691.100-1lt*
    - 2-Propanol, GC 99.5% Technical grade, *ITW Reagents: 211090.1211-1lt*
    - Ethanol absolute for analysis, GC 99.8%, *ITW Reagents: 131086.1212-2.5lt*
    - UltraPure Distilled Water
    - Microcentrifuge tubes (1.5ml)
    - Pulsing vortex
    - Pestles
    - Dry heat block
    - Centrifuge
    - Pipettes 200µl
    - Tips 200µl
    - Biosafety cabinet
5. **Reagents (extraction)**
    - CTAB solution, 2%
    - Chloroform 99.4%
    - 2-Propanol, 99.5%
    - Ethanol, 70%

Reag. (2% CTAB prep)	Vol.	Final conc.

1M Tris-HCL, Ph 8.0	100mL	100mM
0.5 EDTA	20mL	10mM
NaCl	81.8g	1.4M
CTAB	20g	2%
Sterile water	bring total volume to 1L	

4.3. **Procedure: (whole sample/ headthorax extraction)**

1. Preheat the dry heat block 65ºC at the beginning of each work day
2. Add 100µL 2% CTAB in each tube
3. Grind the mosquito using pestle
4. Incubate all tubes at 65ºC for 30minutes
5. Add 100µl choloform to each tube
6. Cap tubes tightly, mix by pulse-voltexing for 5s then centrifuge at 1200rpm for 5minutes
7. While tubes are centrifuging, label a second set of 1.5ml tube to every sample for next step
8. Remove tubes from centrifuge and pipette **ONLY supernatant** just 70µl into the clean labelled tube
9. Add 100µl isopropanol (2-propanol) to each tube.
10. Cap tubes tightly, mix by pulse-voltexing for 5s, then centrifuge at 1200 rpm for 20minutes at 4ºC
11. Dispose of the flow 2-propanol into waste collection beaker, and then blot on paper towel to remove excess liquid.
12. Add 100µl of the 70% ethanol to each sample tube.
13. Cap tubes tightly, centrifuge at 12000rpm for 5 minutes at room temperature.
14. Dispose of the flow ethanol, and them blot on paper towel to remove excess ethanol.
15. Place uncapped tubes on a paper towel in incubator set at 40ºC for 15 minutes or overnight in a biosafety cabinet. Allow to air dry DNA pellet
16. After completion, add 50µl Ultrapure Distilled water to each tube. Briefly centrifuge to be sure that the DNA pellet is completely resuspended.
17. Cap and place the tubes in 55ºC heat block for 5 minutes to facilitate solubilisation.
18. Store the extracted gDNA at -20ºC until use

*Mosquito specimen can be left at room temperature in CTAB overnight if necessary.

Appendix 2: WHO cylinder susceptibility record sheet

WHO CYLINDER SUSCEPTIBILITY RECORD
SHEET_DiscriminatingDose

Test number: 01 Date: 31/08/2021
Investigator name: NEEMA BENDERA
Area information
Region: TANGA District: MUHEZA

Sample information
Species tested: AEDES Species Control: FEMALE AEDES
Sex: FEMALE Age (days): 4 DAYS

Collection method (tick)

Larval collection: ☐ Others (specify): Progeny F1:

Physiological stage (tick): non-blood fed: ☐ Semi Gravid or Gravid:

Insecticide information
Insecticide tested:DELTAMETHRIN Concentration: 0.05% Batch: DE 342
Impregnated papers prepared by: WHO Number of times this paper was used: 01X
Date of Expiry: FEB 2022 Date box first opened: 15/08/2021
Storage conditions (tick): Room temperature: Refrigerated: ☐

Test conditions
Period of exposure (minutes): 60

Test conditions	Time	Temperature (ºC)	Relative humidity(%)
Holding period pre-test: **Start**	16:00	24.8	81
Exposure period: **Start**	17:00	24.8	81
Exposure period: **End**	18:00	25	80
Holding period 24h: **Start**	17:00	26	75
Holding period 24h: **End**	18:00	27	75

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Total test	Total control
Number of mosquitoes exposed	20	20	20	20			20

Test results

Number knocked down after exposure for minutes

	Time	No	Time	No	Time	No	Time	No	Time	No	Time	No
START	17:03		17:06		17:09		17:12				17:00	0
10	17:13	0	17:16	0	17:19	2	17:21	4			17:10	6
15	17:18	3	17:21	5	17:24	8	17:27	15			17:15	31
20	17:23	10	17:26	10	17:29	13	17:31	18			17:20	58
30	17:33	14	17:36	17	17:39	16	17:42	18			17:30	65
40	17:43	17	17:46	18	17:49	18	17:52	18			17:40	71
50	17:53	18	17:56	18	17:59	19	18:02	19			17:50	74
60	18:03	18	18:06	18	18:09	19	18:12	19			18:00	74

Number of dead / alive mosquitoes at 24hours post exposure

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Total test	Total Control
Number dead	18	18	19	19			0
Number alive	2	2	1	1			20

WHO CYLINDER SUSCEPTIBILITY RECORD
SHEET_DiscriminatingDose

Test number: 02 Date: 31/08/2021
Investigator name: NEEMA ALLY BENDERA
Area information
Region: TANGA District: MUHEZA

Sample information
Species tested: AEDES Species Control: FEMALE AEDES
Sex: FEMALE Age (days): 4 DAYS

Collection method (tick)

Larval collection: ☐ Others (specify): Progeny F1:

Physiological stage (tick): non-blood fed: ☐ Semi Gravid or Gravid:

Insecticide information
Insecticide tested: PERMETHRIN Concentration: 0.75% Batch:AL 098
Impregnated papers prepared by: WHO Number of times this paper was used: 01X
Date of Expiry: JAN 2020 Date box first opened: 31/08/2021
Storage conditions (tick): Room temperature: Refrigerated: ☐

Test conditions
Period of exposure (minutes): 60

Test conditions	Time	Temperature (ºC)	Relative humidity(%)
Holding period pre-test: **Start**	14:00	25	81
Exposure period: **Start**	15:00	25	81
Exposure period: **End**	16:00	25	80
Holding period 24h: **Start**	15:00	26	75
Holding period 24h: **End**	16:00	26	75

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Total test	Total control
Number of mosquitoes exposed	20	20	20	20			20

Test results

Number knocked down after exposure for minutes

	Time	No	Time	No	Time	No	Time	No	Time	No	Time	No
START	15:03		15:06		15:09		15:12				15:00	0
10	15:13	2	15:16	3	15:19	4	15:21	7			15:10	16
15	15:18	8	15:21	9	15:24	16	15:27	16			15:15	49
20	15:23	11	15:26	16	15:29	19	15:31	20			15:20	66
30	15:33	16	15:36	18	15:39	19	15:42	20			15:30	73
40	15:43	18	15:46	20	15:49	20	15:52	20			15:40	78
50	15:53	20	15:56	20	15:59	20	16:02	20			15:50	78
60	16:03	20	16:06	20	16:09	20	16:12	20			16:00	78

Number of dead / alive mosquitoes at 24hours post exposure

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Total test	Total Control
Number dead	20	20	20	20			0
Number alive	0	0	0	0			20

WHO CYLINDER SUSCEPTIBILITY RECORD
SHEET_DiscriminatingDose

Test number: 03 Date: 31/08/2021
Investigator name: NEEMA ALLY BENDERA

Area information

Region: TANGA District: MUHEZA

Sample information

Species tested: AEDES Species Control: FEMALE AEDES
Sex: FEMALE Age (days): 4DAYS

Collection method (tick)

Larval collection: ☐ Others (specify): Progeny F1:

Physiological stage (tick): non-blood fed: ☐ Semi Gravid or Gravid:

Insecticide information

Insecticide tested: ALPHACYERMETHRIN Concentration: 0.05% Batch: AL 0.98
Impregnated papers prepared by: WHO Number of times this paper was used: 01X
Date of Expiry: JAN 2022 Date box first opened: 31/08/2021
Storage conditions (tick): Room temperature: Refrigerated: ☐

Test conditions

Period of exposure (minutes): 60

Test conditions	Time	Temperature (ºC)	Relative humidity(%)
Holding period pre-test: **Start**	15:00	25	80
Exposure period: **Start**	16:00	25	80
Exposure period: **End**	17:00	26	72
Holding period 24h: **Start**	16:00	27	75
Holding period 24h: **End**	17:00	28	80

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Total test	Total control
Number of mosquitoes exposed	20	20	20	20			20

Test results

Number knocked down after exposure for minutes

	Time	No	Time	No	Time	No	Time	No	Time	No	Time	No
START	16:03		16:06		16:09		16:12				16:00	0
10	16:13	3	16:16	0	16:19	1	16:21	6			16:10	10
15	16:18	7	16:21	4	16:24	9	16:27	11			16:15	31
20	16:23	19	16:26	16	16:29	18	16:31	16			16:20	69
30	16:33	19	16:36	19	16:39	18	16:42	19			16:30	75
40	16:43	20	16:46	20	16:49	19	16:52	20			16:40	79
50	16:53	20	16:56	20	16:59	19	17:02	20			16:50	79
60	17:03	20	17:06	20	17:09	19	17:12	20			17:00	79

Number of dead / alive mosquitoes at 24hours post exposure

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Total test	Total Control
Number dead	20	20	19	20			0
Number alive	0	0	1	0			20

Appendix 3: Ecological form

Index	Location (Wards)	Coordinates	Habitat characteristics						Number of mosquitoes collected		
			TDS	Algae cover	Temperature	pH	Vegetation	Type	*Ae. aegypti*	Culex	Anopheles
1	Genge	S-5.174421/ E38.792200	0.42	present	29.8	6.21	Absent	temporary	161	150	54
2	Mbaramo	S-5.170549 E38.778731	0.31	present	25.0	7.60	Present	temporary	267	130	68
3	Misozwe	S-5.06458/ E38.77127	0.21	absent	29.1	6.94	Present	temporary	131	120	72
4	Magila	S-5.162510/ E38.760294	0.36	present	30.0	7.12	Absent	permanent	78	89	92
5	Ngomeni	S-5.120646 E38.857403	0.51	absent	28.7	7.08	Present	permanent	153	52	73
6	Kwafungo	S-5.24893/ E38.66999	0.24	absent	28.3	7.04	Present	temporary	236	120	79
7	Zeneti	S-5.225010 E38.660581	0.49	Present	27.7	7.20	Present	Permanent	240	163	44