

THE EFFICACY OF INDOOR RESIDUAL SPRAYING AND INSECTICIDE
SUSCEPTIBILITY STATUS OF THE ANOPHELES MOSQUITO POPULATION
FROM SELECTED MALARIA ENDEMIC REGIONS OF NORTHERN
NAMIBIA

A RESEARCH THESIS SUBMITTED IN FULFILMENT OF THE
REQUIREMENTS FOR
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MASTER OF SCIENCE
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OF
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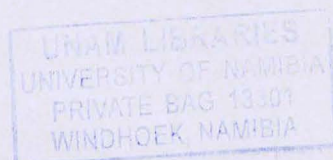
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Abstract

Indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) are the primary malaria vector control interventions recommended by the World Health Organisation (WHO). These interventions have been used in northern Namibia as the main intervention for malaria vector control since the 1965 (IRS) and 2005 (LLINs). However, insecticide resistance (IR) in *Anopheles* mosquitoes to pyrethroids, a major insecticide class approved for use in both LLINs and IRS of modern structures, may threaten continued use of these interventions. This study aimed to evaluate the susceptibility status and composition of *Anopheles* mosquitoes and the efficacy of indoor residual spraying insecticides in northern regions. Data was collected at one point in time and not throughout the year. WHO bioassay tubes were used to investigate the susceptibility status of *Anopheles* mosquitoes from Otjozondjupa, Omusati, Oshana, Ohangwena, Kavango West, Kunene and Oshikoto region to DDT (4 %) and Deltamethrin (0.05 %) insecticide. The *Anopheles* mosquitoes were then morphologically identified; as well as molecularly identified to a species level using the Polymerase Chain Reaction (PCR) method. Residual efficacy of insecticides DDT, Deltamethrin, pirimiphosmethyl (Actellic 300CS), and clothianidin (SumiShield 50WG) sprayed on zinc, modern and traditional structures in Oshana region was also tested with susceptible *Anopheles arabiensis*, using WHO cone bioassays. Findings confirmed IR against Deltamethrin in Oshikoto and Kavango West region (78.75 % and 90 % respectively) according to WHO protocols, with other malaria regions showing reduced susceptibility. There is reduced susceptibility to DDT in Kavango West region (93.75 %), while all other regions showed full susceptibility (100 %). PCR results showed an overall predominance of *Anopheles gambiae* sensu stricto (982) over *Anopheles arabiensis* (600) in northern Namibia; Otjozondjupa (214, 137), Omusati (183, 123), Oshana (196, 48), Ohangwena (163, 167), Kavango West (41, 54), Kunene (113, 0), Oshikoto (72, 71), respectively. Five months after initial application, SumiShield 50WG induced 100 % mortality of mosquitoes exposed on both modern and zinc structures, Deltamethrin killed more than 80 % of mosquitoes on these structures, although Actellic 300CS and DDT were below the threshold recommended. There is a prevalence of pyrethroid resistance in Namibian malaria vectors in Kavango West and Oshikoto regions, which might hinder vector management. However, SumiShield 50WG an environmentally acceptable insecticide

with a different mode of action to pyrethroids may be a suitable alternative insecticide for IRS in Namibia, where resistance to Deltamethrin and DDT insecticide has started to develop. SumiShield 50WG is a novel neonicotinoid insecticide that acts as an agonist of the nicotinic acetylcholine receptor (nAChR). This receptor differs from those of the existing recommended insecticide families (pyrethroids, organophosphates, carbamates and organochlorine). Although, continued IR surveillance is recommended in more than one sentinel site of the malaria endemic regions of Namibia, monitoring should start earlier to avoid incomplete tests due to insufficient sample size. Additionally, it is recommended that the residual efficacy study should be repeated with a sufficient number of structures and sufficient mosquito sample size.

Key words: *Anopheles*, indoor residual spraying, pyrethroids.

List of Conferences and Posters

1. **Joseph R. N.**, Eiseb S. J., Uusiku P., Iitula I., Maliti D. V. and Mumbengegwi D. R. (2019). Investigation into the insecticide susceptibility status of the *Anopheles* mosquito population in malaria endemic regions of northern Namibia. *The 3rd Multi-disciplinary Research Centre Conference*. 23-24 July, 2019. National Institute of Public Administration and Management, Windhoek, Namibia. Oral Presentation.
2. **Joseph R. N.**, Eiseb S. J., Uusiku P., Ogoma-Barasa S., Iitula I., Maliti D. V. and Mumbengegwi D. R. (2019). Evaluation of indoor residual spraying insecticides and the susceptibility status of the *Anopheles* mosquito population in selected malaria-endemic regions of northern Namibia. *The National Students' Research Symposium*. 02-03 October, 2019. National Institute of Public Administration and Management, Namibia. Oral Presentation.
3. **Joseph R. N.**, Eiseb S. J., Uusiku P., Ogoma-Barasa S., Iitula I., Maliti D. V., Katokele S., Mwema T., Gueye S. C., Vajda E., Lobo N., Mumbengegwi D. R. (2019). Investigation into the efficacy of indoor residual spraying and the insecticide susceptibility status of the *Anopheles* mosquito population in selected malaria endemic regions of northern Namibia. *67th American Society of Tropical Medicine and Hygiene Annual meeting*. 20-24 November, 2019. Gaylord National Resort and Convention Centre, Washington D.C, United States of America. Poster Presentation.

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List of Abbreviation

AChE	Acetylcholinesterase
ACTs	Artemisinin-based Combination Therapies
BChE	Butyrylcholinesterase
CCEs	Carboxyl cholinesterases
CS	Capsule Suspension
DDT	Dichloro-diphenyl-trichloroethane
DNA	Deoxyribonucleic acid
EC	Emulsifiable concentrate
EHAPs	Environmental Health Assistant Practitioners
EHPs	Environmental Health Practitioners
GSTs	Glutathione S-Transferases
HIV/AIDS	Human Immuno-deficiency Virus/Acquired Immune Deficiency Syndrome
IR	Insecticide Resistance
IRM	Insecticide Resistance Management
IRS	Indoor Residual Spraying
IVM	Integrated Vector Management
Kdr	Knock-Down Resistance
LLINs	Long-lasting Insecticidal Nets
MOA	Mode of Action
MoHSS	Ministry of Health and Social Services
nAChR	Nicotinic acetylcholine receptors
NADPH	Nicotinamide adenine dinucleotide phosphate
NMSP	National Malaria Strategic Plan
NVDCP	National Vector-borne Diseases Control Programme
PCR	Polymerase Chain Reaction
RDT	Rapid Diagnostic Tests
SADC	Southern African Development Community
SC	Suspension concentrate
SOPs	Standard Operating Procedures
VGSC	Voltage-gated sodium channel

UCSF	University of California San Francisco
WG	Water dispersible Granules
WP	Water dispersible Powder
WHO	World Health Organization
WHOPES	World Health Organization Pesticides Evaluation Scheme

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Dedication

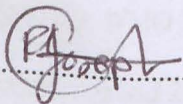
This work is dedicated to my beloved family and mostly my mommy, Ronia Pengevali Shishiveni. I am grateful for your support, motivation and your constant reminder that I am more than capable. I love and appreciate you all very much. You are and will always be my pillars of strength. May you be excessively blessed! Jeremiah 29:11 "Future & Hope".

Declarations

I, Rosalia Nghitalesheni Joseph, declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

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Date. 24 November 2020

Rosalia Nghitalesheni Joseph

CHAPTER ONE: INTRODUCTION

1.1. Orientation of the study

Malaria is a parasitic protozoan disease which is caused by the family Plasmodium parasites (Apicomplexa: Plasmodiidae) (Burke *et al.*, 2017) which are transmitted by the female anopheline mosquitoes (Beck-Johnson *et al.*, 2017). The *Anopheles gambiae* complex and *Anopheles funestus* group constitute the most efficient vectors in the world that are responsible for malaria transmission (Alemu *et al.*, 2012). Malaria is one of the leading causes of deaths in the world. About 228 million cases of malaria were reported in 2018 and 405 000 malaria deaths were reported in 2018, children younger than five years accounted for 67 % of these deaths (Zekar and Sharman, 2020). The WHO South-East Asia Region and WHO Eastern Mediterranean Region reported 5 % and 2 % of the world malaria cases, respectively (WHO, 2019). The Sub-Saharan African region has the highest share of the global malaria burden (Figure 1), with 93% malaria cases and 94% malaria deaths in 2018 (WHO, 2019). In 2018, six African countries accounted for more than half of all malaria cases worldwide: Nigeria (25 %), the Democratic Republic of the Congo (12 %), Uganda (5 %), and Côte d'Ivoire, Mozambique and Niger (4 % each) (Aju-Ameh, 2020).

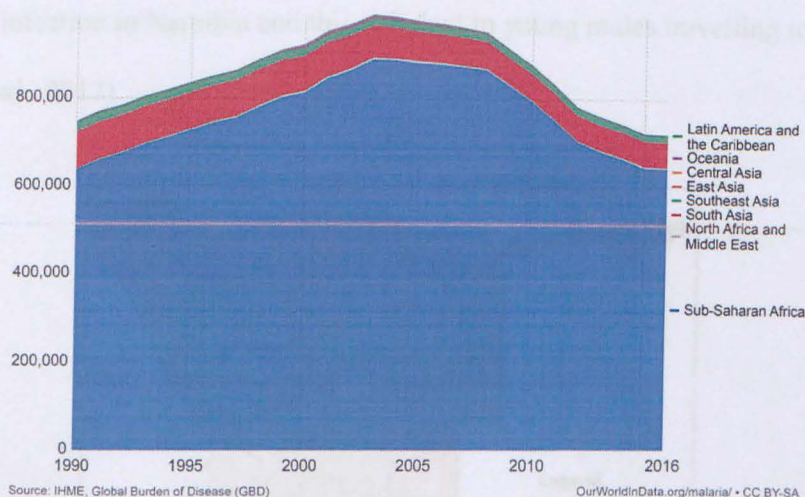


Figure 1. World malaria deaths by region (Roser and Ritchie, 2018)

In southern Africa, malaria and its associated mortality is commonly caused by the *Plasmodium falciparum* parasite transmitted by female anopheline mosquitoes (Beck-Johnson et al., 2017). In Namibia, malaria is one of the major public health challenges, nonetheless it is both avertable and treatable (Ministry of Health and Social Services (MoHSS), 2017). Namibia is considered as a low malaria transmission area and the country has three distinct epidemiological strata, Zone 1 - moderate transmission, zone 2 - low transmission and zone 3 - malaria risk free (shown in Figure 2). The regions in the southern parts of Namibia are generally free of malaria; however, there is a low-to-moderate malaria transmission risk prevailing in the nine northern regions of the country, namely: Zambezi, Kavango East, Kavango West, Kunene, Ohangwena, Oshikoto, Omusati, Oshana, and Otjozondjupa (MoHSS, 2017). Malaria occurs seasonally with periodic focal outbreaks, primarily influenced by rainfall pattern (MoHSS, 2017). Close to 70 % of the Namibian population reside in these malaria endemic regions of the country with the majority located close to the border with Angola (MoHSS, 2017). Young men aged 15-29 years are at higher risk of malaria

infection in Namibia and this is linked to young males travelling to Angola (Smith *et al.*, 2017).

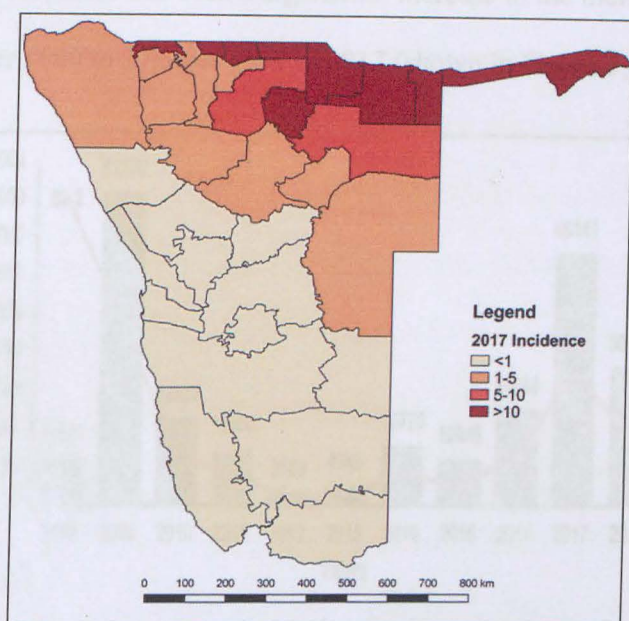


Figure 2. Map of malaria endemicity in Namibia 2017 (MoHSS, 2017)

Malaria elimination involves the implementation of four programme phases in continuum which are: control; pre-elimination; elimination; certification and prevention of reintroduction (WHO, 2016). Namibia is in the elimination phase with a scale-up of Artemisinin-Based Combinations Therapies (ACTs) treatment and indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) for prevention in vulnerable populations. As a result, Namibia was declared in 2007 as one of the four front-line southern African countries that are moving towards elimination of malaria (Auala, 2016). Therefore, in 2010 the National Vector-borne Disease Control Program (NVDCP) adopted a strategy to eliminate malaria from Namibia by the year 2022 (MoHSS, 2017).

Due to these sustained control and preventative efforts, the incidence of malaria came down to 1.4 per 1000 in 2012 from 249.7 cases per 1000 in 2002 (MoHSS, 2018). However, there has been a significant increase in the incidence of malaria from 2013 2.2 per 1000 to 27.3 per 1000 in 2017 (shown in Figure 3) (MoHSS, 2018).

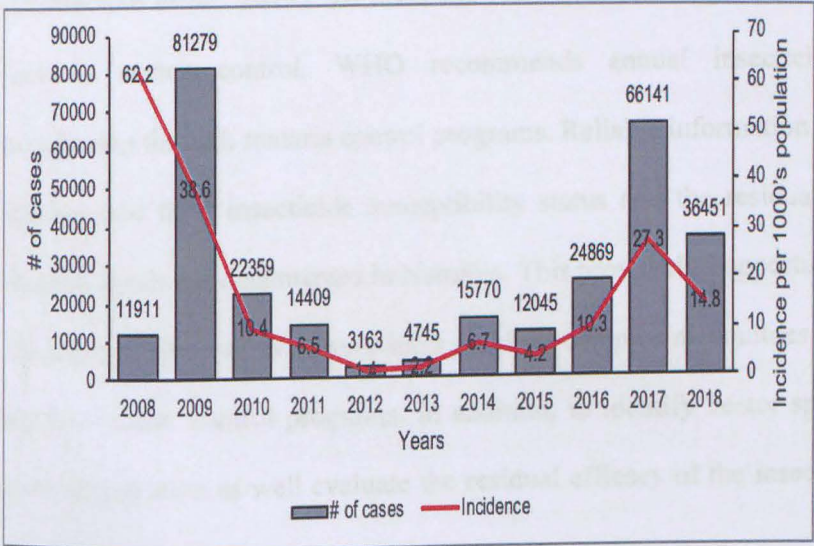


Figure 3. Malaria cases and incidence per 1000 population in Namibia from 2008-2018 (MoHSS, 2018).

This could be attributed to continuous use of ineffective pyrethroid insecticides in IRS and LLINs where there is a widespread pyrethroid resistance (IR) among *Anopheles* mosquito populations. It is possible that pyrethroid resistance has significantly reduced efficacy of IRS and standard LLINs for vector control and personal protection (Oumbouke *et al.*, 2019). To meet the resistance challenge and restore sustainable malaria vector control, WHO recommends annual insecticide susceptibility monitoring through malaria control programs. Reliable information on malaria vector species and their insecticide susceptibility status and the residual efficacy of IRS insecticides is not documented in Namibia. This provided an opportunity to investigate the susceptibility status of *Anopheles gambiae* complex mosquitoes to the insecticides used in vector control programs. In addition, to identify vector species present and their distribution as well evaluate the residual efficacy of the insecticides sprayed in houses.

1.2. Statement of the problem

Despite significant headway made, malaria remains a major public health problem in Namibia, with 66,141 malaria reported cases in 2017 (MoHSS, 2019). This could be attributed to continuous use of ineffective Pyrethroids insecticides in IRS and LLINs. Anopheline mosquito populations in sub-Sahara African countries are reported to show a resistance to Pyrethroids (Ranson and Lissenden, 2016) the only insecticide used in bed nets and IRS of modern structures in Namibia. There are currently a limited number of insecticides available on the market and it is possible that Pyrethroids resistance has reduced the efficacy of LLINs and indoor spraying as vector control strategies. The different malaria vector species present and their susceptibility to residual insecticides which guides the effectiveness of control programs is not known.

Hence, as Namibia is moving towards eliminating malaria, the effective management and prevention of malaria transmission without knowledge of anopheline species susceptibility and potency of insecticides on structures will be limited.

1.3. Objectives of the study

- a. To determine the susceptibility status of *Anopheles* mosquitoes to DDT (4 %) and Deltamethrin (0.05 %) insecticides used for vector control in northern Namibia.
- b. To identify *Anopheles* mosquitoes in northern Namibia to a species level using the Polymerase Chain Reaction (PCR) method.
- c. To evaluate the residual efficacy of insecticides, DDT, Deltamethrin, pirimiphos-methyl (Actellic 300CS), and clothianidin (SumiShield 50WG) on traditional, modern and zinc wall surfaces in Oshana region, using susceptible *Anopheles arabiensis* mosquitoes.

1.4. Hypothesis of the study

- a. Malaria vectors in northern Namibia have developed insecticide resistance against DDT and Deltamethrin insecticides used in vector control strategies.
- b. *Anopheles gambiae* complex is the most prevalent malaria vector in the malaria endemic regions.
- c. There is no significant difference in the efficacy of DDT and Deltamethrin insecticide for IRS as compared to Actellic 300CS and SumiShield WG50 insecticide.

1.5. Significance of the study

In light of the fact that there has been an observed significant increase in the incidence of malaria since 2013, accompanied by malaria epidemics occurring almost annually, vector control is the most effective malaria prevention strategy. Therefore, there is a vast need to strengthen the national capacity to be able to plan, implement, monitor and evaluate the vector control interventions in place.

Insecticide resistance development threatens the efficacy of the insecticides in use and can result in malaria outbreaks if appropriate measures are not taken. This study may provide information that will enable timely detection of insecticide resistance in order to prevent control failure by timely transitioning to an alternative class of insecticide. This information will be useful in guiding the mitigation of future resistance in malaria vectors, as well as guiding the management of IRS and LLINs.

Indoor residual spraying of insecticides is the primary vector control method in Namibia and retaining this intervention will be the most cost-effective approach. The evaluation of insecticides will inform IRS programs on the choice of insecticide to use, when to start spraying and the number of spraying cycles to ensure that the sprayed insecticide remains effective and protective to the household inhabitants. Given the limited number of insecticides available for public health use, evaluation of alternate chemicals such as SumiShield, an insecticide with a new and different mode of action will help to improve the control of resistant mosquito vectors.

Country-wide studies on the composition and distribution of the malaria vectors in Namibia has not been done. Therefore, *Anopheles* vector species present will guide malaria control programs on which respective intervention to use against the different vectors present to reduce transmission.

The results of this study may support the objective of the National Vector-borne Disease Control Programme (NVDCP) of the MoHSS in Namibia to develop an appropriate Insecticide Resistance Management (IRM) and Integrated Vector Management (IVM) plan for sustainability of vector control strategies and to that of the National Malaria Strategic Plan's (NMSP) of eliminating malaria by 2022.

1.6. Limitations of the study

The residual efficacy study presented some limitations in design and interpretation of the findings. The inadequate capacity of the insectary did not allow for a sufficient mosquito colony size of the same age range to be available for every assays in all the malaria regions. Hence, there was a wide range in the standardized age of the mosquitoes as per protocol. Furthermore, the assays were conducted in a few households in one sentinel site in Oshana region. Under natural conditions, it was a challenge to get the required number of unsprayed houses with the representative four wall types used in the experimental design. The susceptible mosquito colony is reared at the Oshakati laboratory and insectary and it was not feasible to transport the mosquito to other regions for the same test.

CHAPTER TWO: LITERATURE REVIEW

2.1. Malaria burden in Africa

Although malaria is preventable and curable, the disease remains a serious health problem in Africa. The most severe form of malaria, with 90 % of deaths attributable to it prevails in Sub-Saharan Africa as shown in figure 4 (WHO, 2018).

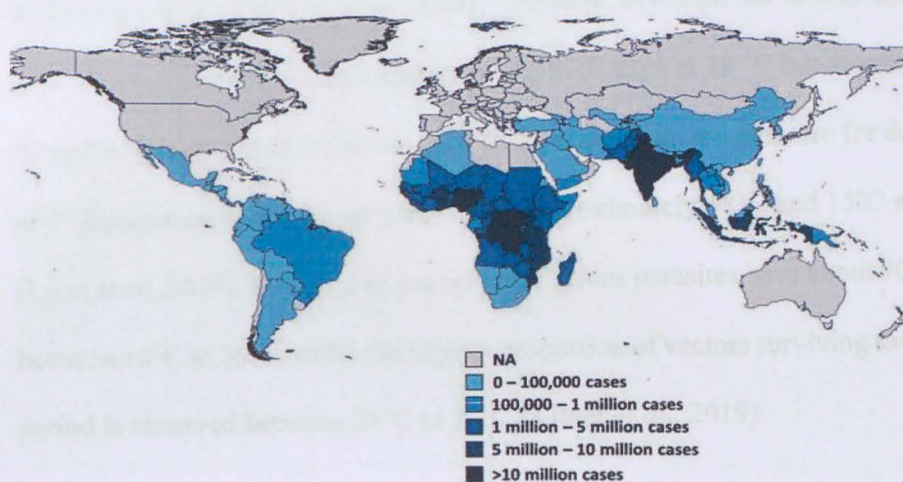


Figure 4. Map showing the estimated number of malaria cases in 2012 (Feachem, Phillips and Targett, 2009)

In high malaria risk, the most affected population groups are pregnant women, and children under the age of five years because of the loss of their maternal immunity (Oladimeji *et al.*, 2019). In regions of low malaria risk like Namibia, young males of 15–29 years that travel to Angola are at a higher risk of getting malaria (Smith *et al.*, 2017). It is likewise in Swaziland, where occupational males aged 24–44 years and often travel to Mozambique have an increased malaria risk infection rate when compared to females (Tejedor-Garavito *et al.*, 2017). This population movement may likely contribute to the importation of parasites. In stable transmission areas, people living with HIV/AIDS and international travelers from non-malaria-endemic areas who lack

immunity and returning immigrants with absent immunity are reported to be at high malaria risk (Calderaro *et al.*, 2018).

Malaria transmission in Africa is easily attributable to the climatic and ecological conditions of the continent (Kibret *et al.*, 2019). Hence weather conditions in the tropical and sub-tropical regions make it favorable for year-round malaria transmission in most parts of Africa (Liatu, 2019). Parasite development within the mosquito depends on the ambient temperature, taking 9-10 days at 28 °C but stopping at 16 °C (Shapiro, Whitehead and Thomas, 2017). The minimum temperature for development of *P. falciparum* and *P. vivax* parasites is approximately 18°C and 15°C respectively (Lyon *et al.*, 2017). Both *P. falciparum* and *P. vivax* parasites have about 90 % survival between 16°C to 36°C, while the highest proportion of vectors surviving the incubation period is observed between 28°C to 32°C (Kibret *et al.*, 2019).

Figure 3. Map of the seasonal rainfall (mm/year) in Namibia (Kibret *et al.*, 2019).

2.2. Malaria transmission in Namibia

Malaria transmission in Namibia is closely related to temperature, rainfall and humidity, with the rain season stretching from November to May (MoHSS, 2017). Malaria transmission is year round in the north-eastern regions as they are characterized by perennial rivers, average high temperatures, humidity and receive more than 500mm of rain per year (Smith *et al.*, 2017). These conditions are conducive for mosquito breeding and parasite development. However, malaria transmission is seasonal in the north-west and central regions due to a low humidity and a lack of standing water which interrupts the transmission cycle. In contrast, the coastal areas and most of the southern part of the country have unsuitable climatic conditions, therefore are believed to be free from malaria transmission (Kibret *et al.*, 2019).

The rainfall data is illustrated in Figure 5 which substantiates the malaria data illustrated in Figure 6.

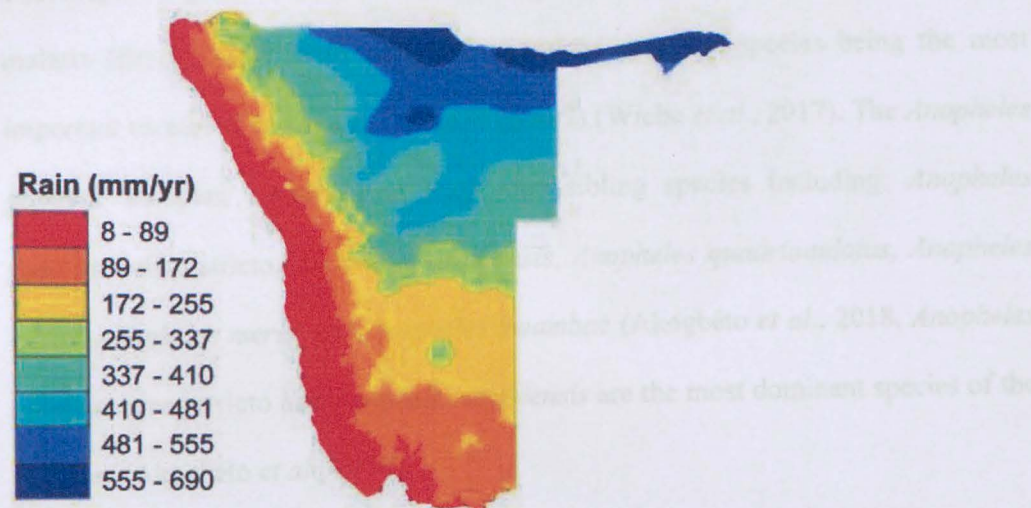


Figure 5. Map of the annual rainfall (millimetres) in Namibia (Kaseke *et al.*, 2016)

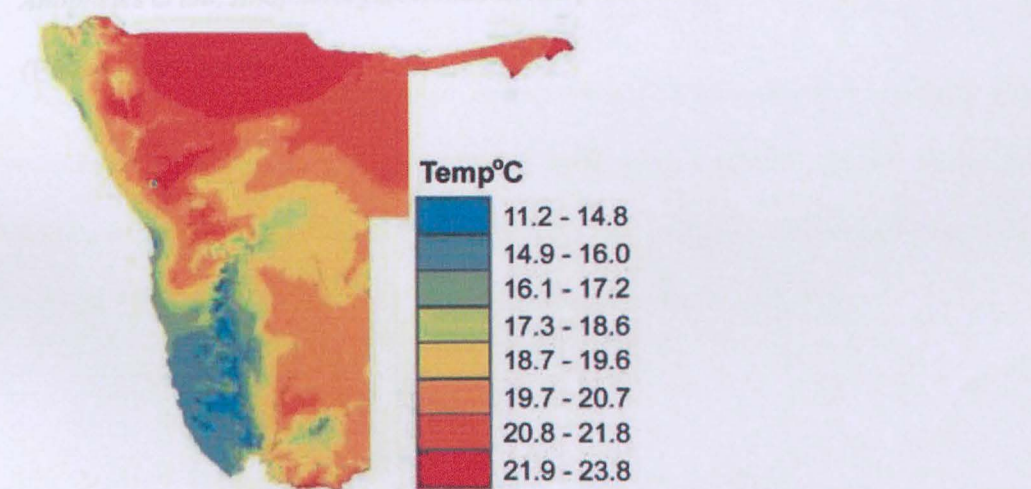


Figure 6. Map of the annual temperature (degrees Celsius) in Namibia (Kaseke *et al.*, 2016)

2.3. Malaria vectors

2.3.1 Malaria vector species and distribution

The *Anopheles* genus (Diptera: Culicidae) is the only group of mosquitoes that transmit malaria (Beier, 2017), with *Anopheles gambiae* complex species being the most important vectors of malaria in Africa (Figure 7) (Wiebe *et al.*, 2017). The *Anopheles gambiae* complex comprises at least nine sibling species including: *Anopheles gambiae* sensu stricto, *Anopheles arabiensis*, *Anopheles quadrianulatus*, *Anopheles melas*, *Anopheles merus* and *Anopheles bwambiae* (Akogbéto *et al.*, 2018). *Anopheles gambiae* sensu stricto and *Anopheles arabiensis* are the most dominant species of the complex (Akogbéto *et al.*, 2018).

The *Anopheles funestus* group comprises nine sibling species including: *Anopheles funestus* sensu stricto, the most dominant species of the group, in addition there is *Anopheles lesoni*, *Anopheles parensis*, *Anopheles vaeedeni*, *Anopheles confusus*, *Anopheles aruni*, *Anopheles fuscivenosus*, *Anopheles brucei* and *Anopheles rivulorum* (Edwin *et al.*, 2018).

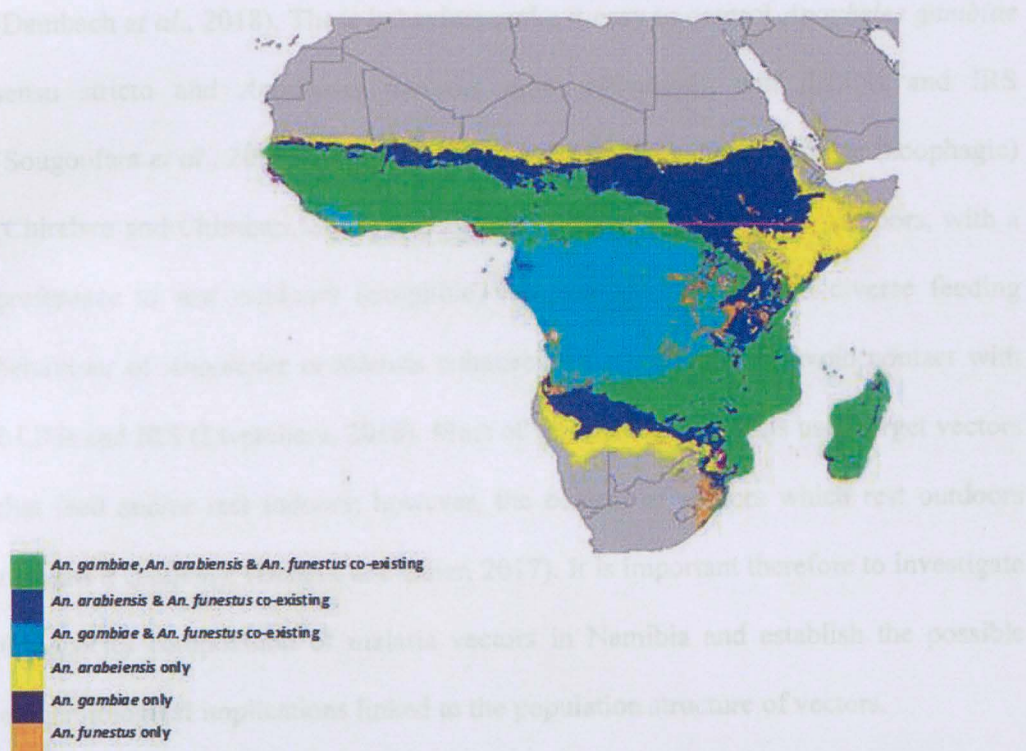


Figure 7. Map of Africa showing the distribution of the three most dominant malaria vectors in Africa (Sinka *et al.*, 2012)

The major malaria vectors *Anopheles gambiae* sensu stricto, *Anopheles funestus* and *Anopheles arabiensis*, have been recorded in Namibia (MoHSS, 2018). Historical entomological data collected prior to the introduction of IRS in 1965 demonstrated the presence of the three malaria vectors in Namibia (Chanda *et al.*, 2015).

2.3.2 Malaria vector species behaviour and breeding habitats

Malaria vectors exhibit different feeding, resting and breeding behaviors as well as host preference which affect their vectoral capacity. *Anopheles gambiae* sensu stricto and *Anopheles funestus* mosquitoes have a preference to feed (endophagic) and rest indoors (endophilic), with stronger preference to feed on humans (anthropophilic)

(Dambach *et al.*, 2018). These behaviors make it easy to control *Anopheles gambiae* sensu stricto and *Anopheles funestus* more effectively with LLINs and IRS (Sougoufara *et al.*, 2016). *Anopheles arabiensis* prefers to feed on cattle (zoophagic) (Chirebvu and Chimbari, 2016) and also bites humans indoors and outdoors, with a preference to rest outdoors (exophilic) (Dhikrullahi, 2017). The diverse feeding behaviour of *Anopheles arabiensis* enhanced its adaptability to avoid contact with LLINs and IRS (Lwetoijera, 2016). Most of the intervention tools used target vectors that feed and/or rest indoors; however, the control of vectors which rest outdoors remains a challenge (Benelli and Beier, 2017). It is important therefore to investigate the species composition of malaria vectors in Namibia and establish the possible epidemiological implications linked to the population structure of vectors.

Anopheles gambiae sensu stricto typically breed in permanent water, such as rice fields and temporary water collections such as sun lit temporary pools (Mattah *et al.*, 2017). *Anopheles funestus* also breed in permanent and semi-permanent water, as well as in swamps, slow streams and ditch edges (Mattah *et al.*, 2017). *Anopheles arabiensis* tends to breed in “Oshana”, which are a flat, low-lying area that collects water during the rainy season and dry out during drought periods (Debebe *et al.*, 2018).

2.3.3 Morphological and molecular identification of the mosquitoes

The correct identification of disease vectors is the first step towards successfully implementing an effective vector control programme. Traditionally, for malaria control, this was based on the morphological differences observed in the adults and larvae between different mosquito species (Erlank, Koekemoer and Coetzee, 2018). Light and dissecting microscopes have been used to morphologically identify specimens, along with a dichotomous key of Coetzee (2020). Morphological

identification is inexpensive, requires little technical equipment and easy to implement in the field and therefore it remains the reference method for both research purposes and operational surveillance.

Morphological identification has three principal limitations, the first being that it relies on expert entomologists performing to identify especially when many species are present in the collection area. The second limitation has to do with the level of preservation of morphological characters used in the sample handling process. The third concerns the species complexes and some morphologically similar species were identification is based on more than one developmental stage, which is not always accessible (Jourdain *et al.*, 2018).

The discovery of species complexes such as *Anopheles gambiae* complex and *Anopheles funestus* group formed the bases of genetic tools to distinguish the sibling species. The Scott *et al.*, (1993) molecular species-diagnostic PCR method is used in identification of *Anopheles gambiae* complex members, and the five most common members of the *Anopheles funestus* group which are identified by the multiplex PCR assay of Koekemoer *et al.*, (2002). Prior to molecular analysis, accurate morphological identification is important in order to correctly interpret the results to be used for vector control programme planning.

2.4. Malaria vector control

Vector control is the key intervention for global malaria control and elimination efforts (Dhiman, 2019). Control methods constitute the use of LLINs and IRS which account for almost 60 % of global investment in malaria control (Gari and Lindtjørn, 2018). LLINs and IRS can be use simultaneously and also supplement one another where one intervention is not feasible. LLINs are distributed in areas that lack sprayable structures or where the population is comprised of mobile people like nomads and cattle herders. In some specific settings, larval control is also used. Personal protection against mosquito bites represents the first line of defense for malaria prevention for individuals.

2.4.1. Indoor Residual Spraying (IRS)

IRS is the primary intervention used in malaria-endemic areas to control malaria vectors (Gari and Lindtjørn, 2018). IRS is the application of a long-lasting insecticide to a potential malaria vector resting surface, such as internal walls and ceilings of a house or structure. IRS targets endophilic malaria vectors such as *Anopheles gambiae* sensu stricto and *Anopheles funestus* group that might rest on the sprayed surfaces and pick up lethal dosage of the insecticide (Desalegn, Wegayehu and Massebo, 2018). IRS application aims to reduce vector longevity than to reduce mosquito density, for the purpose of limiting transmission (Ohm *et al.*, 2018).

The insecticide for the type of sprayable surface is applied as formulations adapted to the type of surface to be sprayed. Residual insecticides for spray application are formulated as: wettable or water-dispersible powder (WP), emulsifiable concentrate (EC), suspension concentrate (SC), water-dispersible granule (WG) and capsule suspension (CS) (WHO, 2015). The major characteristics of the different formulations

and their impact on IRS are described in Table 1 below. WP formulations are the most commonly used insecticides for IRS in rural areas on porous surfaces (e.g. mud and thatch walls) in traditional buildings. EC formulations and SC formulations are used on modern buildings for spraying impervious and painted surfaces because they do not cause spots and stains (WHO, 2015). The Namibian annual spraying cycle begins in October to December just before the onset of the rainy season. As recommended by WHO, annual IRS coverage in Namibia remained above 80 % and more since 2005, except in 2008 where the cover age dropped to 38% (MoHSS, 2017). The main classes of insecticides recommended for IRS in Namibia include organochlorines, organophosphates, carbamates and Pyrethroids (MoHSS, 2017). Selection of insecticides for IRS is guided by the susceptibility status of the local vectors and the residual activity of insecticide (Dengela *et al.*, 2018).

DDT is an Organochlorine insecticide and it was the first insecticide used for residual spraying. DDT has been used in Namibia since 1965 for IRS (MoHSS, 2017). DDT was sprayed in urban houses in northern parts of the country and with time it was sprayed in traditional structures in all malarious areas in Kavango, Omusati, Oshana, Ohangwena and Oshikoto region (MoHSS 2017). DDT binds to the voltage-gated sodium channel (VGSC) of the insect's nervous system, therefore preventing its transition from an activated to an inactivated state (Field *et al.*, 2017). DDT is known to have a residually efficacy for up to six months or more (Himeidan, Kweka and Temu, 2012).

Bendiocarb from the Carbamate class of insecticides was later introduced to replace DDT in urban areas of Namibia in 1993. Bendiocarb reversibly inhibits the acetylcholinesterase enzyme by binding to its active site. As a result, this leads to an accumulation of acetylcholine, which is required for the transmission of nerve

impulses, at nerve muscle sites (Himeidan, Kweka and Temu, 2012). Bendiocarb remains residually effective for 2–6 months (Himeidan, Kweka and Temu, 2012). Deltamethrin replaced Bendiocarb in 1995 for use in modern structures (MoHSS, 2017). From this time DDT has been used for IRS in traditional houses and Deltamethrin in modern houses respectively. Like DDT, Deltamethrin also bind to the voltage-gated sodium channel (VGSC) of the insect's nervous system, as a result preventing its transition from an active to an inactive state (Field *et al.*, 2017). Deltamethrin remains effective for 2–3 months on mud and thatch surfaces, but nine months has been reported for modern surfaces (Himeidan, Kweka and Temu, 2012). From 2018, Namibia changed from Deltamethrin to pirimiphos-methyl (Actellic 300CS) which is an organophosphate. This was necessitated by confirmed cases of insecticide resistance to Deltamethrin in some regions (MoHSS, 2017).

The usefulness of IRS in Namibia is under threat due to increasing resistance of malaria vectors to Pyrethroids and DDT, as reported all over Africa (Niang *et al.*, 2018). This gave rise for a need to evaluate alternative insecticides with a new mode of action (MOA). A neonicotinoid clothianidin, SumiShield 50WG was identified as an insecticide with good potential for use in IRS against malaria. SumiShield is a non-repellent with low mammalian toxicity when compared with Pyrethroids and DDT insecticides; this allows vectors to pick up the lethal dosage (Kweka *et al.*, 2018). SumiShield interrupts the transmission of nerve impulses by irreversibly blocking the nicotinic acetylcholine receptors (nAChR) on the postsynaptic membrane of the insect nerve cell (Ngwej *et al.*, 2019). This eventually leads to an inactive state of the vector. This is advantageous there would be presumably no cross-resistance mechanisms between neonicotinoids and the other IRS classes. SumiShield 50WG is reported to

have a residual efficacy of more than 7 months under field conditions and has proven increased mosquito mortality rates (Ngwej *et al.*, 2019).

Knowledge of residual efficacy of insecticides help inform IRS programmes on when to spray and the number of spraying cycles in areas of malaria transmission. This is done with consideration to the duration of the malaria transmission season of a specific area (Corrêa *et al.*, 2019). The residual efficacy of insecticides is evaluated by performing bioassays as per recommendations by WHO (2018), which should ideally be conducted in actual human habitations. However, factors such operational cost of perform bioassays, difficulty in accessing areas with sprayed houses and ethical considerations compromise the performance of these tests in field conditions (Corrêa *et al.*, 2019). On the other hand, laboratory performed bioassays, under controlled conditions, have demonstrated a more prolonged residual effect, which may lead to a longer interval in the spray cycles (Corrêa *et al.*, 2019). The use of a laboratory test house in place for an actual field house can minimize the differences between the laboratory and the field; it may also decrease the operational costs involved, providing a better solution to define spray cycles for IRS.

2.4.2. Long Lasting Insecticide Nets (LLINs)

The success of indoor residual spraying coupled with insecticide treated bed nets is important in the eradication of the malaria disease (Choi, Pryce and Garner, 2019). LLINs are treated with pyrethroids insecticides only such as Deltamethrin 30–50mg/m² and Permethrin, which binds around polyester fibers (Djènontin *et al.*, 2018). The net retains its biological efficacy activity for at least 20 WHO standard washes under laboratory conditions and 3 years of recommended use under field conditions (WHO, 2018). Treated nets kill and repel mosquitoes that land on them and possibly

diverse them to a non-human blood host. Treated nets also directly protect individuals under the net and reduced transmission from infected individuals to susceptible mosquitoes (Birget and Koella, 2015).

In sub-Saharan Africa, studies showed that reduced malaria incidence rates by 50% and malaria mortality rates by 55% in children under 5 years of age was attributed to the use of LLINs alone (Solomon *et al.*, 2019). Studies also show that the use of LLINs by people at risk remained lower than expected, despite a decline in malaria burden and increased LLIN access (WHO, 2017). In 2016, only 43% of people had access to sufficient LLINs (one net for two people), and only 54% of people at risk for malaria used LLINs (WHO, 2017). Most commonly reported barriers to the use of LLINs are worn out LLINs, a lack of space to hang the LLINs, living away from vector breeding sites, discomfort, and a lack of awareness and perception of low efficacy to prevent malaria (Solomon *et al.*, 2019).

LLINs have been used in northern Namibia since the mid-2000s, with over 625,000 LLINs distributed at health facilities, outreach sites, antenatal clinics and via mass campaigns to villages from 2005 to 2011 (Gueye *et al.*, 2014). Increased net coverage resulted in reduction of malaria cases and mortality between 2001 and 2011 (Gueye *et al.*, 2014). Traditionally nets should be allocated per available sleeping spaces in malaria transmission areas, however in Namibia, one net is allocated per 1.8 persons. The National Vector-borne Disease Control Programme (NVDCP) set a goal to achieve a 95 % LLIN coverage of the entire population at risk of malaria, instead of targeting only vulnerable populations (Gueye *et al.*, 2014).

Table 1. Summary of insecticides used for Long-lasting Insecticidal Nets and Indoor Residual Spraying in Namibia

Insecticide compound	Insecticide Type	Dosage (g a.i./m ²)	Mode of Action	Use	Formulation	Duration of effective action (months)
Pyrethroid	Permethrin			LLIN	Incorporated polyethylene	
Pyrethroid	Deltamethrin			LLIN	Coated polyester	
Pyrethroid	Deltamethrin	0.020-0.025	Contact	IRS	Wettable powder (WP)	3-6
Organo-chlorine	DDT	1-2	Contact	IRS	Wettable powder (WP)	>6
Organo-phosphate	Pirimiphos-methyl	1	Contact & airborne	IRS	Capsule suspension (CS)	4-6
Carbamate	Bendiocarb	0.1-0.4	Contact & airborne	IRS	Wettable powder (WP)	2-6
Carbamate	Propoxur	1-2	Contact & airborne	IRS	Wettable powder (WP)	3-6

2.4.3. Larviciding

Larviciding is making use of chemical or microbial agents such as *Bacillus thuringiensis* (Bti) and *Bacillus sphaericus* to water bodies to control mosquito larvae and pupae. This is done to reduce the number of mosquitoes that reach adulthood (Choi and Wilson, 2017). In Namibia, larviciding is done with an organophosphate chemical known as Temephos. It has a proven efficacy, environmentally safe and approved by WHOPES (MoHSS, 2014). Temephos acts by inhibiting cholinesterase and affects the central nervous system of the mosquito (dos Santos Dias *et al.*, 2017). Larval habitats between different mosquito species makes it difficult to implement larviciding successfully across a wide geographic area, this intervention is only appropriate in settings where mosquito breeding sites are easy to identify and treat (Antonio-Nkondjio *et al.*, 2018). Hence in Namibia, larviciding is carried out during the dry winter months, when the water bodies are fewer, easier to identify and larvae populations are concentrated than during the wet season (MoHSS, 2014).

Larviciding has the potential to overcome some challenges currently facing malaria vector control, such as controlling mosquitoes where LLINs and IRS coverage is insufficient to prevent malaria transmission (Choi and Wilson, 2017). In Namibia, larviciding is the core vector control intervention in Omaheke region, but it is a supplementary intervention to IRS and LLINs in the other malaria regions (MoHSS, 2014). It is also done in the event of active case detection and malaria outbreaks.

2.5. The Global Technical Strategy (GTS)

The Global Technical Strategy (GTS) provides a comprehensive framework that allows countries to develop tailored programmes for accelerating towards elimination of malaria. It emphasizes that progression towards a malaria-free status is a continuous process that involves a structuring of programmes in line with sub national stratification by malaria risk. It highlights the need to ensure that there is a universal coverage of the main malaria interventions, and proposes milestones and goals for 2020, 2025 and 2030. It also identifies regions in which innovative solutions will be essential to achieve its goals, and outlines the global financial implications that are incurred to implement the strategy. This strategy is built on three pillars with two supporting elements; ensuring universal access to malaria prevention, diagnosis and treatment, accelerating efforts towards elimination and attainment of malaria-free status and strengthening malaria surveillance.

2.5.1. Pillar 1: Ensure universal access to malaria prevention, diagnosis and treatment

Pillar one emphasizes that universal coverage is a key principle of the strategy, therefore all the main interventions such as vector control, chemoprevention, diagnostic testing and treatment, needs to be scaled up to cover all populations at risk. In order to tailor interventions to local contexts and ensure the efficient use of resources, the stratification should be based on the malaria risks. Countries are also urged to intensify efforts to prevent and manage challenges such as drug and insecticide resistance and diverse vector behavior; to remove all ineffective antimalarial medicines and substandard vector-control products from markets; to expand community-based diagnostic testing and treatment; and to enforce pre- and

post-shipment quality testing of vector control products. This is proposed to ensure the long-term effectiveness of the main interventions.

2.5.2. Pillar 2: Accelerate efforts towards elimination and attainment of malaria-free status

In addition to covering all populations at risk with the main interventions, all countries are urged to intensify efforts to eliminate malaria, especially in low transmission areas. The strategy highlights the importance of renewing political commitment and strengthening cross-border collaboration. Likewise, countries are urged to lower malaria transmission by reducing the pool of infections through transmission-blocking chemotherapy for *Plasmodium falciparum* malaria and radical cure for *Plasmodium vivax* malaria.

2.5.3. Pillar 3: Transform malaria surveillance into a core intervention

Strengthening of malaria surveillance is fundamental to programme planning and implementation and is a crucial factor for accelerating elimination. There should be a malaria surveillance system in malaria-endemic and malaria-receptive countries. In order to direct resources to the most affected populations, identify gaps in programme coverage, detect outbreaks, and assess the impact of interventions to guide changes in programme planning and implementation. The strategy also emphasizes the need for countries to strengthen malaria surveillance so that it functions as a core intervention.

2.5.4. Supporting element 1: Harnessing innovation and expanding research

In support of the three pillars, affected countries and the global malaria community need to harness innovation and expand both basic and implementation research.

Successful innovation in product development and service delivery will greatly eliminate. Basic research is important to better understand the vectors and parasite, and to develop more effective diagnostics and antimalarial medicines, and improved and innovative vector control methods. Implementation research is important to optimize impact and cost-effectiveness, and to facilitate the rapid uptake of novel tools.

2.5.5. Supporting element 2: Strengthening the enabling environment

Supporting element two emphasizes the need for strong political commitment, sustainable financing and increased multi-sectoral collaboration for further progress. To optimize national malaria responses, and enable the adoption and introduction of novel tools and strategies in a timely manner, health systems should be strengthened and improved in the enabling environment. Expanding malaria interventions can be used as an entry point for strengthening health systems, inclusive of maternal and child health and laboratory services, and to build stronger health information and surveillance systems. In addition, communities should be empowered, health workforce should be skilled and regulatory frameworks should be strengthened.

2.6. The Four Phase Elimination-Continuum

The Southern African Development Community (SADC) elimination Strategic Program had pledged that in 2007 to eliminate malaria from Southern Africa by the year 2015. Eight countries that showed greater potential to eliminate malaria were chosen, that being Namibia, Botswana, South Africa and Swaziland which were the front line countries. The second in line are: Angola, Mozambique, Zambia and

Zimbabwe. A four-phase continuum was implemented by the WHO in order for these countries to achieve a malaria free status. The four phases are shown in Figure 8 are: control; pre-elimination; elimination; certification and prevention of reintroduction (WHO, 2016a). Figure 9 shows the countries that are malaria-free, that are eliminating and controlling malaria.

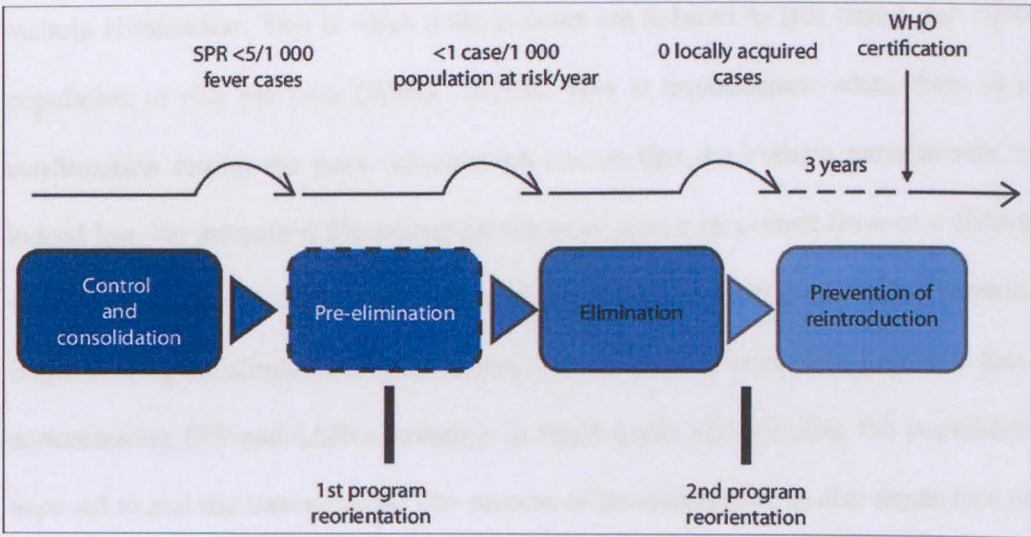


Figure 8. The WHO malaria elimination continuum (WHO, 2016a)

2.6.1. Control

The first phase of the continuum is the control of malaria. It involves the control of the malaria vector country wide with the main aim of reducing morbidity, mortality and the burden of malaria (WHO, 2016a). The main control strategies include the use of IRS and the distribution of LLINs (Russell *et al.*, 2013), prompt and effective management of cases as they come into the health facilities, use of appropriate anti-malarials for treatment and prevention in vulnerable populations (Patouillard *et al.*, 2011). This is done in order to reduce malaria cases to a level where malaria is no longer a major public health problem. Once a country is able to bring malaria incidence

down to less than 5 indigenous cases per 1000 population per year, then they are considered ready to move on to the pre-elimination phase of the continuum.

2.6.2. Pre-Elimination

Pre-elimination refers to a phase of programme reorientation from malaria control to malaria elimination. This is when malaria cases are reduced to less than 1 per 1000 population at risk per year (WHO, 2016a). This is implemented when there is a confirmation during the peak transmission season that the malaria parasite rate is indeed low, for instance $< 5\%$ among people of all ages with current fever or a history of fever in the past 2–3 days. Strategies for this phase include: preparations towards implementing an elimination programme, identifying and eliminating malaria foci, concentrating IRS and LLINs coverage in target areas and reducing the population exposed to malaria transmission. The success of pre-elimination is also dependent on intensifying inter-country and cross border collaborations between countries with varying levels of malaria transmission improving ease of access of population whether local or non-local to private and/or public health-care facilities to increase health coverage (Yangzom *et al.*, 2012).

2.6.3. Elimination

Elimination programmes may start once pre-elimination reorientation has been completed and the malaria incidence in the targeted areas is very low. Elimination is achieved when local mosquito-borne malaria transmission has been interrupted and the incidence of malaria has been reduced to zero in a defined geographical area as a result of deliberate efforts; continued measures to prevent reestablishment of transmission are required (Cohen *et al.*, 2010). Strategies aimed at achieving this,

involves the rapid identification, locating and elimination of any malaria transmission through a monitoring and surveillance strategy (Kelly *et al.*, 2012). Strengthening vector control efforts to reduce human–vector contact; improving on personal protection and environmental management methods; and further strengthening surveillance systems to one that is able to detect future malaria incidences.

2.6.4. Certification and prevention of re-introduction

Once a country has succeeded in having zero locally acquired malaria cases for three consecutive years, a request to certify its malaria free status can be sent to WHO (WHO, 2016a). Certification can only be granted by WHO after the country demonstrates surveillance and health systems that are strong to convince a skeptical observer that there is no longer any malaria transmission occurring in the country (Cohen *et al.*, 2010). Strategies are then redirected from elimination to preventing re-introduction of malaria in the area. This can be achieved by reducing onward transmission from imported cases, targeting vector control to areas of probable transmission, screening of immigrants for malaria when importation of malaria is highly suspected.

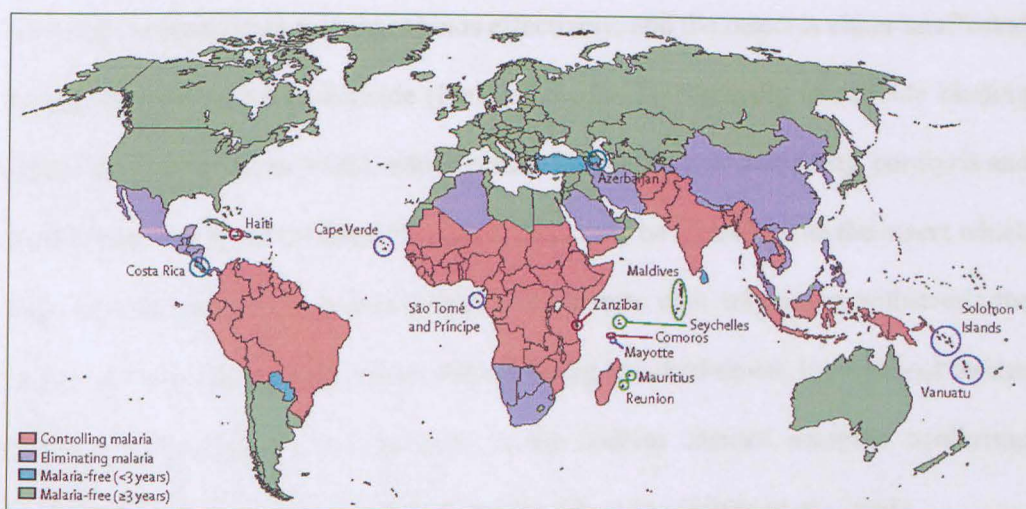


Figure 9. Categorization of countries as malaria free, eliminating malaria, or controlling malaria, 2016 (Newby *et al.*, 2016)

2.7. Insecticide resistance and mechanism

Insecticide resistance involves an alteration in one or more genes, which leads to a decrease sensitivity of the disease vector (WHO, 2016). WHO classifies mosquitoes as being insecticide resistant if the population mortality is less than 90 % 24-hours after exposure to an insecticide in standardized bioassays (WHO, 2016). This widespread resistance is particularly to pyrethroid insecticides, the only class of insecticides approved for use in LLINs (Ranson and Lissenden, 2016). Insecticide resistance is divided in two groups, which are target site and metabolic resistance mechanisms, both can occur in the same vector population or within the same individual mosquito (Dang *et al.*, 2017).

2.7.1. Target site resistance mechanism

Target site resistance to pyrethroids and organochlorines, is associated with mutations in the voltage-gated sodium channel (VGSC) of the mosquito's nerve membranes,

such that the insecticide no longer binds effectively, and the insect is either unaffected or less affected by the insecticide (Field *et al.*, 2017). Normally insecticide binding delays the closing of the VGSC which results in repetitive neuron firing, paralysis and the eventual death of the insect. However, there may be alterations in the insect which results to resistance, this is manifested when insects with *kdr* alleles withstands the prolonged exposure to insecticides without being knocked-down. In Organochlorines (DDT) and Pyrethroids, the mutation in the sodium channel receptor, conferring knockdown resistance (*kdr*), mediated by the *kdr* genes (Silver *et al.*, 2014).

The *kdr* genes have been reported in many places in Africa. Two *kdr* alleles have been described as West (1014F) and East (1014S). The 1014F mutation was first detected in *Anopheles gambiae* sensu stricto from Cote d'Ivoire against pyrethroids (Figure 10) (Edi *et al.*, 2017); then 1014S was detected in *Anopheles gambiae* sensu stricto from Kenya (Ondeto *et al.*, 2017). This led to these two mutations being referred to as *kdr*-West and *kdr*-East, although, these alleles are not restricted to either side of the continent. In *Anopheles gambiae* sensu stricto the 1014F mutation has been detected as far East as Uganda (Riveron *et al.*, 2018) and the 1014S mutation has been found in Angola (Chouaïbou, Fodjo and Djogbenou, 2017) and several countries in central Africa (Ndjemaï *et al.*, 2009). There are also several reports of 1014F/1014S hybrids in *Anopheles gambiae* sensu stricto and both 1014F and 1014S alleles have been detected in the sister taxa, *Anopheles arabiensis* (Maliti *et al.*, 2014). Resistance mutations, can also affect acetylcholinesterase (*Ace-1*), which is the molecular target of organophosphate and carbamate insecticides (Keïta *et al.*, 2020). Organophosphates and carbamates insecticides target the acetylcholinesterase (AChE) enzyme which hydrolyzes the excitatory neurotransmitter acetylcholine on the post-synaptic nerve membrane. An insect's AChE has a substrate specificity intermediate between

vertebrate AChE and butyrylcholinesterase (BChE) (Karunaratne *et al.*, 2018). Alterations in AChE in organophosphate-and carbamate-resistant insects result in a decreased sensitivity to inhibition of the enzyme by these insecticides (Alizadeh *et al.*, 2014).

2.7.2. Metabolic resistance

Metabolic resistance is mediated by elevated activities or changes in a mosquito's enzyme system that results in a sufficient proportion of the insecticide being sequestered before it reaches the target site to impair the toxicity of the insecticide (Gleave *et al.*, 2018). There are three major enzyme groups responsible for metabolic resistance to classes of insecticides. Carboxylcholinesterases (CCEs) are the primary enzymes responsible for organophosphate and carbamate metabolism in anophelines. Monooxygenases are involved in the metabolism of Pyrethroids; and glutathione S-transferase (GSTs) are responsible for DDT metabolism (Ondeto *et al.*, 2017).

The carboxylcholinesterases-based resistance mechanisms have mostly been studied in *Culex* mosquitoes, although, there is work in progress in a range of *Anopheles* species. CCEs act by rapidly binding to the insecticide and slowly turning over the insecticide; they sequester the insecticide rather than rapidly metabolize it (Nkya *et al.*, 2014). In contrast, a number of *Anopheles* species have a non-elevated esterase mechanism that confers resistance specifically to Malathion through increased rates of metabolism (Karunaratne *et al.*, 2018).

Studies have shown that insecticide-resistant insects have elevated levels of glutathione S-transferase activity in crude homogenates. GSTs are dimeric multifunctional enzymes that play a role in the detoxification of a broad range of

insecticides (Marimo, Hayeshi and Mukanganyama, 2016). GSTs confer resistance against DDT and secondarily to organophosphates by catalyzing the nucleophilic attack of reduced glutathione (GSH) on the electrophilic centers of lipophilic compounds (Panini *et al.*, 2016). GST-based DDT resistance is common in anopheline species, which could reflect the heavy use of this insecticide for malaria control over several decades. Molecular characterization of GSTs is mostly developed in *Anopheles gambiae*, although work on *Anopheles dirus* from Thailand also suggests a similar arrangement of GSTs occurs. In *Anopheles gambiae* a large number of different GSTs are elevated, some of which are class I GSTs (Panini *et al.*, 2016), these GSTs are also constitutively over-expressed. Likewise, the GST-2 of *Aedes aegypti* is over-expressed in all tissues except the ovaries of resistant insects (Panini *et al.*, 2016).

The P450 monooxygenase are commonly the rate-limiting enzyme step in the chain which leads to the detoxification of organophosphorus insecticides (Corbel and N'Guessan *et al.*, 2014). P450 enzymes bind molecular oxygen and receive electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to introduce an oxygen molecule into the substrate. Elevation in activities of monooxygenase is linked to pyrethroid resistance in *Anopheles stephensi*, *Anopheles subpictus* and *Anopheles gambiae* (Singh *et al.*, 2017). P450s in insects have so far been assigned to six families: five are insect-specific and one, CYP4, has sequence homologies with families in other organisms (Panini *et al.*, 2016).

2.7.3. Cross resistance

An added complication to insecticide resistance management arises as a result of cross-resistance between different classes of insecticides that share the same mode of action

(MOA) (WHO, 2016). Thus, vectors that are resistant to DDT because they possess the *kdr* gene will possibly be resistant to certain pyrethroid insecticides. Likewise, the *Ace-1* mutation can confer target-site resistance to both carbamate and organophosphate insecticides (WHO, 2016). Cross-resistance also occurs when insecticides of two or more classes of insecticides are metabolized by the same enzyme (WHO, 2016). Furthermore, the prevalence of multiple insecticide resistance mechanisms that co-occur in single populations as well as in individual mosquitoes is increasing in malaria affected countries, therefore, this restricts the choice of alternative insecticides in situations where insecticide resistance has been detected (WHO, 2012).

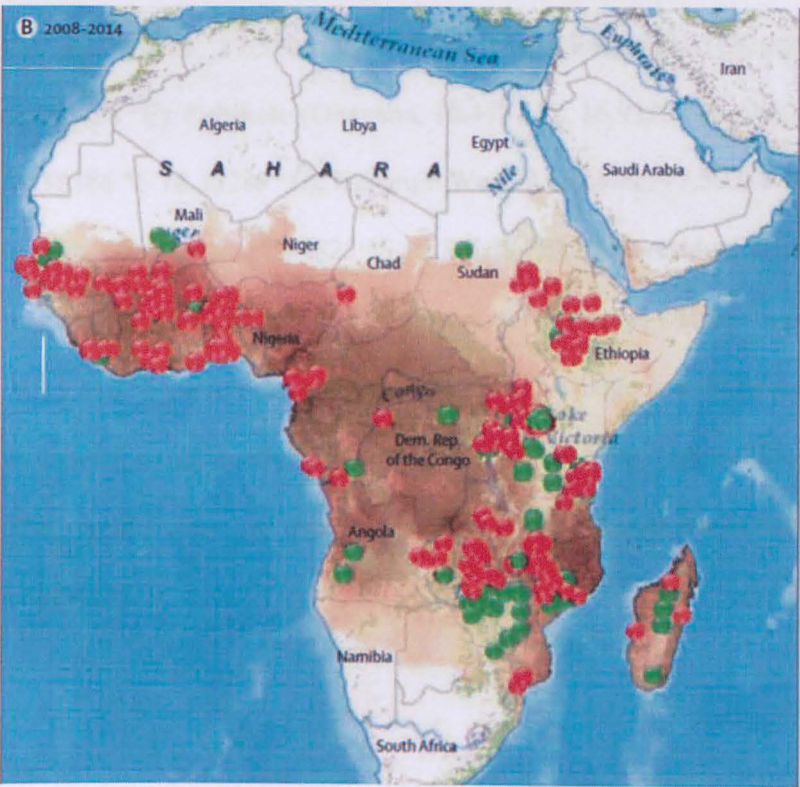


Figure 10. Map of Africa showing the distribution of pyrethroid resistance (Hemingway *et al.*, 2016)

CHAPTER THREE: MATERIALS AND METHODS

3.1. Research Design

This was a quantitative study that employed the interpretation of results from WHO tube and wall cone bioassays and molecular species-diagnostic Polymerase Chain Reaction assays.

3.1.1. Study Area

Mosquito larvae collections were conducted from April to May 2018, in seven sentinel sites located in seven malaria-endemic regions (Figure 11). The seven regions with the sentinel sites (in brackets) were: Kunene (Otjimuhaka, 17.19763 °S 13.49722 °E), Omusati (Etaka, 17.3108 °S 14.5910 °E), Ohangwena (Okanghudi, 17.44806 °S 16.112428 °E), Oshikoto (Onayena, 18.4153 °S, 16.9123 °E), Otjozondjupa (Otjituuu, 19.38386 °S 18.35289 °E), Kavango West (Mukekete, 17.50341 °S 19.10347 °E) and Oshana (Onamutai, 17.74825 °S 15.84066 °E). The residual efficacy of insecticides was conducted in June 2019 in one sentinel site only, Oshana region (Okaku, 17.480 °S 15.510 °E). Each sentinel site constitutes a village in a malaria endemic region and includes a sampling area of about 10 km radius from a central location of the village. The sentinel sites are used for routine entomological surveillance by the NVDCP (Appendix J).



Figure 11. Map of the regions in Namibia where larvae were collected for the insecticide susceptibility test; and the residual efficacy tests were conducted

3.2. Mosquito sampling and procedure

3.2.1. Susceptibility bioassays

Using WHO recommended procedures (WHO, 2018); anopheline larvae were collected towards the end of the rainy season during the period of 12 April to 5 May 2018 from the seven sentinel sites in Namibia. Larval collections included searching for mosquito larva from breeding sites within 10km radius of each of the sentinel sites in the seven malaria endemic regions of Namibia. Larval sampling was done using larval scoops dipped at the edges of breeding sites. Sampled larvae were counted on site and transferred into larvae bottle with water from the same breeding site to Oshakati laboratory and insectary.

Upon arriving at the insectary, the temperature in the larval bottles was recorded. Forms detailing larvae numbers, collection sites, type of breeding sites, and number of breeding sites and date of collection were filled in. Field collected larvae were transferred into larval rearing trays and reared into adults. Larvae were reared at temperatures of 26 - 28 °C and humidity of 70 – 80 %. The larvae were fed with a

mixture of Beeno biscuits and brewer’s yeast (3:1) and the adult mosquitoes fed with a 10 % sugar solution. Only adult mosquitoes aged 3–5 days were used for susceptibility tests using recommended WHO procedures (WHO, 2018).

Insecticide resistance testing was done using the WHO insecticide resistance test kits following the recommended procedures (WHO, 2018). A complete test included 4 replicates (each with 20 adult female mosquitoes aged 3–5 days) and two controls, therefore each trial used 80 mosquitoes. In total 1680 female mosquitoes were used for the insecticide resistance monitoring study from all seven sentinel sites. Mosquitoes were exposed to a dosage of 4 % DDT and 0.05 % Deltamethrin; 560 laboratory-reared *Anopheles arabiensis*, first generation mosquitoes were used as controls. Test mosquitoes were exposed to the insecticides for a period of 1 hour at 26⁰C–29⁰C and relative humidity range of 70 % – 80 %.

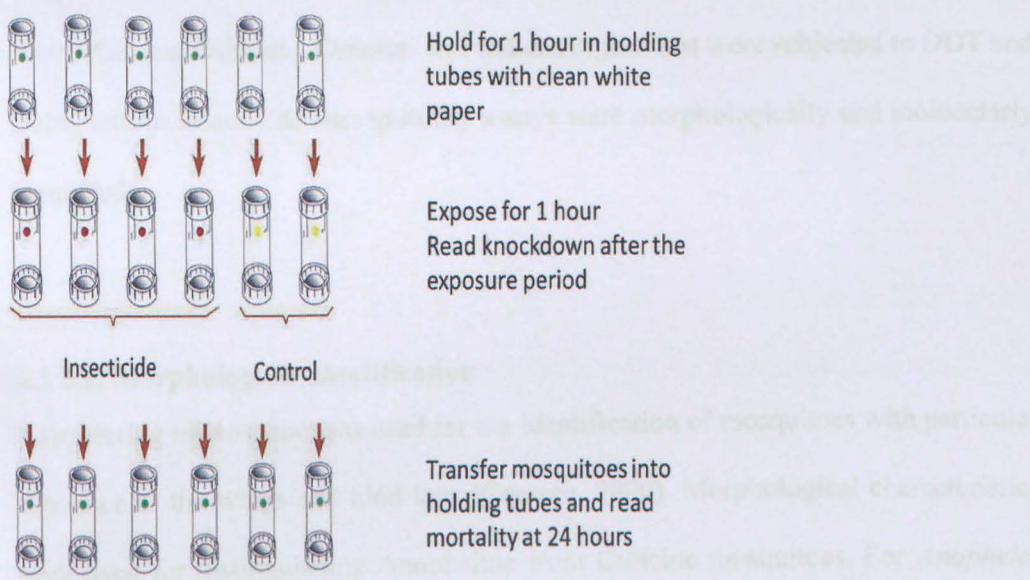


Figure 12. WHO tube bioassay test procedures

Mosquito mortality was scored after 24 hours post exposure. The observed mortality of the test sample was calculated by summing the number of dead mosquitoes across

all exposure replicates and then expressing this as a percentage of the total number of exposed mosquitoes:

Equation 1. Equation for the mortality of the test sample from the WHO insecticide resistant tube bioassays

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

After the susceptibility test, live and dead mosquitoes were separated and were preserved in Eppendorf tubes with silica until needed for molecular assays.

3.2.2. *Anopheles* species composition

Anopheles mosquitoes from breeding sites in Otjozondjupa, Ohangwena, Kavango West, Kunene, Oshikoto, Omusati and Oshana region that were subjected to DDT and Deltamethrin insecticide susceptibility assays were morphologically and molecularly identified.

3.2.2.1. Morphological identification

A dissecting microscope was used for the identification of mosquitoes with particular reference to the wings and hind legs (Coetzee, 2020). Morphological characteristics were used for distinguishing Anopheline from Culicine mosquitoes. For *Anopheles gambiae* complex, the identification focused on the mosquito's abdomen which does not have lateral projecting tufts of scales, the scaling on the abdomen was scanting and confined to the 8th tergum. The identification also focused on speckled legs with tarsi 1-4 having conspicuous pale bands on the apices. The palps were smooth or shaggy

with pale bands and the 3rd preapical dark area on vein 1 with a pale interruption, which sometimes fused with proceeding pale spots. The mosquito was also identified according to a dark spot at the upper margins of the wings which is common to the complex. The checklist for morphological identification is attached in the Appendix I.

3.2.2.2. Molecular identification

Mosquitoes were identified to subspecies level using the PCR (Polymerase Chain Reaction) method (Scott *et al.*, 1993). PCR amplification was done in 12.5µls reaction volumes which included 1.25µls of 10x PCR buffer, 1.25µls of dNTP mix, 0.5 µls of MgCl₂, 1 µls of *Anopheles gambiae* sensu stricto (GA) forward primer (5'-CTGGTTTGGTCGGCACGTTT-3'), 1 µls of *Anopheles arabiensis* (AR) forward primer (5'-AAGTGTCTTCTCCATCCTA-3'), 0.5 µls *Anopheles quadriannulatus* (QD) forward primer (5'-CAGACCAAGATGGTTAGTAT-3'), 1 µl *Anopheles merus* (ME) forward primer (5'-TGACCAACCCACTCCCTTGA-3'), 1 µls of Universal reverse primer (UN) (5'-GTGTGCCCCTTCCTCGATGT-3'), 0.1 µls of Kappa Taq, 4.9 µls of PCR quality water.

DNA was amplified by a thermo cycler through 30 cycles set at 92°C initial denaturation for 2 minutes, denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 30 seconds. Final extension was done at 72°C for 5 minutes. 5µls of the PCR products were mixed with 1µls of loading dye and loaded into agarose gel. The first lane was loaded with 1kb ladder. Separation of the PCR products was done on a 2.5 % TAE agarose gel by electrophoresis run at 100V for 30 minutes in 1x TAE buffer. DNA bands were visualized using trans-illuminator GBox (SynGene, UK) connected to a computer with software Gene Snap.

Table 2. Summary of primer names and sequences used to amplify the different *Anopheles* species (Scott *et al.*, 1993)

Primer name	Primer sequence (5' to 3')	Sizes
UN	GTG TGC CCC TTC CTC GAT GT	-
GA	CTG GTT TGG TCG GCA CGT TT	390 bp
ME	TGA CCA ACC CAC TCC CTT GA	464 bp
AR	AAG TGT CCT TCT CCA TCC TA	315 bp
QD	CAG ACC AAG ATG GTT AGT AT	153 bp

3.2.3. Residual efficacy bioassays

Insecticide susceptible *Anopheles arabiensis* mosquitoes (University of the Witwatersrand strain) were used. The mosquito colony was maintained at the Oshakati laboratory and insectary. Larvae were fed on Beeno biscuits and brewer's yeast (3:1) while adults were fed on human blood and 10 % sugar solution *ad libitum*. Temperature and humidity within the insectary were maintained between 26 – 29°C and 70 – 80 % respectively. Female nulliparous mosquitoes aged 5 – 12 days' old that have never had a blood meal were used for the cone bioassays.

This study was conducted in houses and before that consent was sought from the head of each household that was involved to allow the technician to conduct bioassays on their walls. Participating households were selected randomly according to the material used to construct walls that is: modern, zinc and traditional material. The modern

structures were made of cemented walls that were either painted while the roof was made of zinc. Walls and roofs of zinc structures were made of corrugated iron sheets while traditional structures were made of mud walls and grass thatched roofs. All study houses were located in Okaku village, Oshana region.

The residual efficacy of insecticide was measured using WHO cone bioassays which are used to assess the mortality rate of mosquitoes exposed to insecticide-treated walls following the method described in the Guidelines for efficacy testing of insecticides for indoor and outdoor ground-applied space spray applications (WHO, 2018). The two insecticides used in Namibia (DDT 750WP and Deltamethrin WP250), were evaluated against alternative insecticides, Actellic 300CS, and the novel chemical SumiShield 50WG. A limited number of structures were sprayed in December 2018 and January 2019 (Table 3), all modern and zinc structures were sprayed on 21st of December 2018 while all mud structures were sprayed on 3rd of January 2019.



Figure 13. Representative pictures of wall structures used for the WHO cone bioassays; showing examples of traditional/mud, zinc and modern/cement wall structure

Table 3. The number of structures and type of insecticides used in the residual efficacy study

Insecticides	Formulation	mg/m ²	Residual time (months)	Number of houses by type of structure		
				Traditional	Modern	Zinc
DDT	750 WP	2	6-9	1	0	0
Deltamethrin	WP 250	0.05	3-6	1	1	0
Actellic 300CS	300 CS	1	4-6	1	1	0
SumiShield 50WG	50WG	0.3	6-9	0	1	1
Control				1	1	1

World Health Organization (WHO) cone bioassays were used to measure immediate and delayed percentage mortality of susceptible *Anopheles arabiensis* mosquitoes exposed to selected study structures. Assays were conducted after approximately 5 months of spraying (5th and 6th June 2019). On a single wall surface, 3 cones were fixed at different heights using steel pins and masking tape at the lower (40cm above the ground), middle, and upper parts (40cm below the roof). 10 susceptible female mosquitoes were gently transferred into each cone and exposed for 30 minutes. In total 300 female mosquitoes were exposed.

At the end of the exposure time, the mosquitoes were transferred into insecticide-free paper cup and were supplied with 10 % sugar solution on a cotton ball. Paper cups

were kept in the insectary with appropriate temperature and humidity 26 – 29°C and 70 – 80 %, respectively. Mortality of mosquitoes previously exposed to Deltamethrin, DDT and Actellic 300CS was recorded after 24- hours while that of mosquitoes exposed to SumiShield 50WG was record at 24-hour intervals for 3 days because it is a slow acting insecticide (Dagg *et al.*, 2019). Mosquitoes were classified as dead if they were immobile.

Equation 2. The formulae for calculating the mortality of the test sample from the WHO cone bioassays

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

3.3. Data Analysis

3.3.1. Susceptibility bioassays

The WHO criteria (WHO, 2018) was used to determine susceptibility or resistance of mosquitoes after 24 hours' exposure in which a percentage mortality of $> 98 \%$ indicates susceptibility, while mortalities between $90 \% - 98 \%$ indicate possibility of resistance that requires more investigation and a mortality of $< 90 \%$ indicates resistance.

3.3.2. Anopheles species composition

Data was analysed using the R statistical software, version 3.5.2 using a Pearson's Chi-square test to determine the difference in anopheline mosquito species composition between the regions. A p-value of < 0.05 was considered significant.

3.3.3. Residual efficacy bioassays

The WHO criterion (WHO, 2018) was used to determine residual efficacy of insecticides after 24 hours in which a percentage mortality of $< 80 \%$ was a loss of residual efficacy and a mortality of $> 80 \%$ was residually effective.

3.4. Research Ethics

This study was conducted under Ministry of Health and Social Services (MoHSS), National Vector-borne Disease Control Programme (NVDCP), as part of routine entomological surveillance. The university of Namibia was supporting the ministry in operational research and daily entomological activities, therefore a research permission was not provided for this study. The study involved human volunteers, who donated blood to the susceptible *Anopheles arabiensis* colony four days weekly. This was done by inserting their arm in one or two cages with mosquitoes for 15 minutes in order for the mosquitoes to feed on their arm.

Ethical clearance was sought from the Ministry of Health and Social Services Ethics Department and the University of Namibia Health Research Ethics Committee. Informed consent forms were provided to all the blood volunteers and they were required to read and understand the content of the consent and ask questions and clarification. The volunteers were tested for malaria each Monday of the week; all the rapid diagnostic tests (RDT) for malaria were conducted by a qualified nurse from the MoHSS. The participants were also compensated for their time and effort.

Therefore, it is unlikely that any human contact with sprayed walls is harmful. All records were kept confidential and data was stored on a secure computer drive, with no personal identifiers of the study participants. Participants were identified primarily by their form serial number, personal identifiers for participants were not entered into the computerized database and no individual identities were used in any reports or publications resulting from the study. Copies of the consent forms involved in this study are attached in the Appendix D.

CHAPTER FOUR: RESULTS

4.1. Susceptibility bioassays

A total of 1680 female *Anopheles* mosquitoes were successfully reared and tested for insecticide resistance using WHO tube bioassays.

Table 4. Insecticide resistance test results using the WHO tube discriminating dose

Sentinel site	Oshana	Omusati	Ohangwena	Oshikoto	Otjozondjupa	Kunene	Kavango West
DDT (4 %)	100.0	100.00	100.00	100.00	100.00	100.00	93.75
Deltamethrin (0.05 %)	100.0 0	97.50	93.80	78.75	93.75	91.25	90.00
<u>Interpretation of results</u>							
Mortality							
≥ 98 %		No insecticide resistance					
91 to 97 %		Possible resistance					
≤ 90 %		Confirmed resistance					

The findings confirmed insecticide resistance against Deltamethrin 0.05 % in Oshikoto and Kavango West (78.75 % and 90 % respectively); while for the rest of the regions there is reduced susceptibility. There is a reduced susceptibility to DDT 4 % in Kavango West (93.75 %) region whilst the other regions showed full susceptibility (100 %) to DDT (Table 4).

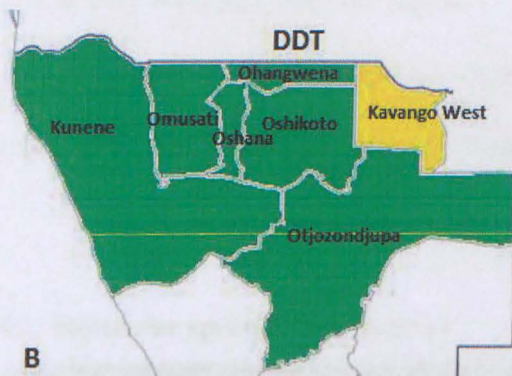


Figure 14. Map A and B shows the insecticide susceptibility of *Anopheles gambiae* complex to Deltamethrin and DDT

The maps show the distribution of the susceptibility status in malaria vectors in the seven sentinel sites. Green color indicates that mosquitoes are susceptible to the tested insecticide, yellow color shows possibility of resistance and red color indicates confirmed resistance.

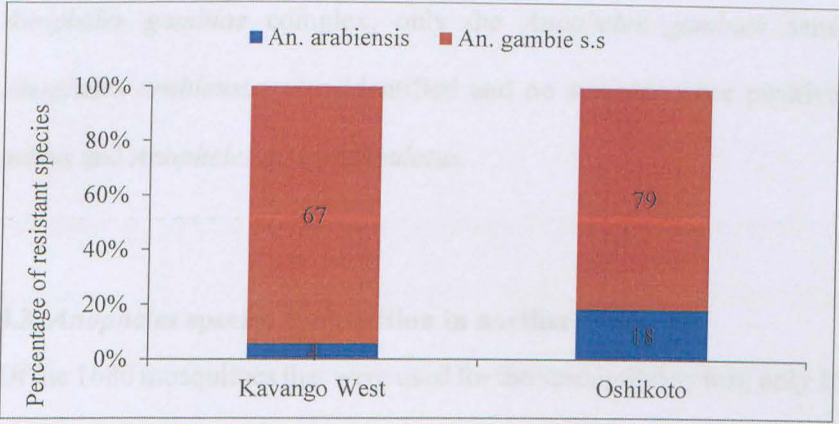


Figure 15. The proportion of live (resistant) mosquitoes after exposure to Deltamethrin in Kavango West and Oshikoto regions

Anopheles gambiae sensu stricto was found to be more resistant to Deltamethrin in both Oshikoto and Kavango West, 79 % and 67 % respectively.

4.2. Molecular species identification

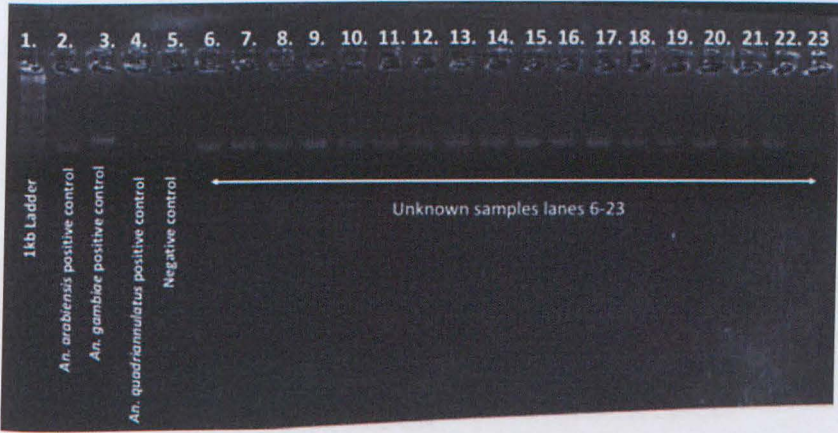


Figure 16. A gel representative showing amplified DNA bands (lanes 6-23 were confirmed as *An. arabiensis*, amplicon size x kb)

Lane 1 = 1kb Ladder; lane 2 = Positive control *Anopheles arabiensis*; lane 3 = Positive control *Anopheles gambiae* sensu stricto, lanes 4 = Positive control *Anopheles quadrimaculatus* and lanes 5 = Negative control, under the samples identified as

Anopheles gambiae complex, only the *Anopheles gambiae* sensu stricto and *Anopheles arabiensis* were identified and no samples were positive for *Anopheles merus* and *Anopheles quadriannulatus*.

4.3. *Anopheles* species composition in northern Namibia

Of the 1680 mosquitoes that were used for the susceptibility test, only 1582 mosquitoes amplified after they were subjected to molecular species identification and were all found to be members of the *Anopheles gambiae* complex, *Anopheles gambiae* sensu stricto showed higher preponderance over *Anopheles arabiensis* of which 982 (62 %) mosquitoes were *Anopheles gambiae* sensu stricto and 600 (38 %) mosquitoes were identified as *Anopheles arabiensis*.

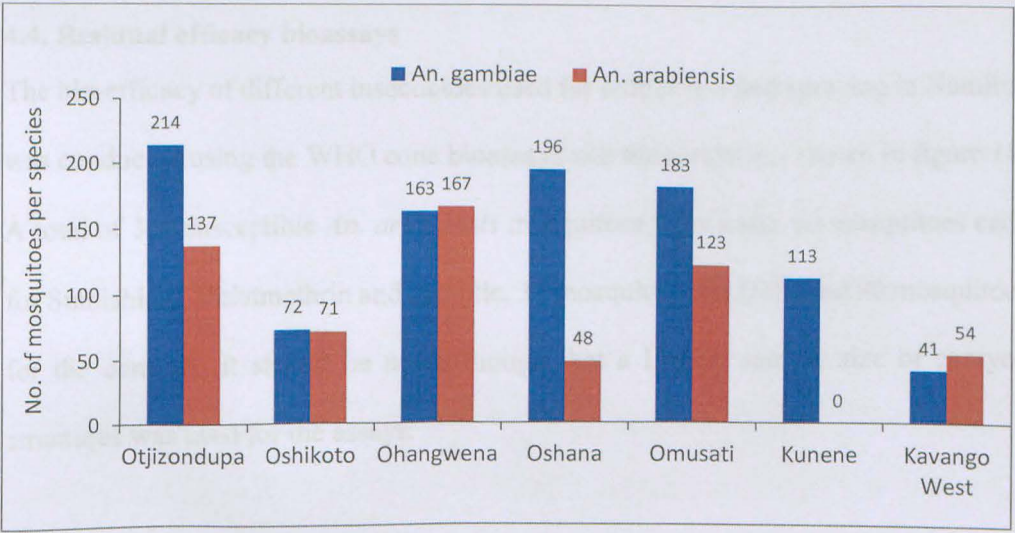


Figure 17. Species composition and distribution of *Anopheles gambiae* sensu stricto and *Anopheles arabiensis* mosquitoes across the sentinel sites

A higher number of *Anopheles gambiae* sensu stricto mosquitoes (214) were identified in Otjozondjupa than other sampled regions, followed by Oshana (196) and Omusati (183), the number of *Anopheles arabiensis* mosquitoes was highest in Ohangwena followed by Otjozondjupa and Omusati. Conversely, the numbers of *Anopheles arabiensis* (54) mosquitoes were highest in Kavango West than *Anopheles gambiae* sensu stricto (41) and no *Anopheles arabiensis* mosquitoes were identified in Kunene region. The results suggest that *Anopheles gambiae* sensu stricto is predominant in west to central north of the country and *Anopheles arabiensis* predominant is the northeast of the country. This difference was statistically significant (Pearson χ^2 , $df=6$, $P=0.001$).

4.4. Residual efficacy bioassays

The bio-efficacy of different insecticides used for indoor residual spraying in Namibia was conducted using the WHO cone bioassays and the results are shown in figure 18. A total of 300 susceptible *An. arabiensis* mosquitoes were used; 60 mosquitoes each for Sumishield, Delatmethrin and Actellic, 30 mosquitoes for DDT and 90 mosquitoes for the controls. It should be noted though that a limited sample size of sprayed structures was used for the assays.

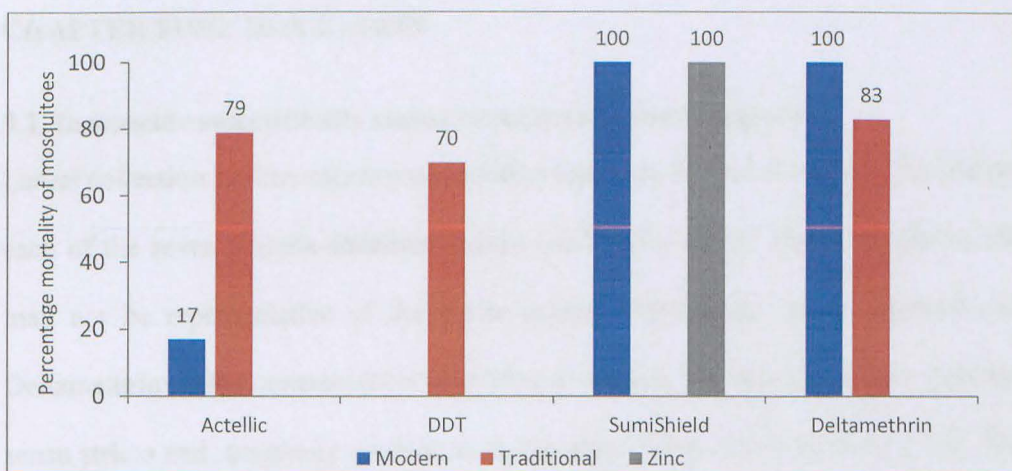


Figure 18. Residual efficacy of insecticides on different wall structures at 5 months after spraying

Five months after initial application, this study demonstrated that SumiShield 50WG induced 100 % mortality of mosquitoes exposed on both modern and zinc structures. Notably, complete mortality of mosquitoes was slightly delayed for 3 days after mosquitoes had been exposed to walls. Similarly, at 5 months, Deltamethrin WP250 effectively killed more than 80 % of the mosquitoes exposed on modern and traditional structures. Residual efficacy of Actellic 300CS (sprayed on modern and traditional) and DDT 750WP (sprayed on traditional) was below the threshold (> 80 % mortality) recommended by the World Health Organization Pesticides Evaluation Scheme (WHOPES). It should be noted though, that a limited sample size of sprayed structures was used for the WHO cone assays.

CHAPTER FIVE: DISCUSSION

5.1. Insecticide susceptibility status in malaria-endemic regions

Larval collection for insecticide susceptibility tests was limited to one sentinel site per each of the seven malaria-endemic regions used in the study. Therefore, the results may not be representative of the entire region. Nonetheless results showed that Deltamethrin of the concentration of 0.05% is too low for both *Anopheles gambiae* sensu stricto and *Anopheles arabiensis* in Kavango West and Oshikoto regions. The mortality was below the threshold (> 90 % mortality) recommended (WHO, 2018). Pyrethroid resistance was mostly found in *Anopheles gambiae* sensu stricto and a minor percentage in *Anopheles arabiensis*. The reduced susceptibility was found equally in both *Anopheles gambiae* sensu and *Anopheles arabiensis*.

These findings are consistent with reports on the resistant status of *Anopheles gambiae* complex to Pyrethroids in Gambia (Ochieng'Opondo *et al.*, 2019). Similarly, insecticide resistance has been detected in three countries neighbouring the malaria regions of Namibia, that being Angola, Zambia and Zimbabwe. Pyrethroid and DDT resistance was associated to knock down resistance (*kdr*) west (L1014F) and *kdr* east (L1014S) as well as an over expression of cytochrome P450s in *Anopheles gambiae* sensu stricto in all three countries (Silva *et al.*, 2016; Chanda *et al.*, 2011; Thomsen *et al.*, 2014). Additionally, pyrethroid resistance linked to high activity levels of P450s in *Anopheles arabiensis* was reported in Zimbabwe and South Africa (Munhenga *et al.*, 2008; Mouatcho *et al.*, 2009). Pyrethroids resistance was reported in Gokwe, Zimbabwe *Anopheles arabiensis*, linked to *kdr* (Munhenga *et al.*, 2008). Reports have linked Pyrethroids-DDT cross resistance in *Anopheles gambiae* mosquitoes to *kdr* mechanism (Matiya *et al.*, 2019), this mechanism may be conferring resistance in Namibia. In addition to target-site resistance mechanism, metabolic resistance

involving esterases and mono-oxygenases may be involved against Deltamethrin (Pyrethroid), and Glutathione S-Transferases (GSTs) against DDT (organochlorine) (Nkya *et al.*, 2013). The existence of cross- resistance and multiple resistances restricts the choice of alternative insecticides in situations where resistance has been detected (Hancock *et al.*, 2018).

The observed resistance of mosquitoes to Pyrethroids may be caused by the overuse of Pyrethroids in Namibia for malaria control, which might have possibly led to a cross resistance in DDT (Hancock *et al.*, 2018). Localized use and the massive scale up of LLINs distribution in Namibia may have inevitably increased Pyrethroid tolerance of *Anopheles gambiae* complex populations. Additionally, a lot of household products used in homes as insect repellents contain organophosphates and Pyrethroids and may be an important cause of insecticide resistance (Hancock *et al.*, 2018).

In this study, resistance to Pyrethroid in *Anopheles gambiae* complex was found in Oshikoto and Kavango West regions, which are areas with high agricultural activities. The extensive use of pesticides for agriculture in these areas might have contributed to the selective pressure on anopheline mosquitoes (Fodjo *et al.*, 2018). Some of the agricultural pesticides used contain the same chemical active ingredients like those found in insecticides used for public health purposes (Fodjo *et al.*, 2018). Hence, the impact of agricultural pesticide usage has been highly indexed as a source of resistance to insecticides (Chouaïbou *et al.*, 2016; Luc *et al.*, 2016). When mosquitoes are regularly exposed to different pesticides applied as a mosaic, it possibly leads to different responses and mutations on the mosquito vector. As a result, the mosquito vector adapts better to the environment and resistant alleles become fixed (WHO, 2016).

The situation in Namibia and bordering countries highlights the seriousness of the rapid spread of insecticide resistance in major malaria vectors in Southern Africa. Insecticide resistance threatens the dependence of vector control programmes on Pyrethroids in IRS (Sherrard-Smith *et al.*, 2018). It may pose implications to the future of malaria transmission prevention and set back elimination. The clearest example of control failure directly attributed to pyrethroid resistance and multi-resistance in major malaria vectors may have implications for the protective efficacy of IRS. Only four classes of insecticides are used for IRS and they only have two modes of action, these results in switching from DDT and pyrethroids as IRS insecticides to the Carbamates and Organophosphates insecticide classes which share a mode of action by inhibiting acetylcholinesterase.

There is currently no evidence that using LLINs will result in failure, even in areas with pyrethroid resistance. This is because even without insecticides, nets are a physical barrier between the sleeper and the mosquito vector (Dhiman, 2019). Even though malaria vectors are resistant to pyrethroids, the contact irritancy and physical barrier of provided by LLINs is likely to provide users with some protection against resistant mosquitoes seeking a blood meal. However, a larger study would be more helpful and informative to determine the impact of this resistance on the protective efficacy of LLINs. Further implication can arise, such as the reemergence of *Anopheles funestus* which had previously been eradicated in Namibia. This species may contribute significantly to the transmission of malaria in Namibia. A few studies reported insecticide resistance in male *Anopheles gambiae* sensu stricto which potentially affect it's mating competitiveness in nature (Platt *et al.*, 2015). Insecticide susceptible males are reported more likely to mate than insecticide resistant males, highlighting its limitation on the spread and persistence of resistant alleles (Matowo *et*

al., 2019). However, if such fitness advantage was present in resistant male mosquitoes, it may represent a serious challenge to any malaria control program.

The determination of vector diversity and biogeography in malaria endemic regions, and therefore, *Anopheles* mosquitoes were collected in seven malaria endemic regions. The results of this study represent a snap-shot of the population structure towards the end of the rainy season. The species composition of the adult mosquitoes revealed only the presence of *Anopheles gambiae* complex members, 38 % *Anopheles arabiensis* and 62 % *Anopheles gambiae sensu stricto*. These percentages might change throughout the course of the year, because of ecological changes and changes from the dry season to rainy season that has an impact on the vector population structure (Odiang'anya, Ferguson and Ghani, 2018).

Anopheles gambiae sensu stricto is the most abundant of the species encountered and this concurs with earlier reports. A study in Angola reported *Anopheles gambiae sensu stricto* as overwhelmingly predominant around Luanda and Namibe which is close to the northern extension of the Namib Desert (Cuamba, Choi and Townsend, 2006). A similar study in Angola reported only 3 *Anopheles arabiensis* and more *Anopheles gambiae sensu stricto* mosquitoes from the larval collection in three western localities.

This is suggestive of a low abundance of *Anopheles arabiensis* in the western parts of the country (Calzetta et al., 2008). Similarly, *Anopheles gambiae sensu stricto* was reported the most prevalent vector when compared to *Anopheles coluzzii* and *Anopheles fowleri* in Zimbabwe (Webe et al., 2017). The findings are similar to those of (Ogawa et al., 2007; Ogawa and Ikpara, 2008) who in Lagos and Kano regions, only reported that *Anopheles gambiae* complex was the predominant species.

Anopheles gambiae sensu stricto was expected to be in abundance in the wet north eastern region (Gutierrez et al., 2014), but it was common in drier northwest regions of

5.2. *Anopheles* species composition and distribution in malaria-endemic regions

A key factor in the mitigation and prevention of malaria transmission is the determination of vector diversity and bionomics in malaria endemic regions, and therefore, *Anopheles* mosquitoes were collected in seven malaria-endemic regions. The results of this study represent a snap-shot of the population structure towards the end of the rainy season. The species composition of the adult mosquitoes revealed only the presence of *Anopheles gambiae* complex members, 38 % *Anopheles arabiensis* and 62 % *Anopheles gambiae* sensu stricto. These percentages might change throughout the course of the year, because of ecological changes and changes from the dry season to rainy season that has an impact on the vector population structure (Magombedze, Ferguson and Ghani, 2018).

Anopheles gambiae sensu stricto is the most abundant of the species encountered and this concurs with earlier reports. A study in Angola reported *Anopheles gambiae* sensu stricto as overwhelmingly predominant around Luanda and Namibe which is close to the northern extension of the Namib Desert (Cuamba, Choi and Townson, 2006). A similar study in Angola reported only 3 *Anopheles arabiensis* and more *Anopheles gambiae* sensu stricto mosquitoes from the larval collection in these western localities. This is suggestive of a low abundance of *Anopheles arabiensis* in the western parts of the country (Calzetta *et al.*, 2008). Similarly, *Anopheles gambiae* sensu stricto was reported the most prevalent vector when compared to *Anopheles coluzzii* and *Anopheles funestus* in Zimbabwe (Wiebe *et al.*, 2017). The findings are similar to those of (Okwa *et al.*, 2007; Oguoma and Ikpeze, 2008) who in Lagos and Kano respectively, reported that *Anopheles gambiae* complex was the predominant species.

Anopheles gambiae sensu stricto was expected to be in abundant in the wet north eastern regions (Gueye *et al.*, 2014), but it was common in drier northwest regions of

Oshana, Omusati, Kunene and Otjozondjupa. This may suggest better fitness by *Anopheles gambiae* sensu stricto (Sinka *et al.*, 2011). Its overall preponderance is explained in light of its ability to adapt to nearly all habitats, as conferred by its genetic heterogeneity (Gimba and Idris, 2014). *Anopheles arabiensis* is known to be common in areas with lower rainfall. However, the findings of this study revealed *Anopheles arabiensis* to be abundant in north eastern regions, Kavango West and Ohangwena. This is similar to a study that reported a higher abundance of *Anopheles arabiensis* in Zambezi (Kalimbeza) and Kavango East (Andara) (Kamwi, 2005). Therefore, *Anopheles arabiensis* remains abundant in the north east of Namibia.

The re-emergence of *Anopheles gambiae* sensu stricto, the most anthropophilic and endophilic vector despite over 50 years of IRS practice in Namibia may suggest low IRS quality and coverage. The presence of these vector species highlights the importance of scaling up IRS in malaria hotspots, with 85 % or more coverage, in order to target and reduce the endophilic *Anopheles gambiae* sensu stricto. This should be coupled with LLINs to serve a barrier between the human host and the highly anthropophilic *Anopheles gambiae* sensu stricto (Sherrard-Smith *et al.*, 2019). *Anopheles arabiensis* is more exophilic and feeds on both animals and humans and can rest outdoors unlike *Anopheles gambiae* sensu stricto. Its presence increases relative outdoor biting, it is also capable of maintaining malaria transmission even at low levels (Musiime *et al.*, 2019). Therefore, it is necessary to make provision of personal protection measures and intensive larviciding to prevent outdoor-biting in addition to indoor interventions such as LLINs and IRS (Burke *et al.*, 2019).

Furthermore, this study confirms the presence of two members of the *Anopheles gambiae* complex that may account for ongoing local transmission in Namibia throughout the year, with some seasonal peaks.

5.3. Residual efficacy of insecticides towards the end of the malaria transmission season

When choosing insecticides to be used for Indoor residual spraying (IRS), considerations should be based on its proven residual effectiveness on the target vector species: the period which sprayed walls remain effective by killing at least 80 % of the mosquitoes. In the present study, the effectiveness of the two insecticide formulations, DDT 750WP and Deltamethrin WP250 were assessed, in addition SumiShield 50WG and Actellic 300CS alternatives were also evaluated. Each insecticide was only evaluated 5 months after it had been sprayed, in order to determine if it was still effective towards the end of the malaria transmission season in Namibia. Insecticide efficacy decays with time and this may be a major weakness in the results obtained in this study (Corrêa *et al.*, 2019). This study was conducted in a programmatic setting where IRS is the main vector control tool, therefore, only a limited number of sprayed structures were found for the study. The researcher had no influence on the type of insecticide sprayed, nor the type and number of structures. It should be noted that due to the low number of test structures, these results may not be generalized for decision making. Further studies should be done on a monthly basis; preferably a week after spraying is conducted to assess insecticide decay.

Nevertheless, this study demonstrated that SumiShield 50WG induced 100 % mortality of *Anopheles arabiensis* exposed on both modern and zinc structures. Similar to previous studies, complete mortality of mosquitoes was slightly delayed for 3 days after mosquitoes had been exposed to walls (Ngwej *et al.*, 2019; Agossa *et al.*, 2018). SumiShield 50WG efficacy could be attributed to its non-repellent nature unlike Pyrethroids which increases the possibility of the vector getting the peak lethal dose of the insecticide from the treated surface. The survival estimates of SumiShield 50WG on modern and zinc structures cannot clearly define the duration of spray

cycles, due to the shortage of the observations. However, with complete coverage, SumiShield 50WG may provide for a longer residual protection.

Due to short observations of the residual efficacy, it is not clear when the decay of Actellic 300CS may have started on both modern and traditional structures. Nonetheless, Actellic 300CS induced a mortality below the WHOPES threshold ($\geq 80\%$) on modern structures, while was still potent on traditional structures, when compared to DDT 750WP. The drastic drop of Actellic 300CS efficacy on modern structures by the 5th may inform IRS campaigns on when to begin spraying considering that IRS is conducted from September to end of November annually. The peak of malaria transmission in Namibia is in May and Actellic 300CS might decay by then leaving people unprotected.

This study revealed a higher estimation of residual efficacy of Deltamethrin WP250 (used on modern structures only in Namibia) on modern and traditional structures against *Anopheles arabiensis* mosquitoes. Thus, Deltamethrin WP250 may be used on these wall surfaces, in areas where malaria vectors are still susceptible to Pyrethroids, when applied in cycles every five–six months. Deltamethrin WP250 might still also provide protection against resistant mosquito populations as it may reduce their fecundity or longevity (Sherrard-Smith *et al.*, 2018).

The short-lived residual activity of DDT in this study (< 5 months) compared to standard knowledge (> 6 months) could be due to the formulation obtained sprayed on the mud substrate. Mud surfaces are very porous and as a result they fast absorb insecticides when applied on them, and they often tend to degrade the molecule of insecticides faster (Etang *et al.*, 2011; Kassem *et al.*, 2019). This sheds light on the role surfaces might play in the efficacy of insecticides, particularly in settings where

mud surfaces are widely used as building material (Radford *et al.*, 2018). Therefore, building material should be included as a risk factor in the epidemiological formula of vector-borne diseases as it influences the success of vector control.

The ideal compound for IRS would come from an entirely different class of insecticide to Pyrethroids or Organochlorines. SumiShield 50WG is a suitable alternative for Deltamethrin WP250, while Actellic 300CS is recommendable to DDT. Both SumiShield 50WG and Actellic 300CS insecticides are reported to show no cross resistance to DDT or Pyrethroids in *Anopheles* mosquitoes and could lead to reversal of Pyrethroids resistance by eliminating resistant vector populations (N'Guessan *et al.*, 2009). SumiShield 50WG and Actellic 300CS insecticides will be essential to keep vector control and malaria elimination targets on track.

CHAPTER SIX: CONCLUSION AND RECCOMENDATIONS

6.1. Conclusion

There is a prevalence of pyrethroid resistance in Namibian *Anopheles* malaria vectors, which might hinder vector management in Oshikoto and Kavango West regions delaying achievement of the goal of eliminating malaria. The study suggests that, SumiShield 50WG with a good residual activity and a different mode of action to pyrethroids may be a suitable alternative insecticide for IRS in Namibia, where resistance to Deltamethrin and DDT insecticide has started to develop. Incorporating SumiShield 50WG in the IRS strategy may reduce the selective pressure generated by pyrethroids and help preserve the continual use of LLINs in Namibia.

The results of this study provide baseline data on the malaria vector species present in northern malaria endemic regions of Namibia. This informs us on the effectiveness of vector control interventions in place and suggests that IRS should be scaled up due to high number of indoor biting and resting *An. gambiae* sensu stricto with complementary larviciding for *An. arabiensis*. The results also do highlight certain regional peculiarities, such as the unexpected predominance of *Anopheles gambiae* sensu stricto over *Anopheles arabiensis* in dry areas, which calls for further site-specific research on the bionomics of the Namibian *Anopheles gambiae* complex species.

6.2. Recommendations

As Deltamethrin efficacy has been proved to be compromised in almost all malaria endemic regions, halting Deltamethrin use in IRS campaigns will help to prevent further selection for resistance genes against the compound. It is recommended that insecticide susceptibility tests should be monitored during the peak of malaria transmission season to avoid incomplete tests due to an insufficient sample size of mosquitoes. This should be conducted in a wider sampling area, with an increase in sentinel site numbers. DDT and Deltamethrin insecticides should still be monitored for susceptibility annually to check if there is a reversal of the susceptibility status. Additionally, biochemical assays such as *kdr* test and mono-oxygenase assays should be conducted on mosquitoes to determine mechanisms conferring resistance in these mosquitoes against DDT and Pyrethroids.

Larval collection was done at one point in time and this resulted in only identifying species that exist towards the end of a rainy season and excluding species that thrive in other seasons of the year. It is therefore recommended to collect anopheline larvae at different points in time throughout the whole course of the year. This gives a better representation of the population structure throughout the year. The study should be done with a larger sample size and in more than one sentinel site of the malaria-endemic regions, to give a better representation of the country.

Furthermore, the residual efficacy study should be repeated in all malaria-endemic regions, with a sufficient and equal number of modern, zinc and traditional structures. Three structures of each wall type should be sprayed with each insecticide. Insecticides are known to decay with time, therefore this study should be conducted ideally a week after spraying to monitor when the insecticide starts to decay.

7.0. References

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8. Appendices

Appendix A: Volunteer consent form for the blood volunteers

CONSENT FORM

VOLUNTEERING FOR MOSQUITO BLOOD FEEDING

Title of activity: Volunteering to blood feed insectary reared mosquitoes

IRB: Research Division, Ministry of Health and Social Services

Principal investigators:

- Hans Angula NVDCP, 0811243435
- litula litula NVDCP, 0812191136
- Deodatus Maliti CHAI, 0814124900

Sponsor: National Vector-borne Diseases Control Program

Version of proposal: Version 1

This Informed Consent Form has two parts:

- Information Sheet (to share information about the research with you)
- Certificate of Consent (for signatures if you agree to take part)

You will be given a copy of the full Informed Consent Form

Date of issue: April, 2018

PART I: Information Sheet

Introduction

We are from the National Vector-borne Disease Control Program (NVDCP) doing research on malaria, a disease that affects many people in Namibia. We are going to give you information and invite you to be part of the research that we will explain to you. You feel free to decide whether or not you will participate in the research. Before you decide, you can talk to anyone you feel comfortable with about the research.

There may be some words that you do not understand. Please ask us to stop as we go through the information and we will take time to explain. If you have questions to ask now or later, you can ask them to Mr. Hans Angula (0811243435), Mr. litula litula (0812191136) or Dr. Deodatus Maliti (0814124900).

Purpose of the research

Malaria is transmitted by mosquito bites. Knowing the type of mosquitoes that transmit malaria, their abundance and behavior is important in controlling mosquitoes in order to prevent malaria transmission to persons. Sampling of mosquitoes that transmit malaria is important for achieving good understanding of how mosquitoes transmit malaria. These mosquitoes will be reared in the insectary for various purposes of research including determination of insecticide resistance status and bio-efficacy of insecticides used for control against malaria vectors.

Type of Research and procedures

You will be asked to volunteer in blood feeding a colony of mosquitoes which are free from malaria parasites. As part of your volunteering, you will be helping in the following:

- You will be given instruction on how to feed live mosquitoes in cages using your hand.
- The mosquitoes reared in the insectary are malaria-free and so cannot infect you with malaria parasites
- You will insert your arm into a cage containing mosquitoes to allow mosquitoes to feed on your blood for 15 minutes.
- You may be required to feed up to 4 mosquito cages a day
- You will not bring or apply any kind of insecticide on your skin or cloth during your participation in this study.
- You will not smoke cigarettes or drink alcohol 4 hours before participating.
- You will be asked not to apply some kinds of lotion or soaps that repel mosquitoes, including any commercial or traditional mosquito repellents when participating in the feeding activity.
- You will need to take malaria test every week for all the period you will be participating in the study and sign a form to show that you have taken the test. The test will be paid for by the study.
- If you become sick we will provide you with the appropriate medical care and you will not participate in the study during this time. Medical costs will be paid by the study.
- You can leave the study at any time as you wish and you will not be obliged to state why you leave – it is your choice to take part.
- If you choose to participate in the study, you will have to comply with the instructions given by the researcher; otherwise the researcher will exclude you from the study.

Voluntary Participation

Your participation in this study is completely upon your wish. You can choose to participate or not to participate. You can withdraw from the study any time you want. When you leave the study, your payment for participation including any other benefits such as clinical care will cease.

Risks

When you participate in feeding mosquitoes, you may encounter body reactions such as skin rashes, swelling or allergic reactions. While these reactions are common, you will take rest when such effects are beyond regular encounters and we will seek medical help for you in case these side effects are high and need to be treated in hospital.

Benefits

If you participate in this study you will be paid **NS\$200** for your work each day you participate in feeding mosquitoes. This amount will be summed and paid at the end of the month. You will be screened for malaria every week during your entire participation in this study and will be treated with appropriate anti-malarial in case you are found positive.

Right to Refuse or Withdraw

This is a reconfirmation that participation is voluntary and includes the right to withdraw. You don't have to participate in this study if you do not wish to do so. You may stop participating in the research at any time you choose and all your rights will be still be respected. Payment for your participation will cease when you withdraw and you will be paid only for the days you participated.

Who to Contact

You will have contacts of persons in charge of this study to communicate any information or ask questions before, during and after the study. These persons are: Mr. Hans Angula (0811243435), Mr. Jitula Jitula (0812191136) or Dr. Deodatus Maliti (0814124900).

You can ask any more questions about any part of the research study, if you wish to. Do you have any questions? Please do not hesitate to ask.

PART II: Certificate of Consent

I, clearly understand the aims of the project entitled "*Volunteering for mosquito blood feeding*", and I agree to participate in the study. During my participation in this study, I understand that mosquitoes can bite me and they may cause allergic reactions on my body and that I need to take malaria test every week. I understand that I may revoke my consent and leave the study at any stage.

Participant Name: _____

Participant Signature: _____

_____ Date _____ (dd/mm/yyyy)

Witness Name: _____

Witness Signature: _____ Date _____ (dd/mm/yyyy)

Appendix B: Form of basic consent form for potential research subjects

If participant is illiterate

A literate witness must sign (if possible, this person should be selected by the participant and should have no connection to the research team). Participants who are illiterate should include their thumb-print as well.

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent to participate in the said study freely.

Name of witness _____

Signature of witness _____

Date _____ (dd/mm/yyyy)

Thumb of the participant

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

1. Participant has been requested to refrain from smoking, consuming alcohol and applying insecticides, repellents or such substances on his/her body for the study duration
2. Participant will be provided with malaria screening and treatment for the duration of the study
3. Participant will be paid **NS\$200/day (counted each day a volunteer feeds mosquitoes)** for the duration of enrollment to the study.

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this this form has been provided to the participant.

Name of Researcher/person taking consent _____

Signature of Researcher /person taking the consent _____

Date _____ (dd/mm/yyyy)

Appendix B: Head of house consent form for residual efficacy assays

Volunteer Consent Form for Heads of Households

Volunteer Consent Form for Heads of Households

Study Title: Evaluating efficacy and residual activity of indoor residual spraying insecticides on traditional and modern house wall surfaces in Namibia

Investigators: Namibia Ministry of Health and Social Services/National Vector-Borne Disease Control Programme,

Participating Institutions: University of Namibia (UNAM), Clinton Health Access Initiative (CHAI), University of California- San Francisco (UCSF)

Funding Institution: Bill and Melinda Gates Foundation

Purpose of Consent Form: The purpose of this consent form is to record your voluntary participation in this study.

Purpose of the Study: You are being asked to allow your house to be selected for monitoring efficacy of insecticides sprayed on walls of houses to protect people against malaria. The overall goal of the study is to determine how long the stay on the wall and how long this is able to kill mosquitoes. This study will provide the Ministry of Health with information that will be used to determine which insecticides to spray on the different types of walls and how often to spray.

Eligibility: Selected houses will be those with walls made of traditional or modern material. The houses should not have smoke at the time of conducting experiments.

Duration: The study will be conducted for a period of 9 months. Study technicians will conduct tests on walls of houses once every month.

Study procedure

If you accept,

- Your house will be sprayed by safe insecticides that are approved for indoor residual spraying in peoples' houses. The insecticides include (Dichlorodiphenyltrichloroethane) DDT, Deltamethrin, SumiShield and Actellic. Once sprayed the houses will be left to dry for a period of one month.
- Study technicians will visit your house once a month to conduct tests on the walls.
- They will use laboratory reared disease free mosquitoes. Mosquitoes will be held in cages and therefore they will not be left to fly freely in your house.
- The tests will be conducted for approximately one hour each day between 0900 and 1100 hours.

Volunteer Consent Form for Heads of Households

- The areas selected on walls for tests will be marked by temporary markers to allow the technicians to identify test location each month.

Risks and Discomfort: You and your household will not be exposed to any risk. However, you may experience minimal disturbance when the technicians are conducting tests.

Compensation: You will not be compensated for participating in the study.

Medical Care for Study Related Injury: If you are injured as a result of taking part in this study, you will be given medical care at no cost to you for that injury. You will not receive any money for any injury compensation, only medical care.

Right to refuse or withdraw: You have the right to withdraw from the study or discontinue your participation at any time without penalty or loss of benefits to which you are entitled. If you choose to withdraw, you will be instructed to notify the field liaisons or the study Coordinator of the impending withdrawal.

Termination of your participation: In case new information pertaining to the study arises, you will be informed immediately by one of the study coordinators or their staff.

Confidentiality: All information associated with this study will remain confidential to the extent possible. Data will be stored for 3 years upon study completion (2018-2020). The results of this study will be provided to Ministry of Health, CHAI, UCSF, and other relevant partners. Research & clinical information relating to you may be shared with other researchers and the scientific community through lectures or publications. You will not be identified by name.

Questions: If you have any questions about your rights as a participant please contact the Study coordinator: Iitula S. Iitula: iitulaititula@yahoo.com , NVDCP, phone number..... The Study coordinator will answer questions regarding study and injury, and the IRBs (.....) will address your concerns/questions regarding your rights.

You can contact the Secretary of the/National Ethics Review Committee, Address: (Telephone numbers:).

NOTE: IF THERE IS ANY PORTION OF THIS CONSENT EXPLANATION SHEET THAT YOU DO NOT UNDERSTAND, PLEASE ASK THE INVESTIGATORS BEFORE SIGNING.

Consent: BY SIGNING BELOW, YOU AGREE THAT YOU HAVE READ THE INFORMATION PROVIDED ABOVE. YOU ALSO AGREE THAT YOU HAVE BEEN GIVEN AN OPPORTUNITY TO ASK QUESTIONS AND YOUR QUESTIONS HAVE BEEN ANSWERED TO YOUR SATISFACTION.

YOU WILL BE GIVEN A COPY OF THIS FORM.

Appendix C: Larval Collection form

[illegible]

Appendix D: Larval transport standard operating procedures

STANDARD OPERATING PROCEDURES NATIONAL VECTOR-BORNE DISEASES CONTROL PROGRAM (NVDCP)	Ref: SOP LARVAE-SURVEY-SAMPLING
	Version : 1
	Date : 15/02/2019

Purpose

This Standard Operating Procedure outlines the procedures to be followed for ground transportation of live mosquito larvae to insectary in order to:

- To test for insecticide susceptibility in malaria vectors
- To establish a wild colony of mosquitoes in the insectary

2. Scope

This protocol should be used in entomological surveillance settings when transporting live mosquito larvae to insectary or laboratory. This document may be used during routine surveillance and operational research. It is of vital importance that this procedure be followed appropriately. New staff needs to be trained and deemed competent after being assessed by a qualified NVDCP officer before performing this procedure independently.

3. Principle

Entomological surveillance is an important part of vector control activities. This may involve sampling of malaria vectors at adult or aquatic stages and transport of the specimen to laboratory or insectary for various tests. For establishment of mosquito colonies from wild mosquitoes, it is essential that live larvae collected from a site arrive at the insectary in a good condition that will enable rearing the larvae into adults. Also live and health larvae may be needed for laboratory tests which are carried out away from the site of collection. This document explains essential steps and precautions for successful live mosquito larvae transportation using ground equipment.

4. Responsible Personnel

- a. EHPs
- b. Licenced drivers
- c. Laboratory/insectary technicians
- d. Laboratory/insectary managers
- e. Entomologists

STANDARD OPERATING PROCEDURES NATIONAL VECTOR-BORNE DISEASES CONTROL PROGRAM (NVDCP)	Ref: SOP LARVAE-SURVEY-SAMPLING
	Version : 1
	Date : 15/02/2019

5. Equipment for larval survey

The equipment required for larval collections is dependent on the method of collection. The most common equipment and materials include:

- a) Cooler box
- b) Clothing or packaging material
- c) Ice slabs
- d) Vials or jars with caps
- e) Thermometer
- f) Marker, pen and pencil for labeling and filling forms
- g) Standard data recording forms/sheets and field notebooks

6. Suitable time for larvae collection

Larvae should be collected during the morning hours and transported to the insectary on the same day. Where it is impossible to collect larvae in the morning and transport them on the same day, an evening collection is done, larvae are kept overnight and transported on the next day to the insectary. In any case larvae should not spend more than 24 hours in the vials before reaching the insectary.

7. Procedures

Before transportation

- a. The vials/jars with live larvae should be filled only up to a half leaving air column for the larvae to breath
- b. Pack the vials/jars with live larvae into the cooler box keeping the vials/jars in upright position
- c. Make sure a packaging cloth or material is included at the bottom of the cooler box
- d. Place the ice slab into the box making sure it does not touch the vials or the thermometer

STANDARD OPERATING PROCEDURES NATIONAL VECTOR-BORNE DISEASES CONTROL PROGRAM (NVDCP)	Ref: SOP LARVAE-SURVEY-SAMPLING
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- e. Place a thermometer into the cooler box ensuring no contact between thermometer and the ice slab. This can be done using separating clothing materials
- f. Ice slabs should be prepared a night before the transportation day by freezing the slabs at -20C

During transportation

- a. Place the cooler box either in the front or second cabing. The cooler box with larvae should not be placed in the back cabin to avoid too much agitation.
- b. Driving to the insectary/lab should be careful to avoid bumping and agitation of the larvae
- c. After every 1-2 hours the driver must stop to check and record the temperature on the sheet provided. Also should record any other annomally as on **Appendix A** form.
- d. The driver/technician should open the vials or jars containing the larvae after every 1-2 hours to alllow fresh air to rush into the vials
- e. Make sure temperature within the cooler box is not less than 18C and does not exceed 30C.

At the insectary/lab

- a. On arrival to the insectary contact the receiving person (insectary manager/technician)
- b. Handle the cooler box with the larvae to the designated person in the lab/insectary
- c. Record temperature of the cooler box on the laboratory forms provided
- d. Fill in the sample receiving form which will be given to you by the insectary/lab personnel
- e. Repack the cooler box with the equipment before leaving the lab

8. References

WHO Test procedures for insecticide resistance monitoring in malaria vector mosquitoes – 2nd ed., 2018.

STANDARD OPERATING PROCEDURES NATIONAL VECTOR-BORNE DISEASES CONTROL PROGRAM (NVDCP)	Ref: SOP LARVAE-SURVEY-SAMPLING
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9. Appendix A: Timesheet of sample maintenance activities during ground transport of larvae to the laboratory

Driver's Name: _____ Date of Departure: __/__/__ at __:__ AM/PM Date of Arrival: __/__/__ at __:__ AM/PM

Activity 1	Activity 2	Activity 3	Time of check	Location of check	Notes/Incidents	Signature
Replenish air in vials	Check on placement of coolers	Temperature of water in vials	Indicate all incidents and any other relevant observations in the Notes/Incidents section of this timesheet during execution of maintenance activities.			
Done	Done	27.5 °C	2:37pm	Rundu town	Temp reached 30°C at Enhana, Vials bumped at Ondagwa	Johannes

Date Samples Received in Lab: __/__/__ Name and signature (Lab Member Receiving Samples): _____

Appendix B: SOP Attestation form

Attestation Record (general staff SOP Review-At least annually)		
{Please ensure that you have read and understood before signing}		
Names	Signature	Date

Appendix E: WHO tube bioassay mortality data form

INSECTICIDE RESISTANCE MONITORING FORM

Form Row(FR)	Project name/ID:	Form Serial Number(SRN):									
1	Date:	Insecticide tested:									
2	Sentinel site ID:	Village code/ID:						Taxon:			
3		Replicate 1	Replicate 2	Replicate 3	Replicate 4	Control 1	Control 2	Temp(°C)	RH(%)		
4	No. exposed										
5	Number of knock down mosquitoes after exposure period										
6	Start										
7	15 mins										
8	30 mins										
9	45 mins										
10	End										
11	Number of dead and alive mosquitoes at the end of holding period (24hours)										
13*Dead	No. dead										
14*Alive	No. alive										
15	Observed mortality(%)										
16	Corrected mortality(%)										
17*Control	Control mortality [average of control1 & control2 mortalities(%)]										
18	Final results (average mortalities of all replicates, observed or corrected mortality)										
19	Observed mortality(%)										
20	Corrected mortality(%)										
21	<i>*These counts need to be by species once dead and alive mosquitoes have been examined for taxonomic determination</i>										
22	To be completed by technician/supervisor at the end of the test										
23	Name/code of technician/supervisor										
24	Comments										
25											
26	I confirm that the test was										
27	completed	Name				Signature					
28	To be completed by data entry clerk during data entry										
29	Name										
30	Signature					Date					

*Row 13=number of dead mosquitoes, 14=number alive, 17=number in the control. These numbers should appear in the Eppendorf tubes for storage records

Appendix F: WHO cone bioassay mortality data form

Date		Village		House ID		GPS Coordinate	
Mosquito species				Insecticide*			
Source of mosquitoes							
Wall type ^s	Replicate	Wall height	Time after exposure (days)	No. Exposed	No. Live	No. Dead	
		Low	1				
		Low	2				
		Low	3				
		Low	4				
		Low	5				
		Low	6				
		Low	7				
		Medium	1				
		Medium	2				
		Medium	3				
		Medium	4				
		Medium	5				
		Medium	6				
		Medium	7				
		High	1				
		High	2				
		High	3				
		High	4				
		High	5				
		High	6				
		High	7				

Appendix G: Morphological species identification key

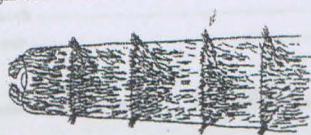
KEY TO THE FAMILIES

The present key is essentially as presented in Gillies and De Meillon (1968) with the addition of a small number of newly described species. The main difference is the provision of figures to illustrate diagnostic points, so that the key can be used by the beginner. The illustrations for the first half of each couplet are on the left and for the second half on the right. The species, *ethiopia* and *speciosa* are included under the first half of each couplet.

Abdominal segments with bristles projecting from the sides

Abdominal segments II-VII

Abdominal segments VIII-X



2. Hind tarsus with at least last 2 segments entirely paleSection II
 — Hind tarsus not so3



3. Hind tarsus 5 mainly or entirely dark, tarsus 4 whiteSection III
 — Hind tarsus not so4



4. Legs speckled, sometimes sparselySection IV
 — Legs not speckled5



5. Wing entirely dark or with pale spots confined to costa and vein 1Section V
 — Wing not so6



6. Wing without a pale spot on basal half of costa Section VI
 — Wing with at least 1 pale spot on basal half of costa 7



7. Palps with apex dark Section VII
 — Palps with apex pale 8



8. Palps with 4 pale bands Section VIII
 — Palps with 3 pale bands 9



9. Wing with pale interruption on 3rd main dark area of vein 1, sometimes fused with preceding pale area Section IX
 — 3rd main dark area with no pale interruption 10



10. Wing with 2 pale spots on vein 5.1 Section X
 — Wing with 1 pale spot on vein 5.1 Section XI



SECTION I. Mosquitoes with laterally projecting tufts of abdominal scales.

1. Wing almost entirely dark, costa without any pale spots *brumpti*
 — Wing with abundant pale areas, costa with at least 4 pale spots 2



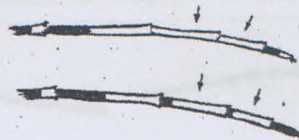
2. Hind tarsus 1-5 entirely dark *argenteolobatus*
 — Hind tarsus 1-4, at least, with apical pale bands 3



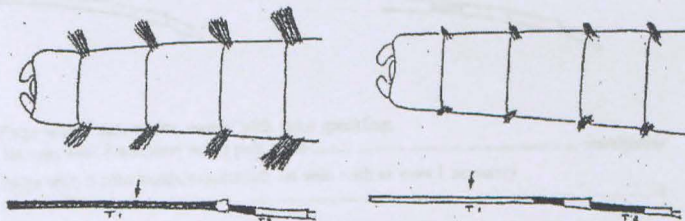
3. Hind tarsus 1 and 2 with definite light and dark rings in addition to apical pale band *cinctus*
 — Hind tarsus 1 and 2 with pale bands at apices only 4



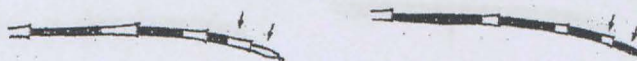
4. Hind tarsus 3 and 4 all white or narrowly dark basally, 5 all dark or at least basal half dark 5
 — Hind tarsus not so 6



5. Very large species, abdominal segments 1-7 with long lateral tufts of yellowish and dark scales; hind tarsus 1 largely dark *implexus*
 — Moderate sized species; abdominal scale tufts short and dark; half or more of hind tarsus 1 pale *crispipalpis*



6. Hind tarsus 5 and about apical half of 4 pale *pharoensis*
 — Hind tarsus 5 all dark and 4 with much less than apical half pale 7



Small to moderate species upper brown first vein either

entirely dark apart from apex or with a few scattered
pale scales only



SECTION II. Mosquitoes with hind tarsal segments 4 and 5 entirely white; abdomen without laterally projecting tufts of scales.

1. Legs speckled 2
— Legs not speckled 3



2. Hind tarsus 3-5 entirely pale 3
— Hind tarsus 3 dark at base 5



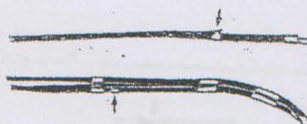
3. Palps with 3 pale bands, usually with some speckling;
1st vein with 2 accessory sector pale spots *maculipalpis*
— Palps with 4 pale bands, unspeckled; 1st vein with at most 1 accessory
sector pale spot 4



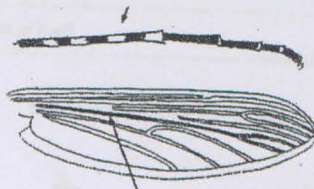
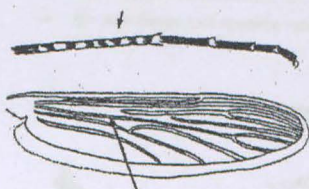
- Mid tarsus 2-4 entirely dark; vein 1 dark at base, basal half of stem of vein 4 with small pale stem *disjunctus*
 Mid tarsus 2-4 with pale apices; vein 1 pale at base, basal half of stem of vein 4 entirely pale *disjunctus*



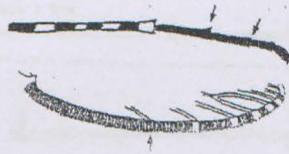
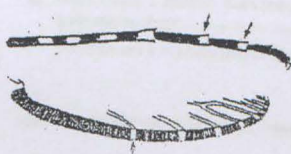
5. Hind tarsus 1 broadly pale at apex; 1st vein with 2 accessory sector pale spots *pretoriusis*
 — Hind tarsus 1 narrowly pale or dark at apex; 1st vein with 1 accessory sector spot 6



6. Fore tarsus 1 with 5-9 pale rings; stem of 4th vein largely pale *macharayi*
 — Fore tarsus 1 with 2-4 pale rings; stem of 4th vein largely dark 7



7. Fore and mid tarsus 2 and 3 pale-ringed apically; 6th fringe spot present on wing *natalensis* (in part)
 — Fore and mid tarsus 2 and 3 dark apically; 6th fringe spot absent *buxtoni*



2. Palps very shaggy, not intended as help 8
 irregular narrow pale bands 8
 Palps smooth with 3 pale bands, the 2 outer ones broad or rarely fused 12



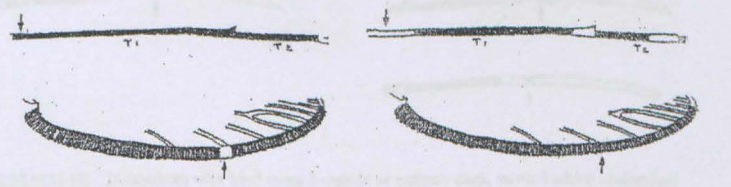
3. Palps without pale bands, or pale spot at apex of hind tibia or base of tarsus 1 11
 Palps with 1-4 pale bands, apex of hind tibia broadly or narrowly pale 12



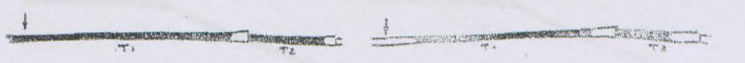
10. Hind tarsus 3 entirely pale 11
 — Hind tarsus 3 dark at base 12



11. Pale fringe spot present opposite vein 5.2; base of hind tarsus 1 dark *paludis*
 — No pale fringe spot opposite vein 5.2; base of hind tarsus 1 broadly pale *constant* (in part)



12. Hind tarsus 1 entirely dark basally or with only a few pale scales there *tenebrosus* (in part)
 — Hind tarsus 1 broadly pale at base 13



14. Hind tibia with a pale streak as long as 1/3 of the length of the segment; on hind tarsus 2 apical band to two-fifths the length of segment *rufipes* (in part)
- Pale streak on tibia 1/3 the length of the segment; on hind tarsus 2 apical band to two-fifths the length of segment *theileri* (in part)

14. 3rd main dark area (preapical dark spot) on vein 1 without a pale interruption; fore tarsus 1-3 without distinct apical pale bands *rufipes* (in part)
- 3rd main dark area on vein 1 with a pale interruption, or with a short extension of the subcostal pale spot into the dark area on vein 1; fore tarsus 1-3 with apical pale bands 15



15. Hind tarsus 3 entirely pale *hancocki*
- Hind tarsus 3 not so *brohieri* (in part)
- *brohieri* (in part) W. Africa
- *theileri* mainly E. and S. Africa



SECTION III. Mosquitoes with hind tarsus 5 mainly or entirely dark, tarsus 4 white; abdominal segments without laterally projecting tufts of scales.

1. Femora and tibiae speckled *kingi*
- Femora and tibiae with at most apical bands only 2



2. Palps snaggy; costae and 1st vein (without basal main dark area) *gambiae*
 Palps smooth; 2nd main dark area (preapical dark spot) of 1st vein dark; 2nd vein with 2 pale interruptions *gambiae* (in part)



SECTION IV. Mosquitoes with speckled legs, hind tarsus 5 and 6 not entirely pale; subcostal segments without laterally projecting tufts of scales.

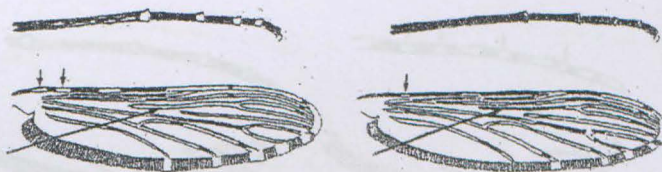
1. Palps with 3 pale bands 2
 — Palps with 4 pale bands 4



2. 3rd main dark area (preapical dark spot) of 1st vein without a pale interruption; abdominal terga fairly heavily clothed with cream or yellowish scales, especially on segments 6 and 7 3
 — 3rd main dark area of vein 1 with a pale interruption, sometimes fused with preceding pale spot; scaling on abdomen very scanty, confined to 8th or rarely 7th terga *gambiae* complex (in part)



3. Base of costa with 2 pale spots; stem of vein 2 entirely pale; fore tarsus 1 with some speckling *salvini*
 — Base of costa with 1 pale spot; stem of vein 2 extensively dark; fore tarsus 1 not speckled *dancalicus*



4. All tarsi completely dark; wing with pale fringe spots present
 3rd vein *ardensis* (in part)
 — Tarsi 1-4 with conspicuous pale bands on at least the apices;
 wing with pale fringe spots up to vein 6



5. 3rd main dark area (preapical dark spot) of vein 1 with a pale interruption, sometimes fused with preceding pale area *gambiae* complex (in part)
 — 3rd main dark area with no pale interruption 6



6. Hind tarsus 2 with about apical two-fifths to half white and the rest dark *natalensis* (in part)
 — Hind tarsus 2 either with less than apical two-fifths white or else prominently marked with dark and pale bands 7



7. No pale fringe spot opposite vein 6; hind tarsus 2-4 with apical pale rings and otherwise dark except for 1 to 2 pale spots *ardensis*
 — Pale fringe spots present opposite vein 6; hind tarsus 2-4 with conspicuous dark and pale rings in addition to apical pale bands 8



2. Thorax and head pale with narrow dark markings 2
 ... Thorax and head dark with narrow pale markings 3

3. Scales on the wings dense and dark 4
 ... Scales on the wings sparse and light 5
 ... Scales on the wings absent 6



SECTION V. Mosquitoes with wings entirely dark or with pale spots confined to costa and vein 1; legs not speckled, hind tarsus 4 and 5 not entirely pale; abdominal segments without laterally projecting tufts of scales.

1. Wings entirely dark or uni-colored 2
 — Wings with at least some areas of paler scales on costa or vein 1, these being sometimes inconspicuous 5



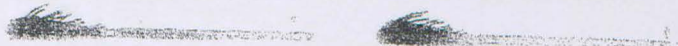
2. Palps and legs entirely dark 8
 — Palps with 2 well marked pale bands; hind femur and tibia narrowly pale at apex concolor



3. Large species, wing length 4.5 mm or more trivittatus
 — Small species, wing length 3.5 mm or less 4

- Very pale brown specimen with dark mesonotal area, small white regions only *athali* (in part)
 General aspect dark brown, mesonotum not
 or cave-dwelling *carani* (in part)

- Large white & pale heads, pale or tan
 Palps with or without pale bands, dark at apex



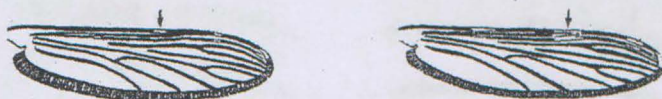
6. Head scales narrow, rodlike, yellowish throughout;
 semi-arid regions only *athali* (in part)
 Head scales broader, white on vertex, dark laterally; all regions



7. Light and dark areas on wings poorly contrasted; semi-arid
 regions only *rhodensis rupicolus* (in part)
 Contrast between light and dark areas on wing well marked



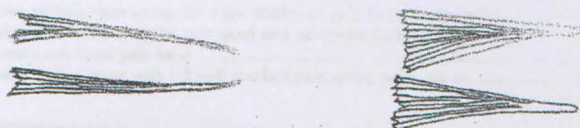
8. Pale areas on wing very narrow, subcostal pale spot present on
 costa only; cave-dwelling *rodhaini*
 Pale areas on wing broader, subcostal pale spot on costa and vein 1 *rhodensis rhodensis*
laumibesi



9. Palps with 3 pale bands, dark at apex *smithii* (in part)
 Palps unbanded or banding indistinct



16. Pale brown species with poorly contrasting light and dark areas *sereni* (in part)
 semi-arid regions *sereni*
 Colour and contrast of dark and light areas variable;
 never dwelling *sereni*
 17. Head scales distinct, and like palpi *sereni*
 Head scales broadest, white on *sereni*
 laterally *sereni*

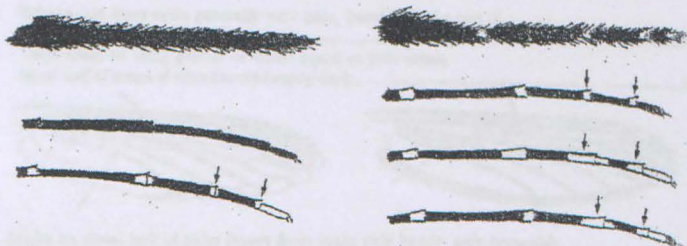


SECTION VI. Mosquitoes without a pale spot on basal half of costa; pale spots not confined to costa and vein 1; legs not speckled; hind tarsus 4 and 5 not entirely pale; abdomen without projecting tufts of scales.

1. Palps shaggy to near tip 2
 — Palps smooth except at extreme base 3



2. Palps entirely dark; hind tarsus 3 and 4 dark or narrowly pale at apices *obscurus* (in part)
 — Palps with pale scales forming more or less definite pale bands;
 hind tarsus 3 and 4 narrowly or broadly pale at apices *lunulatus* (in part)



3. Palps with apex dark, sometimes only narrowly so 4
 — Palps with apex pale 5



4. Stem of 4th vein largely pale, vein 5.1 with 2 pale spots & largely pale, fringe spots present opposite veins 3-5.1 *grisea*
 — Stem of vein 4 largely dark, vein 5.1 with 1 narrow pale area, pale fringe spots entirely absent *smithi* (in part)



5. Costa entirely dark except for a few indistinct pale spots subapically; palps with a broad apical pale band and otherwise dark except for a narrow sub-basal pale band *doni*
 — Outer half of costa with 1-3 well marked pale areas; palps not so *smithi*



6. Palps with 3 pale bands, the subapical pale band broad and about equal to apical band 7
 — Palps either with 4 bands or else with 3 bands, the subapical of these being much narrower than apical band 16



7. Wing apart from costa generally very pale, basal half of stems of veins 2 and 4 entirely pale 8
 — Dark areas on wing greater or about equal to pale areas, basal half of stems of veins 2 and 4 largely dark 9



8. Scales on distal half of palps (apart from main pale bands) pale brownish, those on basal half dark brown; distal half of proboscis prominently pale scaled *wellcomei wellcomei*
 — Scales on palps (apart from main pale bands) uniformly dark brown; proboscis dark-scaled or pale scaling inconspicuous *wellcomei ugandae*



SECTION VII. Mosquitoes with palps dark at the apex or without distinct apical pale band; at least 1 pale spot on basal half of costa, pale scales not confined to costa and vein 1; legs not speckled, hind tarsus 4 and 5 not entirely pale; abdomen without laterally projecting tufts of scales.

- Small pale brown species, pale patches on wings distinct, basal quarter of costa entirely dark; head scales narrow and yellowish *azania* (in part)
 — Wings with well-contrasting light and dark areas, basal quarter of costa with at least 1 pale area, even if narrow; head scales not so 3



3. Costa with humeral pale spot, no subapical (preapical) pale spot on costa and 1st vein *obscurus* (in part)
 — Costa without a humeral pale spot, subapical pale spot present on costa and 1st vein 4



4. Wings with pale fringe spots opposite vein 3 up to vein 5.2 *jebudensis*
 — Wings with no pale fringe spots behind vein 3 *faini*



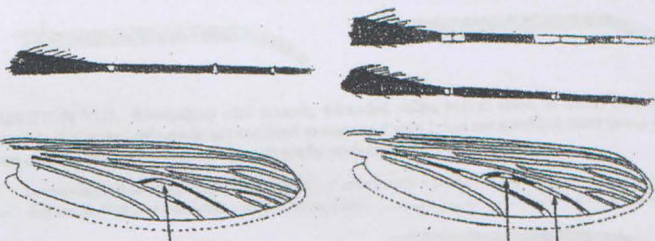
5. Wings generally pale, contrast between pale and dark areas, apart from costa and vein 1 poorly defined; anterior mesonotal scales scanty, not forming conspicuous tuft *turkhu*
 — Wings with well-contrasting light and dark areas; conspicuous anterior mesonotal scale tuft present 6



7. 2nd main dark area (median dark spot) on vein 1 with 1 pale interruption
 2nd main dark area on vein 1 with at most 1 pale interruption



Pale bands on palps very narrow, occupying apices of 1st, 2nd and 3rd segments and overlapping joints on 3, 4 with a single pale spot.
 Pale bands on palps variable in width, basal 5 bands overlapping joints.
 3, 4 with 2 pale spots



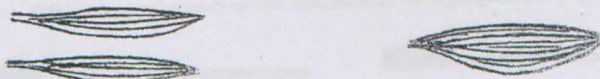
8. Wing field apart from costa and vein 1 predominantly dark, no pale spots on basal quarter of costa9
 — Pale and dark areas on wing about equally distributed, humeral and presector pale spots present on costa11



9. Basal fifth of vein 1 entirely pale10
 — Basal fifth of vein 1 either dark or with a sub-basal pale patch not extending to base.....*smithii* (in part)



10. Wings scantily scaled, all wing scales very narrow*fontinalis*
 — Wings heavily scaled, upstanding scales moderately broad*loretiae*



11. Basal pale band of palps about equal to or slightly narrower than median band *sinensis* (in part)
 broadly overlapping base of 3rd segment 10
 Basal pale band on palps either much narrower than median band, scarcely overlapping
 base of 3rd segment, or both basal and median pale bands very narrow 10

12. Extreme base of costa pale *multicolor* southern Africa only
 Extreme base of costa dark *leptus*
acutus southern Africa only



SECTION VIII. Mosquitoes with smooth, 4-banded palps, pale at apex, at least 1 pale spot on basal half of costa; pale scales not confined to costa and vein 1; legs not speckled; hind tarsus 4 and 5 not entirely pale; abdomen without laterally projecting tufts of scales.

1. 3rd main dark area (preapical dark spot) of vein 1 with a pale interruption 2
 — 3rd main dark area without a pale interruption 3



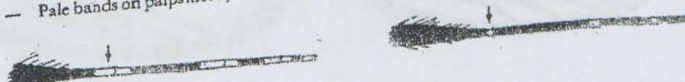
2. Abdominal terga clothed with yellowish scales; hind tarsus 1-4 with very broad apical pale bands *christyi*
 — Abdomen without such scales; hind tarsi entirely dark or with a few pale scales at apices of segments 1-3 *schioetzi* (in part)



3. 2nd main dark area (median dark spot) of vein 1 with 2 pale interruptions; fore and mid femora with a subapical pale spot *wilsoni* (in part)
 — 2nd main dark area of vein 1 with only 1 pale interruption; legs not so 4



4. Pale bands on palps broad, basal pale band overlapping base of 3rd segment *cinereus* (in part)
 — Pale bands on palps mostly narrow, basal band not overlapping base of 3rd segment 5



5. No pale fringe spots posterior to vein 3, femora and tibiae inconspicuously speckled *dennis* (in part)
 — Pale fringe spots present opposite all main veins up to 5.2 or 6, femora and tibiae not speckled 6



6. Stem of vein 5 and vein 5.2 pale at, and adjacent to, the fork *garrahi* (in part)
 — Fork of vein 5 dark 7



7. Wing 4mm or less; flattened mesonotal scales not extending onto scutellum *demeilloni* (Berg River form)
 — Wing 4.4mm or more; some flattened scales present on scutellum as well as mesonotum *carteri* (in part)

SECTION IX. Mosquitoes with a pale interruption on 3rd main dark area (preapical dark spot) of vein 1 or else this area entirely absent; costa with at least 1 pale spot on basal half, pale scales not confined to costa and vein 1; palps 3-banded, pale at apex; legs not speckled, hind tarsus 4 and 5 not entirely pale; abdomen without laterally projecting tufts of scales.

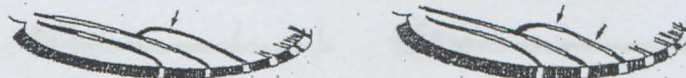
1. 2nd and 3rd main dark areas (median and preapical dark spots) absent from vein 1 *teellcomeni unguis*
 — 2nd and 3rd main dark areas present on vein 1 2



2. Hind tarsus 5 entirely white, tarsus 4 white except for a broad median dark band *seydali*
 — Hind tarsus 5 entirely dark 3



3. Vein 5.1 with 1 pale spot, sometimes with a vestigial 2nd pale spot 4
 — Vein 5.1 with 2 well developed pale spots 5



4. Pale fringe spot present opposite vein 6; fore tarsus 1-4 with conspicuous basal and apical pale bands *mortiauxi*
 — No pale fringe spot opposite vein 6; fore tarsus 1-4 narrowly pale apically only *berghei*



5. Subapical pale band on palps very narrow, confined to apex of 3rd segment 6
 — Subapical pale band on palps broad, overlapping apex of 3rd and base of 4th segment 7



6. Base of costa with 2 pale interruptions *brunneipes*
 — Basal quarter of costa entirely dark *walravensi* (in part)



7. Hind tarsus either all dark or with apical pale bands on segments 1 and 2 only 8
 — Hind tarsus 1-4 with well marked apical pale bands 10



8. Scutal fossae and lateral areas of mesonotum above wing root with scattered or abundant broadish scales 9
 — Fossae and lateral areas of mesonotum without scales *harperi*

9. Subapical pale band on palps about equal to or slightly narrower than apical band *ngambiensis*
 — Subapical pale band on palps much narrower than apical band *walravensi* (in part)



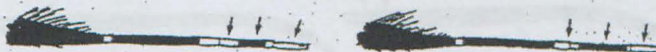
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1. Apical and subapical pale bands on palps broad so that intervening dark band is either slightly broader, about equal to or narrower than either pale band.....2

— Apical and subapical pale bands narrower, intervening dark band much broader than pale bands.....16



2. Apical pale bands on hind tarsus 4, and sometimes on tarsus 2 and 3 also,
extending onto bases of succeeding segments.....3
— Bases of hind tarsus 5 and of other segments dark.....6



3. 2nd main dark area (median dark spot) on vein 1 with 2 pale interruptions;
bases of hind tarsus 4 and 5 sometimes broadly pale.....*rufipes brasseurii* (in part)
— 2nd main dark area on vein 1 with 1 pale interruption; bases of hind tarsus
4 and 5 at most narrowly pale.....4



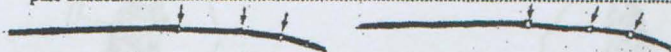
4. Base of costa with 1 pale interruption, 3rd main dark area (preapical dark spot)
on costa and vein 1 much broader than subcostal pale spot.....*domicolus*
— Base of costa with 2 pale interruptions; 3rd main dark area equal to or narrower
than subcostal pale spot.....5



5. Pale fringe spot present opposite vein 6.....*lloreti*
— No pale fringe spot opposite vein 6.....*barberellus*



6. Apices of hind tarsus 3 and 4 dark or at most with a few
pale scales.....7
— Apices of hind tarsus 1-3, and sometimes 1-4, distinctly
pale-banded.....12



7. Base of costa with 1 or no pale interruption.....8
— Base of costa with 2 pale interruptions.....9



8. Vein 6 either with pale fringe spot or with pale scales at apex *brucei* (in part)
 — Vein 6 without pale fringe spot and with no pale scales at its apex *riculorum* (in part)



9. Mesonotal scales fairly broad, extending over whole scutum and onto scutellum *carleri* (in part)
 — Mesonotal scales variable, but flattened scales confined to at most anterior two thirds of scutum 10
 10. Very small species, wing length 2.8mm or less *brucei* (in part)
 — Small or moderate species, wing length 2.9mm or more 11

11. Hind tarsus entirely dark; preaccessory dark spot on vein 1 usually absent *fretoumensis*
 — Hind tarsus 1 and 2 narrowly but distinctly pale apically; pre-accessory dark spot present *demeillani* (in part)



12. 3rd main dark area (preapical dark spot) equal to or narrower than subapical pale spot *flavicauda* (in part)
 — 3rd main dark area much broader than subapical pale spot 13



13. Mesonotal scales broadish and white, only slightly less dense on posterior third of scutum than anteriorly, and extending onto scutellum *flavicauda* (in part)
 — Mesonotal scales on posterior third of scutum scanty, narrow and yellowish-brown 14



14. Moderate-sized species, wing more than 3.2mm *keniensis* (in part)
 — Small species, wing 3mm or less.....15
15. Fore tarsus 4 dark or indistinctly pale at apex; pale fringe spot
 opposite vein 6 usually absent* *moucheti*
 — Fore tarsus 4 with well marked apical pale band; 6th fringe spot present* *bervoetsi*



16. Large species; fork of vein 5 pale..... *garnhami* (in part)
 — Small or moderate-sized species; vein 5 dark at fork.....17



17. Base of costa with 1 or no pale interruption18
 — Base of costa with 2 pale interruptions..... *demeilloni* (in part)



18. Small species, wing about 2.5-3.3mm *rivulorum* (in part)
 — Small or moderate-sized species, wing 2.9-4.2mm..... *demeilloni* (in part)

SECTION XI. Mosquitoes with 1 pale spot on vein 5.1, no pale interruption on 3rd main dark area (preapical dark spot) of vein 1, costa with at least 1 pale spot on basal half; palps with less than 4 bands, pale at apex; legs not speckled, hind tarsus 4 and 5 not entirely pale; abdomen without laterally projecting tufts of scales.

1. Palps with apex pale and no other pale bands2
 — Palps with 3 pale bands3



*But see note under *moucheti nigeriensis* in Gillies and De Meillon, (1968).

2. Subapical pale spot on costa and vein 1 about equal to or broader than 4th main dark area (apical dark spot), pale fringe spots present opposite veins 3, 4.2, 5.1 and 5.2.....*nili* Congo form
 — Subapical pale spot narrower, usually much narrower, than 4th main dark areas, no pale fringe spot present opposite vein 5.1*nili somaticus*



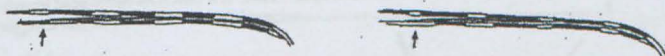
3. Joints of hind tarsal segments narrowly or broadly enveloped in pale bands,
 • at least tarsus 5 pale-ringed basally.....*longipalpis*
 — Pale banding on hind tarsus narrow and apical only.....4



4. Preaccessory dark spot on vein 1 about twice as broad as pale spots on either side of it.....*fuscivenosus* (in part)
 — Preaccessory dark spot absent or, if present, narrower or only slightly broader than adjoining pale spots.....5



5. Basal area of vein 1 proximal to 1st main dark area (presector dark spot), pale with a broad dark spot.....*cuticifacies*
 — Basal area of vein 1 entirely pale.....6



6. Subapical pale band on palps broader than or slightly narrower than subapical dark band and 3rd main dark area (preapical dark spot) of costa and vein 1 equal to or narrower than sub-costal pale spot.....*aruni* (in part)
 — Subapical pale band on palps much narrower than subapical dark band, or 3rd main dark area broader than subcostal pale spot.....7



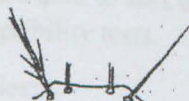
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The following species, known only as adults, are not included: *caliginosus*, *vermus*, *dermingsi*, *fontinalis*, *fuscovenosus*, *ethiopicus*, *berghiei*, *mortiauxi*, *upemba*, *daudi*, *brumpti*, and *cristipalpis*.

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- A diagram showing a horizontal beam balanced on a central fulcrum. Two weights are suspended from the beam, one on each side of the fulcrum. Arrows indicate the forces acting on the weights.



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Appendix H: SOPs_Field Procedure-Mosquitoes larval collection for IR test

1. Purpose

This Standard Operating Procedure outlines the procedure to be followed when sampling Anopheline mosquito larvae for insecticide susceptibility test:

- Collect larvae for routine monitoring of insecticide resistance in the malaria endemic regions.

2. Scope

This protocol should be used in entomological surveillance settings – specifically in sampling of Anopheline mosquito larvae for insecticide susceptibility tests. It is of vital importance that this procedure be followed appropriately. New staff needs to be trained and deemed competent after being assessed by a qualified NVDCP officer before performing this procedure independently.

3. Principle

Routine entomological surveillance involves sampling mosquitoes at aquatic and adult stages in order to determine various entomological parameters such as mosquito biting and resting behaviours, mosquito species composition and distribution, mosquito density and biting rate, and mosquito susceptibility to insecticides used in malaria vector control programs. For insecticide susceptibility tests, only Anopheline mosquitoes are sampled as they are the only genus of mosquitoes that transmit malaria. So it is important that field surveillance teams are able to sort out Anopheline larvae from non-anopheline larvae for successful susceptibility tests.

Larval sampling is comprised of two basic activities:

- Identifying larval habitats,
- Sampling larvae
- Identifying anopheline larvae from other mosquito larvae

4. Responsible Personnel

- a) Entomologists
- b) Foci investigators
- c) Entomologist technicians
- d) Environmental Health Officers and practitioners (EHOs/EHPs)
- e) Insectary and laboratory technicians

5. Equipment for larval sampling

The equipment required for larval collections is dependent on the method of collection. The most common equipment and materials include:

- a) GPS
- b) Pair of boots used as personal protection equipment
- c) Tray for collecting and sorting samples
- d) Dippers varying in shape and size, light-colored to see larvae easily
- e) Net for collection (netting method)
- f) Pipettes for sample sorting and transfer (pipetting method)
- g) Different mesh sieves for sample sorting
- h) Large sealed bottles or suitable containers to transport live larvae
- i) Cooler box
- j) Thermometer
- k) Packaging materials e.g. clothing, for shock absorbing
- l) Ice blocks
- m) Marker, pen and pencil for labeling and filling forms
- n) Standard data recording forms/sheets and field notebooks

6. Optimal periods for larval sampling

Immature mosquito development and activities depend on environmental factors, especially water temperature and it is important to schedule larval prospection when the weather conditions are favorable. Typically, larval surveys should be organized during sunny days (avoid rainy or cloudy days), in the morning between 10am and 1pm and in the afternoon between 3pm and 5pm.

Conduct routine larval surveys every depending on rainfall, characteristics of the site and the survey objectives.

7. Procedures

7.1. Identifying and mapping potential larval habitats

Note that preferred (but not restricted) sites where *Anopheles* larvae can be found are:

- a) Sunlit water bodies or the sun-exposed area of a water body,
- b) Edges of water bodies,
- c) Around low vegetation e.g. grass tufts, round swimming debris and leaves,
- d) In-between floating vegetation.

7.1.1. Identifying potential larval habitats

- a) Check for presence of standing water bodies;
- b) Cautiously approach any encountered standing water and check with naked eyes to see if larvae and/or pupae are present;
- c) Avoid projecting shadow onto the water; otherwise larvae react by swimming down to hide;
- d) Take particular care around small debris accumulated on the water surface or where vegetation rises, when searching larvae.

7.1.2. Localizing and numerating larval habitats

- a) Check and mark any sources of water encountered using an adequate map of the investigated area or GPS device to locate potential breeding sites;
- b) Give all breeding sites a unique number and report it in the field form, notebook and database.

7.2. Characterizing and monitoring larval populations and their habitats

7.2.1. Characterizing larval habitats

- a) Categorize the breeding site (permanent, semi-permanent, temporary);
- b) Determine the nature of the water body (puddle, rice field, ditch, lake, stream);
- c) Determine the origin of the water (rain, river, lagoon, irrigation scheme, drainage system);
- d) Record the physical and chemical properties of the water (clear, turbid, polluted, dark, temperature, pH, salinity);
- e) Evaluate the exposure to sunlight (shaded, full sunlight);
- f) Note the type and presence of vegetation and (emergent, sub-merged, floating).

7.2.2. Monitoring larval populations and their habitats

7.2.2.1. Collecting larvae and pupae

The most commonly used methods to collect larval samples are the dipping, netting and pipetting methods.

7.2.2.1.1. Dipping

The dipping method is most frequently used for the collection of anopheline mosquito larvae and pupae. The essential equipment for this method consist of a ladle, enamel bowls, pipettes, larval vials, bigger sealed bottles or suitable containers, recording forms and material for labeling.

Steps in dipping

- a) Estimate the active breeding surface area of the breeding site (e.g. area preferentially occupied by larvae).

- b) Dip at the rate of 10 dips (at least 10) per breeding site or area of active breeding surface for large breeding sites. In the case of water channels, drains, large swamps, walk along/around the habitat and take up to 60 dips per habitat to investigate for the presence of larvae.
- c) During dipping, avoid shadowing (e.g. dipping person, ladle)
- d) Immerse the ladle in the breeding place (e.g. river bed pools, river margins, streams, edge of swamps, rice fields, gem pits) at an angle of 45°.
- e) Let the ladle fill $\frac{3}{4}$ of water (with larvae, if any) and withdraw quickly (If the dipper is immersed too slowly, the larvae get disturbed and escape).
- f) Keep 2-3 minutes' intervals between each dip to allow larvae to come to the surface again.
- g) If the surface water is covered with dense floating vegetation or organic debris, agitate the water surface to cause the larvae to sink, clear away the vegetation and then wait for 3-5 min for larvae to come to the surface and dip.
- h) Count the 1st, 2nd, 3rd and 4th stage larvae, and pupae in each dip and record in the appropriate forms.
- i) Calculate the larval density per 10 or more dips for each larval breeding habitat separately as indicated on the sampling forms.

7.2.2.1.2. Netting

Larvae may be collected from large stretches of water along the edge of rivers, streams, ponds, wells, and other large water bodies using the netting collection method. The minimal essential equipment for this method consists of a larval net, enamel bowls, pipettes, larval vials, bigger sealed bottles or suitable containers, recording forms and material for labeling.

Note: A larval net consists of a ring of iron frame (diameter 20-25 cm) to which a nylon/ muslin cloth net (10 cm long) is attached. A long wooden handle is attached to the ring.

Steps in netting

- a) Hold the net at an angle of 30° and skim rapidly through the surface water near emerging or floating vegetation to collect larvae
- b) Invert and wash out the net in a bowl of water to recover collected larvae
- c) Collect larvae and pupae using a pipette and transfer to a labeled container
- d) Grow larvae and pupae until they emerge to adults and then identify using standard identification guides.
- e) The larval density should be given as number of larvae per larval net for each species for each breeding habitat.

7.2.2.1.3. Pipetting

Small pipettes can be used for collection of larvae from tree holes or other small breeding sites. The minimal essential equipment for this method consists of small pipettes, enamel bowls, larval vials, bigger sealed bottles or suitable containers, recording forms and material for labeling.

7.2.2.2. Larval morphological identification

After collecting larvae from the breeding sites, the larvae should be morphologically identified to sort out Anopheline larvae from other mosquito larvae such as Culicine larvae. This procedure should be done on site as it is quick and easy with least requirement of equipment and expertise.

- Place the larvae in a water tray with a white background
- Allow the larvae and the water to settle
- Carefully examine the morphology of the larvae
- Anopheline larvae stay parallel with the water surface while Culicine larvae hung with their heads pointing into the water
- Culicine larvae can also be distinguished from Anopheline larvae by the presence of a long and narrow breathing tube which they use to attach to the water surface.

Refer to figure 1 below for morphological difference between anopheline and culicine larvae.

- Sort out Anopheline larvae from Culicine larvae and place Anopheline larvae in a container provided.
- Discard Culicine larvae
- Make sure the larvae container is at least half filled with water from the breeding site where the larvae were sampled

For transportation of the larvae to the insectary please refer to the SOP for larva transportation to the insectary.

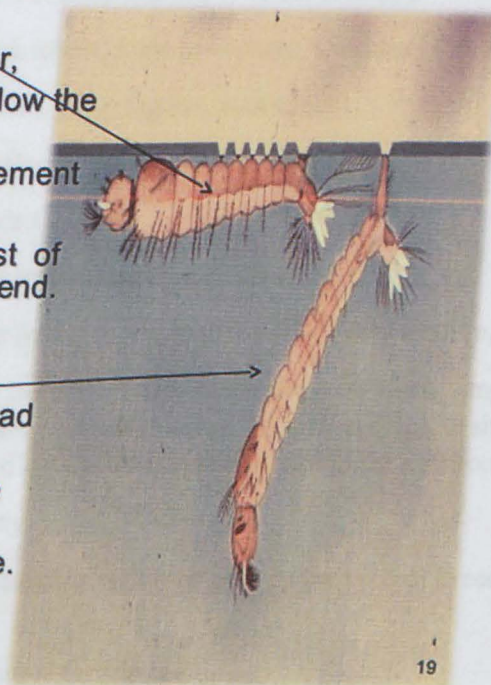
Figure 1: Morphological differences between anopheline and culicine larvae

Anopheles larva:

- Exclusively surface feeder,
- Floats horizontally just below the surface of water,
- very active with swift movement
- No siphon tube, but
- breathing apparatus consist of parallel air tube at the tail end.

Culicines Larva:

- suspended in water with head downwards,
- much slower with snake like movement
- Has long narrow siphon tube.



7.2.2.3. Preserving and transporting larvae and pupae

Transporting live larvae and pupae

- a) Place all collected specimens from a particular breeding site in one bottle or vial and label it. The label must be written in pencil on a piece of paper and dropped into the specimen bottle. Do not use a ballpoint pen as the ink dissolves in water.
- b) The collected larvae and pupae must arrive alive and undamaged at the laboratory. Cap each bottle or vial tightly so that water cannot spill. Make sure that there is air in the top of the container so larvae and pupae can breathe for a few hours.
- c) If the journey to the laboratory takes more than 2–3 hours, remove the stoppers of each container every 2 hours to provide the specimens with fresh air. Pack bottles and vials carefully so that they are not jolted during transport. If the larvae are to be used in insecticide susceptibility tests they should be transported in water in a large vacuum flask or other large container.

7.3. Records of collection

Use a GPS to locate and number the breeding sites sampled. The collected data is recorded in a specific larval collection data form (Annex 2). The information includes namely:

- a) Name of collector
- b) Date and time of collection

- c) Geographic location (GPS coordinates, name of the locality).
- d) Type of breeding site (permanent, semi-permanent, temporary).
- e) Origin of the water (e.g. rain, river, lagoon, man-made).
- f) Nature of the water collection (e.g. puddle, rice field, ditch).
- g) Exposure to sunlight (shaded, sunlit).
- h) Presence of vegetation (emergent, submerged, floating).
- i) Characteristics of the water (clear, turbid, polluted, dark, temperature, pH).

Data recorded should also include the number of dips made, the time spent sampling and the date of sampling. All vials/bottles containing larvae from a breeding site should be labeled with the number of the breeding site that was annotated in the notebook.

7.4. Estimation of larval density

- **Larval density:** is the average number of larvae per dip per type of breeding site.

$$\text{Larval density} = \frac{\text{Total number of larvae and/or pupae}}{\text{Total number of dips}}$$

8. References

- USAID (2012) Training Manual on Malaria Entomology for Entomology and Vector Control Technicians (Basic Level)
- WHO (2013) *Malaria entomology and vector control*. Guide for participants. ISBN 978 92 4 150581 9.
- WHO (1975a) *Manual of Practical Entomology in Malaria*. Part I., WHO Offset Publication, No. 13.
- WHO (1975b) *Manual of Practical Entomology in Malaria*. Part II. WHO Offset Publication No. 13.