

https://dspace.org/

Comparative insecticide resistance intensity studies between the pyrethroid resistant wild Zambian and the laboratory reared Mozambique Anopheles funestus

Segoea, Godira

2015
http://hdl.handle.net/10539/18654

Downloaded from a DSpace repository. https://dspace.org/

Comparative Insecticide Resistance Intensity Studies between the Pyrethroid Resistant Wild Zambian and the Laboratory Reared Mozambique *Anopheles funestus*

GODIRA SEGOEA

A research report submitted to the Faculty of Health Science, University of the Witwatersrand, Johannesburg, in partial fulfillment of the requirements for the degree of Master of Science in Medicine (Biology and Control of African Disease Vectors)

Johannesburg, 2015

Declaration

I, Godira Segoea, declare that this research report is my own, unaided work. It is being submitted in partial fulfillment for the degree of Master of Science in Medicine in the Faculty of Health Sciences at the University of Witwatersrand, Johannesburg. This research report has not been submitted before for any degree or examination in any other university.



April 2015

ABSTRACT

Background:

Malaria continues to be a significant cause of morbidity and mortality in the developing world with Africa being the most affected. Malaria vector control with chemical insecticides is the primary intervention to curb transmission. However, the success of chemical based interventions is threatened by the escalating development of insecticide resistance in the major anopheline mosquito vectors.

Methods:

Indoor resting collections of *Anopheles funestus* females were originally obtained from northern Zambia and subsequently from Zimbabwe. The collections were set up under laboratory conditions to promote oviposition. Wild females that laid eggs were identified to clade level using PCR. Females from F_1 generation wild *An. funestus* clades, laboratory pyrethroid resistant *An. funestus* and DDT resistant *An. arabiensis* were tested for deltamethrin and bendiocarb resistance intensities.

Results:

Anopheles funestus clades I and II were detected in Zambia but only clade I in Zimbabwe. The Zimbabwe and Zambia wild caught strains showed no difference in bendiocarb resistance intensities but the Zimbabwe strain had higher deltamethrin resistance intensity than the Zambia strains. When compared with an insecticide susceptible strain of *An. funestus* (FANG), the selected pyrethroid resistant laboratory strain from Mozambique (FUMOZ-R), presented significantly higher resistance intensities compared to both FANG and its baseline colony, FUMOZ-BASE. FUMOZ-R also showed higher deltamethrin and bendiocarb resistance intensities than wild *An. funestus* strains. FUMOZ-BASE showed lower deltamethrin but higher bendiocarb resistance intensities compared to wild strains.

There was no difference in bendiocarb resistance intensities amongst DDT resistant laboratory strains of *An. arabiensis* (SENN-DDT and MBN-DDT) but MBN-DDT had higher deltamethrin resistance intensities than SENN-DDT.

Conclusion:

Wild *An. funestus* strains showed higher deltamethrin resistance intensities compared to the baseline pyrethroid resistant laboratory strain. The high pyrethroid resistance intensities in the wild strains show the seriousness of pyrethroid resistance in Zambia and Zimbabwe. Resistance management is needed urgently in the two study areas. Resistance intensities assays are recommended to be undertaken on a routine basis in the event that resistance is detected in order to assess the possible impact on the vector control interventions being used.

Key words: Resistance intensities, deltamethrin, bendiocarb, *An. funestus*, *An. arabiensis*

DEDICATION

This work is dedicated to my loving wife Tshoaro Jessie Segoea

ACKNOWLEDGEMENTS

I thank the Lord for granting me good health and blessing me with an opportunity to enroll for this degree.

My sincere gratitude goes to my supervisor Prof Basil D. Brooke and the head of Wits Research Institute for Malaria (WRIM) Prof Maureen Coetzee for the study idea, design, and guidance during all phases of this project.

I am grateful for the work done by Prof Richard H. Hunt, Prof Maureen Coetzee, Dr Candice Lyons, Dr Luisa Nardini, Dr Riann Christian, Mr. Michael Samuel, Mr Mbanga Muleba and Mr Aramu Makuwaza in collection of wild samples, assistance with identification and rearing to F_1 generation.

This work forms part of the International Centers of Excellence for Malaria Research (ICEMR) project (U19 AI089680) funded by the US National Institutes for Health. I am therefore indebted to ICEMR for funding this research project.

I also want to thank my employer, NMCP in the Ministry of Health, Botswana for sponsoring my studies.

Much appreciation goes to the National Malaria Control Programme (NMCP) in the Ministry of Health and Child Welfare, Zimbabwe and the NMCP in the Ministry of Health, Zambia for facilitating field collections.

My heartfelt appreciation goes to Ms. Shune Oliver sacrificing her time to ensure I am on track with my experimental work, preliminary data analysis and draft write-up.

I also want to thank the entire staff and students of WRIM for the cordial working relationship and assistance that led to the success of this research project.

I will always be grateful to my loving wife Tshoaro Jessie Segoea for being my pillar of strength during the course of my studies. I am thankful to my parents Mr. and Mrs. Jimmy Mackenzie and my younger brother Olorato for their support during my period of study. My in-laws Mr. and Mrs. Charles David Mapitse and the entire family are always a blessing to me.

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical)

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

Ref: W-CJ-100510-1

10/05/2010

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigators: **Professor Maureen Coetzee**

Project title: **Research on mosquitos.**

Reason: This waiver covers all research using mosquitos and mosquito parasites by Professor Coetzee, her staff and students as long as no humans or human tissues are involved. The waiver lasts 5 years and may be renewed.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2010 -05- 10
JOHANNESBURG

Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

copy: Anisa Keshav, Research Office, Senate House, Wits

TABLE OF CONTENTS

CONTENTS		PAGE
Title Page…….		i
Declaration……..		ii
Abstract……		iii
Dedication……		v
Acknowledgements…….		vi
Ethics waiver…….....		viii
List of Figures……		xii
List of Tables…….		xiv
CHAPTER ONE	**INTRODUCTION**	
1.1	The global burden of malaria……	1
1.2	Malaria parasites……	1
1.3	African malaria vectors……..	1
1.3.1	*Anopheles gambiae* complex……	2
1.3.2	*Anopheles funestus* group……...	4
1.3.2.1	*Anopheles funestus*……..	4
1.4	Malaria situation in Zambia……..	5
1.5	Malaria situation in Zimbabwe…….	6
1.6	Malaria vector control……	8
1.7	The problem of insecticide resistance…….	9
1.7.1	Insecticide resistance in *An. funestus*……..…….....	10

1.7.2 Insecticide resistance in the *An. gambiae* complex ……..……. 11

1.8 Mitochondrial clades in *An. funestus* and role in malaria transmission…… 11

1.9 Resistance monitoring…….. 12

1.10 Rationale for the study…….. 13

1.10.1 Broad objective…….. 13

1.10.2 Specific objectives…….. 14

CHAPTER TWO MATERIALS AND METHODS

2.1 Indoor resting collections…….. 15

2.2 Rearing the F_1 generation…… 16

2.3 PCR identification for *An. funestus* group species……. 18

2.4 PCR identification for *An. funestus* clades…… 19

2.5 Insecticide resistance intensity assays…….. 20

2.6 Data analysis……..……. 22

CHAPTER THREE RESULTS

3.1 Species identification…….. 23

3.2 Knockdown trends over 8 hour exposure on deltamethrin and bendiocarb……..…… 24

3.3 Probit analysis for measure of resistance intensity…… 27

3.4 Deltamethrin and bendiocarb resistance intensities for different strains…….…… 32

3.5 Deltamethrin and bendiocarb resistance ratios ……..…….. 34

3.6 Comparisons of resistance intensity assays and resistance monitoring assays…….. 36

CHAPTER FOUR DISCUSSION

4.1 Important findings…….. 37

4.2 Insecticide resistance and fitness costs in *An. funestus*……..…… 38

4.3 Implications of existing selection pressure in Nchelenge and Honde Valley……. 39

4.4 Resistance intensities on *An. arabiensis* laboratory strains ……. 41

4.5 Importance of insecticide resistance intensity studies ……. 41

4.6 CONCLUSION…… 42

REFERENCES……. 44

APPENDIX…….. 51

LIST OF FIGURES

Figure 1.1 Endemicity of malaria in Zambia……..…… 6

Figure 1.2 Endemicity of malaria in Zimbabwe...……..…… 8

Figure 2.1 Locations of the two sampling sites in Zambia and Zimbabwe..... 15

Figure 2.2 Experimental set up of egg laying vials for wild material…… 17

Figure 2.3 Experimental set-up and resistance intensity assay procedure ……..…..…… 21

Figure 3.1 *Anopheles funestus* species specifc PCR for samples collected in Honde Valley, Zimbabwe……..……..…. 23

Figure 3.2 Deltamethrin induced knockdown trends in wild caught and laboratory *An. funestus* over 8 hour exposure period…..……..…….. 25

Figure 3.3 Bendiocarb induced knockdown trends in wild caught and laboratory *An. funestus* over 8 hour exposure period……..... 25

Figure 3.4 Deltamethrin induced knockdown trends in laboratory *An. arabiensis* over 8 hour exposure period……..…. 26

Figure 3.5 Bendiocarb induced knockdown trends in laboratory *An. arabiensis* over 8 hour exposure period..…… 26

Figure 3.6 Probit transformed bendiocarb induced knockdowns as a function of time…..…….. 28

Figure 3.7 Probit transformed deltamethrin induced knockdowns as a function of time ……..…….…….. 30

Figure 3.8 Bendiocarb lethal times of wild caught and laboratory *Anopheles* strains……..…….... 32

Figure 3.9 Deltamethrin lethal times of wild caught and laboratory *Anopheles* strains……. 33

Figure 3.10 Standard WHO bioassay results for deltamethrin exposure in pyrethroid laboratory *An. funestus* strains …… 36

LIST OF TABLES

Table 3.1: *Anopheles funestus* clades identified from collections made in Zambia and Zimbabwe (February-March 2014)…….. 23

Table 3.2: Deltamethrin and Bendiocarb RIRs……...… 35

CHAPTER 1

INTRODUCTION

1.1 The global burden of malaria

The World Health Organization (WHO, 2013a) estimated a global incidence of 207 million malaria cases and 627 000 deaths in 2013. The African region contributed 81% of these cases and 91% of the total global malaria deaths. Malaria is the leading cause of morbidity and mortality in many countries particularly in Sub-Sahara Africa. Of the population at risk, pregnant women and children under five years old are the most vulnerable. The WHO estimates that an under five year old child dies every minute from malaria in sub-Saharan Africa (WHO, 2013a).

1.2 Malaria Parasites

Malaria in humans is caused by five species of protozoan parasites of the genus *Plasmodium*: *P. falciparum, P. vivax, P. ovale* and *P. malariae* are the most common (Oaks *et al*., 1991). *Plasmodium knowlesi* has been named the fifth human malaria parasite (Singh *et al*., 2004) even though human to human transmission has not been conclusively documented for this primate malaria parasite (Antinori *et al*., 2013). *Plasmodium falciparum* is responsible for the majority of the disease burden and it is the most virulent parasite, while *P. vivax* is geographically the most widely distributed parasite (Bousema and Drakeley, 2011).

1.3 African malaria vectors

The continent of Africa experiences the bulk of the global malaria burden due to the presence of extremely efficient mosquito vectors (Gillies and De Meillon, 1968). This

situation is further exacerbated by the complex vectorial system present in Africa. The *Anopheles gambiae* complex consists of a number of isomorphic species, differing in behaviour and vectorial capacity (Gillies and Coetzee, 1987). *Anopheles gambiae sensu stricto* (herein, referred to as '*Anopheles gambiae*') and *An. coluzzii* are the dominant vector species within the complex, the others being *An. arabiensis* and the salt water breeding species, *An. merus* and *An. melas* (Sinka *et al*., 2010; Coetzee *et al*., 2013a). There are a further three dominant vector species with human biting preferences (anthropophily) in Africa. They are *An. funestus sensu stricto* (herein, referred to as '*Anopheles funestus*'), a member of the *An. funestus* group, *An. moucheti* and *An. nili* species belonging to the *An. moucheti* and *An. nili* groups respectively (Sinka *et al*., 2010). The anthropophilic habits of these dominant vector species are a major contributing factor to their public health impact with *An. funestus* considered to be one of the first species to have adapted to the human host (Charlwood *et al*., 1995).

1.3.1 *Anopheles gambiae* complex

The *Anopheles gambiae* complex contains the most common species of malaria mosquitoes in Africa (Gillies and Coetzee, 1987) and comprises 8 named morphologically identical species that can only be differentiated by DNA analysis. Three members of *An. gambiae* complex are important major malaria vectors in Africa. These are *An. coluzzii, An. gambiae* and *An. arabiensis. Anopheles coluzzii* and *An. gambiae* are preferentially anthropophilic thus being efficient vectors of malaria parasites (Antonio-Nkondjio *et al*., 2002). *Anopheles arabiensis* adults, unlike *An. gambiae* and *An. coluzzii,* show plasticity in their life history with a propensity to feed on both humans and animals. *Anopheles arabiensis* also feeds and rests both indoors and outdoors. The currently effective control interventions, such as indoor residual spraying (IRS) and the use of insecticide treated bed nets (ITNs) that target indoor resting vector populations (White, 1974; Sharp and Le Sueur, 1991) are not as effective against *An. arabiensis.*

Anopheles gambiae and *An. arabiensis* choose relatively similar larval habitats during rainy seasons. These are very temporary, shallow fresh water collections, e.g. rain puddles, animal hoof prints and small ground pools (Service, 1970). In contrast, *An. coluzzii* larval habitats are predominantly permanent water bodies occurring all year round, such as rice paddies and swamps (Gimonneau *et al*., 2012).

Other members of the *An. gambiae* complex that are minor malaria vectors include *An. merus*, *An. melas* and *An. bwambae*. *Anopheles merus* and *An. melas* are saline water breeders (Gillies and De Meillon, 1968) and feed on both humans and domesticated animals (Gillies and Coetzee, 1987). *Anopheles merus* is found mainly in coastal East Africa but has also been detected far inland in Zambia, Zimbabwe, South Africa and Swaziland (Gillies and Coetzee, 1987; Coetzee *et al*., 1993; La Grange, 1995; Masendu *et al*., 2005), while *An. melas* is restricted to coastal West Africa (Gilles and Coetzee, 1987; Moreno *et al*, 2004; Jawara *et al*., 2008). The third minor vector, *An. bwambae*, is found only in Bwamba country, Uganda, where it is found breeding in the mineral springs of the Semliki forest (White, 1985).

There are only two members of the *An. gambiae* complex that play no role on malaria transmission. These are *An. amharicus* and *An. quadriannulatus*. Both are freshwater breeders with feeding preference restricted to animals. *Anopheles quadrannulatus* is mainly distributed in the southern African region (Gillies and Coetzee, 1987) and is mostly found in sympatry with dominant vector species (Coetzee *et al*., 1993). *Anopheles amharicus* is known only from Ethiopia and rests mainly indoors in cattle and human habitations (Hunt *et al*., 1998; Coetzee *et al*., 2013a).

1.3.2 *Anopheles funestus* group

Anopheles funestus mosquitoes are relatively small (i.e. wing length of 3mm or less) compared to the *An. gambiae* complex (Gillies and Coetzee, 1987). This group of species breeds exclusively in permanent water bodies like swamps, river beds, streams and wells (Leeson, 1937). The *An. funestus* group comprises 11 African species divided into two subgroups (Harbach, 2004). The *An. funestus* subgroup comprises *An. funestus, An. vaneedeni, An. parensis, An. aruni, An. confusus* (Harbach, 2004) and the recently discovered and temporarily named *An. funestus*-like (Spillings *et al*., 2009). The other subgroup is the *An. rivulorum* subgroup that consists of *An. rivulorum, An. brucei, An. fuscivenosus and An. rivulorum*-like (Cohuet *et al*., 2003; Harbach, 2004). The 11th species, *An. leesoni* is said to belong to the Asian *An. minimus* subgroup (Harbach, 2004).

Anopheles funestus group adults are extremely difficult to identify morphologically although some species have distinct larval and egg characteristics (Gilllies and De Meillon, 1968). *Anopheles funestus, An. parensis*, *An. aruni* and *An. vaneedeni* cannot be identified morphologically at any life stages (Gilllies and De Meillon, 1968). This necessitates the use molecular techniques for identification to species level (Koekemoer *et al.,* 2002).

1.3.2.1 *Anopheles funestus*

Anopheles funestus is also a major malaria vector species in sub-Saharan Africa. It is highly endophilic and anthropophilic and feeds predominantly in the hours just before and after midnight (Gillies and De Meillon, 1968). Malaria parasite infection rates in populations of *An. funestus* have been reported as high as 22% in South Africa (Gillies and De Meillon, 1968), 4-10.8% (Shiff *et al.*, 1995) and 5.4% (Temu *et al.*, 1998) in Tanzania and 9.2% in Zambia (Choi *et al*., 2014), making it the most important vector in east-southern Africa. This species was also implicated in transmission that resulted in an epidemic in South Africa in 1999/2000 (Hargreaves *et al.*, 2000; Coetzee *et al.,* 2013b). Several studies have shown *An. funestus* to have potential for rapid selection for insecticide resistance that subsequently impacts negatively on the success of control interventions (Brooke *et al*., 2001; Hunt *et al*., 2005, Casimiro *et al*., 2007; Choi et al., 2014)

1.4 Malaria situation in Zambia

Malaria is endemic throughout Zambia especially in the rural areas and *P. falciparum* is responsible for most of the laboratory confirmed cases (ICEMR, 2014, unpublished report). *Anopheles arabiensis* and *An. funestus* are incriminated for transmission (Chanda *et al*., 2007). Malaria transmission in Zambia is seasonal, following rainfall patterns that correlate positively with mosquito vector population abundance and transmission rates. The rains start between September and November and malaria transmission reaches a peak in April/May. Cases drop significantly in June-July when the rainy season ends (Ministry of Health, 2000). The ten year (between 2000 and 2010) review of Zambian malaria epidemiology showed general declining trends in morbidity and mortality, a result of 90% IRS coverage, scale up of ITN coverage from 50 to 70% and nationwide rollout of artemisin-based combination therapy (Masaninga *et al.,* 2013). Malaria transmission in Zambia is not uniform as areas like the Nchelenge district in Luapula Province are still hyperendemic, with significant morbidity in children under 5 years despite good coverage of IRS and ITNs (Das *et al*., 2014). The *An. funestus* and *An. gambiae* populations in Nchelenge are highly endophilic and anthropophilic (Das *et al*., 2014).

Malaria transmission in Zambia ranges from moderate to high in the northern provinces (Northern, Muchinga, Luapula and Eastern Provinces) sharing the border with the Democratic Republic of Congo and Mozambique. The southern part of Zambia ranges from low to moderate in malaria incidence. Only Lusaka Province has very low transmission levels. Figure 1.1 shows malaria endemicity in Zambia and Nchelenge district.

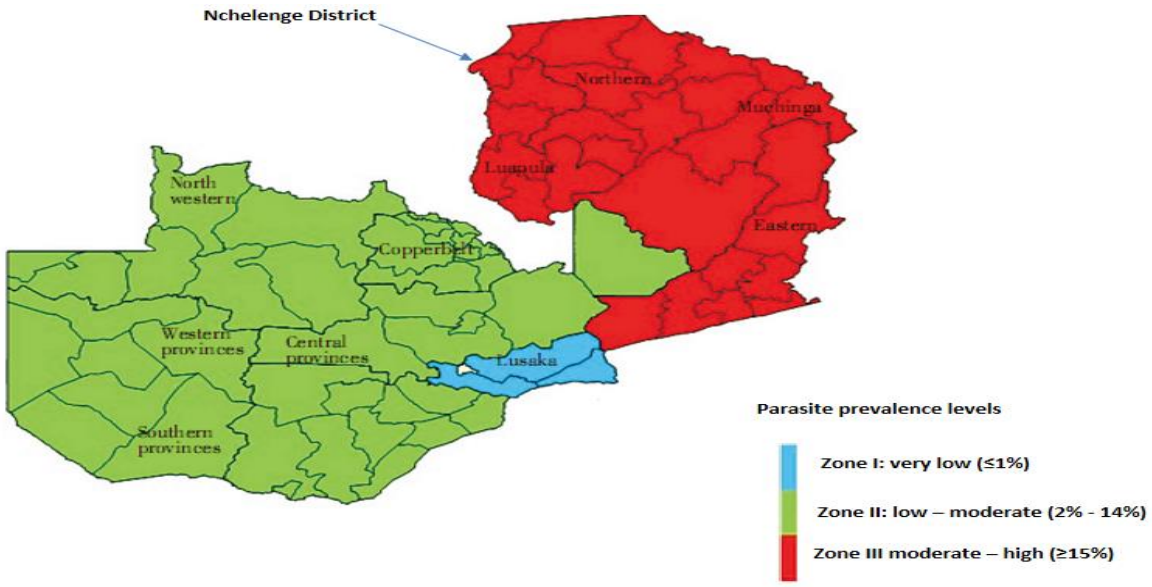


Figure 1.1: Endemicity of malaria in Zambia– from Chanda *et al.*, 2007**.**

1.5 Malaria Situation in Zimbabwe

Malaria is a public health problem in Zimbabwe. The disease contributes significantly to causes of morbidity and mortality in the 45 malaria endemic districts (out of the 62 districts in the country) (WHO, 2013a). The malaria season coincides with the rainy season from November to April with much of the precipitation falling in the Manicaland province (Lukwa *et al*., 2014). It is estimated that 50% of the Zimbabwean population live in malaria endemic areas and 95% of the cases are due to *P. falciparum* infections (WHO, 2013a). The remaining 5% is attributed to *P. malariae* and *P. ovale* infections (Taylor and Mutambu 1986; Midzi *et al*., 2004). Figure 1.2 shows the endemicity of malaria in Zimbabwe and the location of Mutasa district. Mutasa is the most affected district in the most malaria endemic Manicaland Province (Henning *et al*., 2014).

Anopheles arabiensis has been incriminated as the primary vector in the country (Lukwa *et al*., 2014). However, *An. funestus* is the predominant vector species in Mutasa district and

has been incriminated for resurgence of malaria in spite of on-going implementation of vector control interventions (Henning *et al*., 2014). The main vector control interventions in Zimbabwe are IRS and ITNs. The prevalence of malaria in Mutasa has been reported to be declining even though there is evidence of residual infection among age groups above 5 years (Mharakurwa *et al*., 2013). There is a good roll out of vector control interventions in Mutasa district albeit with little success in reduction of malaria morbidity and mortality because of the presence of pyrethroid resistant vectors (Mharakurwa *et al.*, 2013).

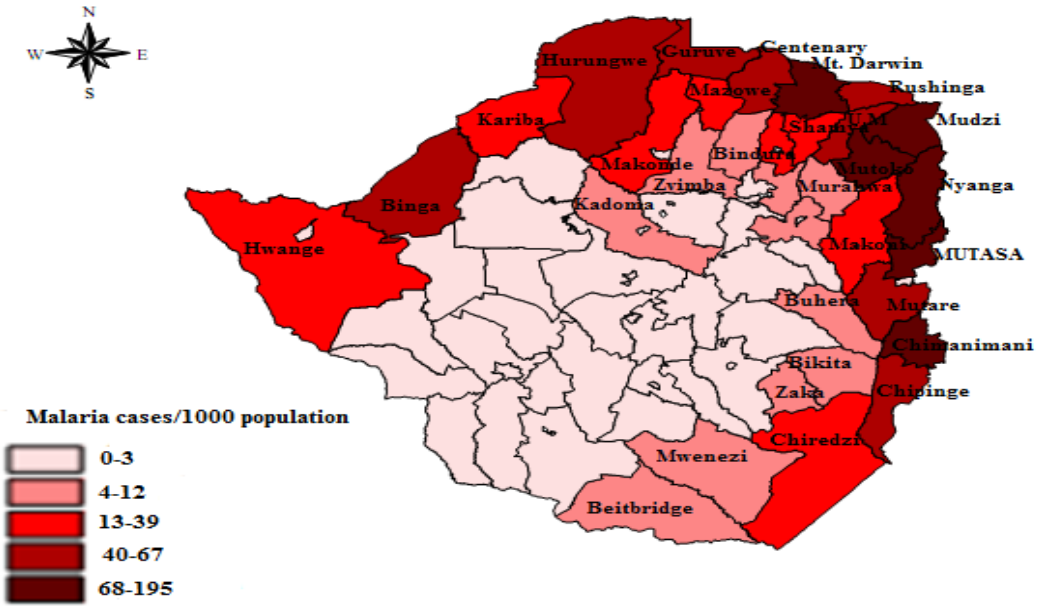


Figure 1.2: Endemicity of malaria in Zimbabwe. Most of the country is considered to be at high risk of malaria infection. Mutasa is among the districts that contribute significantly to the country's incidence of malaria (PMI Publication, 2012, http://www.pmi.gov).

1.6 Vector Control

The purpose of malaria vector control is to protect people against infective malaria mosquito bites by reducing vector density and human-vector contact. This eventually reduces the intensity of local transmission at community level and hence the incidence and prevalence of infection and disease (Gillies and Coetzee, 1987). IRS and ITNs are the current primary malaria vector control interventions (WHO, 2012).

There are four classes of insecticides currently used in the control of malaria vector mosquitoes: organochlorines, organophosphates, carbamates and pyrethroids. There are only 12 insecticides in these four classes that are World Health Organization Pesticide Evaluation Scheme (WHOPES) approved for use in IRS. At present, pyrethroid formulations constitute 50% of the WHOPES approved IRS insecticides and they are also currently the only insecticides approved for use on ITNs (WHO, 2013b). Unfortunately, there is growing evidence of insecticide resistance in many African countries where vector control with chemical insecticides is implemented. In southern Africa alone, resistance in *An. funestus* and *An. arabiensis* has been detected in Mozambique, South Africa, Zimbabwe, Zambia and Malawi (Brooke *et al*., 2001; Hargreaves *et al.,* 2000, 2003; Masendu *et al*., 2005; Casimiro *et al.*, 2006; Cuamba *et al*., 2010; Hunt *et al*., 2010; Kloke *et al.*, 2011; Choi *et al.*, 2014). Resistance to pyrethroids is a particular cause for concern as no other insecticide class can be used for impregnation of ITNs (WHO, 2013b).

IRS targets endophilic and endophagic (indoor feeding) vectors that come into contact with treated surfaces before and/or after feeding on the inhabitants. Therefore, understanding the malaria vector bionomics is very important in informing strategies for vector control programmes. *Anopheles funestus* is endophilic and anthropophilic whereas *An. arabiensis* has shown plasticity in its behaviour, feeding on both animals and humans depending on prevailing conditions (Githeko *et al*., 1996). In drier and hot regions, indoor conditions are

suitable resting sites for mosquitoes as they provide dark, humid environments (WHO, 2006). Treatment of indoor resting surfaces with insecticide would therefore result in a progressive decline in indoor vector density thus reducing overall vectorial capacity. However, the target vector species has to be susceptible to the insecticide used for achievement of impact.

Depending on specific settings and under special circumstances, core vector control interventions can be complemented by other methods, such as larval source management and a scale-up of personal protection measures. The WHO also recommends biolarviciding with the use of environmentally sound bacterial larvicides such as *Bacillus thuringiensis var. israelensis* and *Bacillus sphaericus.* Larval control is recommended in settings where mosquito breeding sites are few, easy to identify, map and treat (WHO, 2013a). Environmental management activities like drainage and vegetation clearing are also emphasised to limit breeding potential of the vectors. Communities, through advocacy and community mobilisation, are also advised to screen houses and use repellents depending on their socioeconomic status (Rozendaal, 1997).

1.7 The problem of insecticide resistance

As insecticides form the basis of vector control initiatives, insecticide resistance has a potential to paralyze the current control efforts that are significantly dependent on chemical insecticides. It is important for sound management that the susceptibility of vector populations is investigated to avoid control programme failure due to unmanageable levels of resistance. The emergence of resistance to pyrethroids in a number of target African mosquito populations has highlighted the importance of research into the effect of resistance on malaria transmission (Hemingway and Ranson, 2000).

There are two principal mechanisms assumed to be responsible for insecticide resistance. These are changes in the structure of the target site that reduces binding of the insecticide

and increases in the rate of insecticide metabolism, which lowers the amount of insecticide reaching the target site (Hemingway and Ranson, 2000). Even though these are the primary mechanisms conferring resistance, consideration of the physiological or behavioral changes in the mosquito population might impact on the efficacy of insecticide based interventions. Thicker cuticles on pyrethroid tolerant or resistant *An. funestus* females (Wood *et al.*, 2010) and behavioral avoidance of insecticide treated surfaces have been recognized even though largely overlooked (Ranson *et al*., 2011). *Anopheles arabiensis* populations in Ethiopia portray evidence of increased exophily with the use of DDT for IRS in the last 40 years (Gatton *et al*., 2013).

1.7.1 Insecticide Resistance in *An. funestus*

Pyrethroid resistance in southern African *An. funestus* populations is attributed to increased levels of P450 monooxygenases (Brooke *et al*., 2001). CYP6P9 and CYP6P13 genes have been identified as being over-expressed in 3-10 days old pyrethroid resistant *An. funestus* females (Amenya *et al*., 2008, Christian *et al*., 2011). Insecticide exposures, synergist and biochemical assays conducted on pyrethroid resistant *An. funestus* strains from northern KwaZulu-Natal (South Africa) and Beluluane (Mozambique), were the first studies to implicate elevated levels of mixed function oxidases (monooxygenases) for the detoxification of pyrethroids in those resistant populations (Brooke *et al*., 2001). The thickness of the cuticle also plays a role in limiting insecticide penetration thus contributing to tolerance (Wood *et al*, 2010). Since its first detection in South Africa in 1999 (Hargreaves *et al*., 2000), pyrethroid resistant *An. funestus* has spread northwards in Mozambique (Cuamba *et al.*, 2010; Kloke *et al*., 2011), Malawi (Hunt *et al*., 2010), Zambia and Zimbabwe (Choi *et al.*, 2014).

1.7.2 Insecticide Resistance in the *An. gambiae* complex

The first report of insecticide resistance in *An. gambiae s.l.* was made in the 1950s when some populations in northern Nigeria were reported to be resistant to organochlorines (Davidson, 1956). Several reports have been published on resistance in this group in southern Africa. In 1974, the Zimbabwe population of *An. arabiensis* was reported resistant to organochlorines (Green, 1981). Populations in South Africa and Mozambique were reported resistant to pyrethroids with cross resistance to carbamates (Hargreaves *et al.,* 2003; Casimiro et al., 2006). Further reports of DDT resistance in a Zimbabwe population of *An. arabiensis* were made by Masendu *et al.,* (2005). Munhenga *et al*., (2008) revealed high levels of monooxygenases, glutathione S-transferases and general esterases as responsible for pyrethroid and DDT resistance in Gwave, Zimbabwe population of *An. arabiensis*. Munhenga and Koekemoer (2011) discovered the up-regulation of CYP6Z1, CYP6Z2, and CYP6M2 P450 genes as responsible for pyrethroid resistance in KWAG-Perm; an *An. arabiensis* laboratory selected strain colonised from material collected in KwaZulu Natal, South Africa.

1.8 Mitochondrial clades in *Anopheles funestus* and role in malaria transmission

There is a need to understand the potential role played by vector populations in malaria transmission and the implications for operational purposes with respect to insecticide resistance (Coetzee and Koekemoer, 2013). It is important to understand the biological differences, if any, between the two clades as they may represent incipient species, both originating from a single highly efficient, highly anthropophilic vector. It is also important to understand the complex and non-equilibrium genetic structure among vector populations to inform control strategies. Two chromosomal forms of partially isolated *An. funestus* populations that exhibited variation in biting and resting behavior, human blood index and

circumsporozoite protein positivity rate were first discovered in Burkina Faso (Costantini *et al*., 1999). Another study revealed significant variation in the mitochondrial DNA (mtDNA) of the two sympatric Burkina Faso *An. funestus* populations, using sequences in the mitochondrial *ND5* gene (Michel *et al*., 2005a). Mitochondrial DNA lineages, designated clade I and clade II in Mozambique, Madagascar and Malawi *An. funestus* populations were differentiated by two fixed differences and an average of 2% divergence, which was thought to indicate that they have evolved independently for approximately 1 million years (Michel *et al*., 2005b; Choi *et al*., 2012). Choi *et al.* (2014) reported the existence of both *An. funestus* clades in Nchelenge District in Zambia but only clade I from the Mutasa district in Zimbabwe. These populations of *An. funestus* are incriminated as malaria vectors and reported to be resistant to pyrethroids and carbamates, but susceptible to organochlorines and organophosphates (Choi *et al*., 2014).

1.9 Resistance Monitoring

The current WHO test procedures for insecticide resistance monitoring in malaria vector mosquitoes stipulate a one hour exposure to diagnostic dosages (except for fenitrothion with a 2 hour exposure period) with a 24 hour recovery period (WHO, 2013b). The rationale is that 100% mortality will be achieved when a susceptible mosquito population is exposed to the diagnostic concentration. A mortality of less than 98% is suggestive of resistance and requires further investigation (WHO, 2013b). If the observed mortality ranges between 90 and 97%, additional bioassay test must be conducted on the same population to confirm the presence of resistant genes. An observed mortality of less than 90% confirms the presence of resistance genes and additional tests may not be necessary provided a minimum of 100 adult females of a species were tested. Provided resistance is detected, investigations for mechanisms and distribution of resistance of the vector populations must be conducted to inform decision making on resistance management.

This protocol only detects the existence of resistance genes in a population but does not give insight on resistance intensities. Resistance intensity tests would give an informed estimate of lethal time (e.g. LT_{50}) of WHO discriminating dosages of a particular insecticide on a resistant population. An adequate exposure time is crucial for evaluation of effectiveness of insecticides (Athanassiou *et al.* 2005). Resistant vector mosquitoes that portray both endophilic and endophagic characteristics, like *An. funestus*, are more likely to be exposed for more than one hour in the event they rest on treated surfaces. There is a need, therefore, to carry out resistance intensity studies in the event that resistance is detected, to evaluate the level of resistance in the population and predict its impact on programme failure.

1.10 Rationale for the study

The distribution of ITNs combined with limited IRS and effective case management has not significantly suppressed the incidence of malaria in Nchelenge District in Zambia nor in Mutasa District (Honde Valley) in Zimbabwe (Choi *et al*., 2014; Mharakurwa *et al*., 2013). Pyrethroid resistant *An. funestus* populations in both localities have contributed to the failure of insecticide based control interventions in these two areas. In as much as resistance is a threat to malaria vector control, it is important to investigate resistance intensities in the event resistance is detected to quantify the magnitude of the insecticide resistance. Comparisons with well characterised laboratory strains will provide data on the magnitude of resistance intensities in the two study areas.

1.10.1 Broad objective

The study proposal was aimed at investigating insecticide resistance intensity in the F_1 progeny from wild caught *An. funestus* from Nchelenge District (Zambia) compared to a pyrethroid resistant *An. funestus* laboratory strain established from material collected in Mozambique in 2002. Although the original study scope was limited to material collected from Zambia, an opportunity arose to expand the study to *An. funestus* material collected

from Honde Valley in the Mutasa District (Zimbabwe) and to include two *An. arabiensis* colonies named MBN-DDT and SENN-DDT.

1.10.2 Specific Objectives

1. To examine the intensity of pyrethroid and carbamate resistance of the two distinct mitochondrial lineages (clade I and clade II) of *An. funestus* originating from Zambia and Zimbabwe.

2. To examine the intensity of pyrethroid and carbamate resistance of *An. funestus* laboratory strains and compare them to the intensities observed in wild caught *An. funestus* strains.

3. To examine the intensity of pyrethroid and carbamate resistance of two *An. arabiensis* laboratory strains artificially selected for high levels of DDT resistance under insectary conditions.

CHAPTER 2

MATERIALS AND METHODS

Wild samples of *An. funestus* were collected by Professor Richard H. Hunt and teams from Nchelenge in Zambia and Honde Valley in Zimbabwe between March and April 2014. Honde Valley is along the Zimbabwe and Mozambique border and Nchelenge is located along the Zambia and Democratic Republic of Congo border (Figure 2.1).



Figure 2.1: Location of the two sampling sites; Nchelenge in Zambia and Honde Valley in Zimbabwe. The collection sites are marked with green icons.

(www.nationsonline.org/oneworld/map/google_map).

2.1 Indoor resting collections

Indoor resting collections of adult *Anopheles* mosquitoes were conducted in rural houses located in Nchelenge District, in Zambia and Honde Valley, in Zimbabwe's Mutasa District. Mosquitoes were caught using manual aspirators with the aid of torches for visualization. These collections were carried out in collaboration with the Zambia Tropical Diseases

Research Centre (TDRC) in Ndola and the National Institute for Health Research, Harare, Zimbabwe. A variety of specific locations which were identified by a local entomologist to have been particularly productive during the 2013 season were revisited. Generally, two sites were visited each day. The first site was visited in the morning at around 09.00hrs, and the second in the afternoon at approximately 13.00hrs. The time spent at each site varied, but lasted two to three hours on average.

Sites identified as more productive during the current collection period were visited on multiple occasions. Interior walls and thatch-roofs were thoroughly searched by torchlight after obtaining permission from house owners. *Anopheles* mosquitoes were collected and sorted morphologically to Funestus group (Gillies and Coetzee, 1987). The *An. funestus* live specimens were then stored in netted plastic cups and given *ad libitum* access to cotton wool dampened with a 10% sucrose solution. At the end of the collection period, the netted plastic cups containing live *An. funestus* were carefully sealed and stored in a backpack for convenient transportation by flight to South Africa. On arrival, the specimens were taken to the Botha De Meillon Insectary at the National Institute for Communicable Diseases (NICD) in, Sandringham, Johannesburg, where subsequent work was carried out.

2.2 Rearing the F_1 generation

Wild-caught females were individually placed into gauze-sealed egg laying glass vials containing a damp piece of filter paper fitted at an angle at the bottom to serve as an oviposition site (Figure 2.2a). The mosquitoes were given *ad libitum* access to 10% sucrose-dampened cotton wool and provided with a blood meal from a guinea pig source twice a week. Monitoring for laid egg batches was done daily. If eggs were present, the filter papers (with eggs laid on it) were carefully removed from the glass vials and placed in separate bowls containing distilled water. Similar codes were allocated for mothers and eggs.

The larvae were fed twice a day on a diet of powdered dog biscuits and brewer's yeast prepared in a ratio of 3:1 as described in Hunt *et al*. (2005). Adults constituting the F_1 generation, having emerged in the larval bowls, were removed and placed in cages where they were given *ad libitum* access to cotton wool dampened in 10% sucrose solution. The adult F_1 progeny from similar clades were pooled into cages to conduct resistance intensity assays.

If the wild-caught adult female in the egg laying vial was found dead, she was removed and stored on silica gel for later processing by PCR. If alive, the adult female was carefully removed during egg transferral and placed into a new tube and offered a blood meal.

The glass vials measuring 45 mm (height) and 25 mm (diameter) that were used to rear individual wild collections of *An. funestus* are shown in Figure 2.2. The vials are held in wooden racks for ease of handling. This method is efficient for promoting oviposition and results in ≥70 % of females ovipositing (Hunt and Coetzee, 1989; Choi *et al*., 2014).



Fig 2.2: Experimental set up of egg laying vials for wild material. A wild-caught female in a glass egg laying vial was allowed to oviposit on a moistened filter paper (A). Egg laying vials are packed into racks to facilitate ease of handling and blood feeding (B).

All wild *An. funestus* group females were identified to species level according to the protocol of Koekemoer *et al*. (2002). Specimens confirmed to be *An. funestus* were further identified to clade level by the hydrolysis probe assay of Choi *et al*. (2013).

2.3 PCR Identification for *An. funestus* group species

Legs or wings were detached from mosquitoes for DNA extraction. A DNA extraction kit (Zygem, New Zealand) was used to extract DNA from samples. The reaction was performed as per manufacturer's specifications. The detached legs or wings were mixed with reagents in labelled PCR tubes and incubated at 75°C for 15 minutes followed by 95°C for 5 minutes. The extracted DNA was subsequently used for species identification following an optimised method by Koekemoer *et al*. (2002).

The PCR reaction mix was prepared using the following final concentrations of reagents in a final volume of 12.5µl: 1X PCR buffer, 1.5mM deoxyribonucleotide (dNTPs), 1.5mM Magnesium Chloride ($MgCl_2$), 1.32pM of each primer (i.e. universal, *An. funestus, An. leesoni, An. parensis, An. rivulorum and An. vaneedeni*), 0.5U of taq polymerase and 2.4µl deionised water. A concentration of 20-200ng of sample DNA was used as template for the reaction. *Anopheles funestus* positive control reactions consisted of a PCR reaction containing 20-200ng of DNA extracted from an *An. funestus* laboratory strain. Two negatives controls were used: a reaction mix without any DNA served as a no template control, and a reaction using an extraction negative control served as an extraction control. These controls ensured there was no contamination in the master mix (no template control) and DNA extraction (extraction control).

All tubes were centrifuged for 5 seconds at 10,000xg to ensure homogenous mixture. Specimens were amplified in an i-Cycler™ thermocycler (Bio-Rad). The cycling conditions were as follows: a 94°C denaturing step for 2 minutes, and thirty cycles of 94°C for 30 seconds, 45°C for 30 seconds and 72°C for 40 seconds. The final step was the extension step set at 72°C for 5 minutes. Then all PCR amplicons were electrophoresed on a 2.5% Tris-Acetate-EDTA (TAE) gel containing ethidium bromide for 90 minutes at a constant voltage of 100V. Band sizes were sized using an O-Range™ 100 base pair ruler (Fermantas-

FESM0623). The samples were then visualised using a ChemiBox Gel documentation system (Syngene).

2.4 PCR Identification for *An. funestus* clades

An. funestus can be divided into two clades (i.e. clade I and clade II) based on the sequence identity of the mtDNA *COI* gene (Michel *et al*., 2005b). All samples were then processed for clade identification according to Choi *et al*. (2013). The technique utilizes single nucleotide polymorphism (SNP) (TaqMan™ hydrolysis probe SNP genotyping) and detects clades I and II nucleotides in *An. funestus.* The sequence for the probe identifying clade I is 5′- TCA GGA ATT **GCT** CAT GCT -3′ and clade II is 5′- TCA GGA ATT **GCC** CAT GCT -3′.

For each test, a PCR hydrolysis probe assay set-up composed of 1X IQ Supermix, Forward primer (10µM), 10µM Reverse primer, 10 µM Clade I probe, 10µM Clade II probe, made up to a volume of 20µl with PCR grade water with 20-200ng of extracted DNA used as template. DNA extracted from standard identified *An. funestus* clade I and clade II individuals served as positive controls and samples without DNA served as negative controls. The specimens were subjected to a cycle of 95°C for 10 minutes denaturing step followed by 45 cycles of 95°C for 10 seconds annealing step and 63°C for 45 seconds extension step. These reactions were performed in a c1000^{tm} thermal cycler (Biorad). The results were interpreted based on the differential detection of fluorescence. A 6-FAM probe was used to detect *An. funestus* clade I and a VIC probe detects *An. funestus* clade II. Fluorescence of either these probes is indicative of positive amplification of the specimen. Sample results are only accepted when the amplification curve for samples is substantially higher than the baseline curve, all positive controls amplified and no amplification on negative control.

2.5 Insecticide resistance intensity assays

Females ranging between 2 and 5 days of age were used for resistance intensity tests (WHO, 2013b). Exposures were conducted using WHO papers impregnated with the pyrethroid, deltamethrin (0.05%) and the carbamate, bendiocarb (0.1%) (Figure 2.3). For each experimental run, a minimum of 100 female mosquitoes of each clade were exposed in 4 replicate standard WHO bioassay tubes (i.e. 25 female mosquitoes per tube) (Figure 2.3c). In addition, *An. funestus* laboratory strains; FUMOZ-R, FUMOZ-BASE and FANG were also tested for resistance intensities to the same insecticides. *Anopheles arabiensis* laboratory strains, SENN-DDT and MBN-DDT, were also tested for deltamethrin and bendiocarb resistance intensities.

The baseline pyrethroid resistant strain, FUMOZ-BASE was established in 2001 from materials collected in southern Mozambique. FUMOZ-R was selected for high pyrethroid resistance from FUMOZ-BASE. FANG was established from material collected from southern Angola in 2003 and is fully susceptible to all insecticides.

SENN-DDT was selected for DDT resistance from SENN-BASE starting from 1995. SENN-BASE was colonized in the 1980s from materials collected in the Sudan´s Sennar region. Similarly, MBN-DDT was selected from MBN-BASE which was established from material collected in Kwa-Zulu Natal province in South Africa in 2002. SENN-BASE and MBN-BASE are susceptible to insecticides.

Unexposed and exposed controls were set up for each experimental run. For *An. funestus* experiments, two unexposed controls were set by exposing 25 FANG females and 25 females of the test strain to untreated papers in standard WHO bioassay tubes. For exposed/susceptible controls, 50 FANG females were exposed to WHO treated papers (25 females per tube). The same protocol was followed for *An. arabiensis* strains, using MBN-BASE and SENN-BASE as susceptible controls.

The mosquitoes were acclimatised for one hour in holding tubes prior to exposure. After an hour, mosquitoes were transferred into exposure tubes which were then laid horizontally (Figure 2.3d). Mosquitoes were exposed to the insecticide for 8 hours with knockdowns scored at various intervals during the 8 hours. The time intervals are described in the data sheet provided in Appendix I. The 8 hour exposure period was set based on the estimated time that resistant populations might rest on treated surfaces after taking a blood meal (Choi *et al*., 2014).

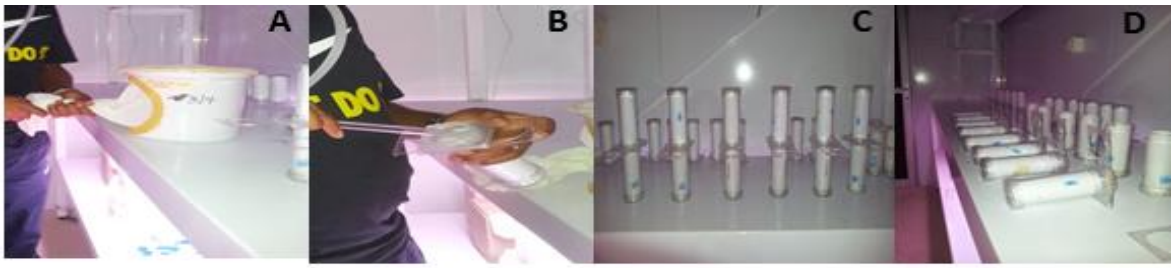


Figure 2.3: Experimental set up and resistance intensity assay procedure. Female mosquitoes were aspirated from cages (A), then introduced into WHO holding tubes (B) and allowed to rest for 1 hour in holding tubes to acclimatise before exposure (C). Resistance intensity assays exposures progressed with the bioassay tubes laid horizontally to ensure that knocked-down individuals continued to receive an insecticide exposure even after knockdown (D).

To determine the importance and difference of resistance intensity experiments from the standard WHO bioassays, an experiment on standard WHO bioassay was conducted on FUMOZ-BASE and FUMOZ-R. Three experimental runs were conducted with 300 females of a strain. For each run, 100 females of a test strain were exposed in four replicate standard WHO bioassay tubes (i.e. each tube holding 25 females). Exposed/susceptible controls were set up using 50 FANG females in 2 replicate standard WHO bioassay tubes. Unexposed controls were composed of one tube with 25 females of the test strain and the other with 25 FANG females. The test females and exposed controls were allowed to rest and acclimatise

for one hour in holding tubes before exposure. After one hour acclimatization period, females were transferred to exposure tubes with 0.05% deltamethrin WHO impregnated papers and tubes were left standing upright. The mosquitoes were left in the exposure tubes for one hour and then transferred back to holding tubes and then given access to 10% sucrose solution. Mortality scores were made 24 hours post exposure.

2.6 Data Analysis

The data were analysed using standard statistical tools including probit analysis (Finney, 1952) to determine the time taken to achieve 50% knockdown (LT_{50}), one way ANOVA to determine difference between mean knockdowns for different strains. A significance level of 5% was set for analysis (i.e. $p \leq 0.05$). Statistics programmes used were Microsoft excel, SPSS version 20 (SPSS Inc., Chicago, USA) and Statistix 8 (Statistics 8 – Talahasee, F1, USA). Resistance intensity ratios (RIR_{50}) were calculated by computing the quotients of resistance intensities (LT_{50}s) of the resistant strain and the corresponding susceptible strain i.e. LT_{50} (resistant strain)/ LT_{50} (susceptible strain).

CHAPTER 3

RESULTS

3.1 Species identification

A total of 471 wild caught females from Zambia and Zimbabwe laid eggs and were identified as *An. funestus* by PCR (Koekemoer *et al*., 2002) (Figure 3.1). The TaqMan™ assay used to identify the mitochondrial clades (Choi *et al*., 2013) showed 79.5% *An. funestus* clade I and 20.5% clade II in Zambian samples. The Zimbabwe samples were 100% *An. funestus* clade I (Table 3.1).

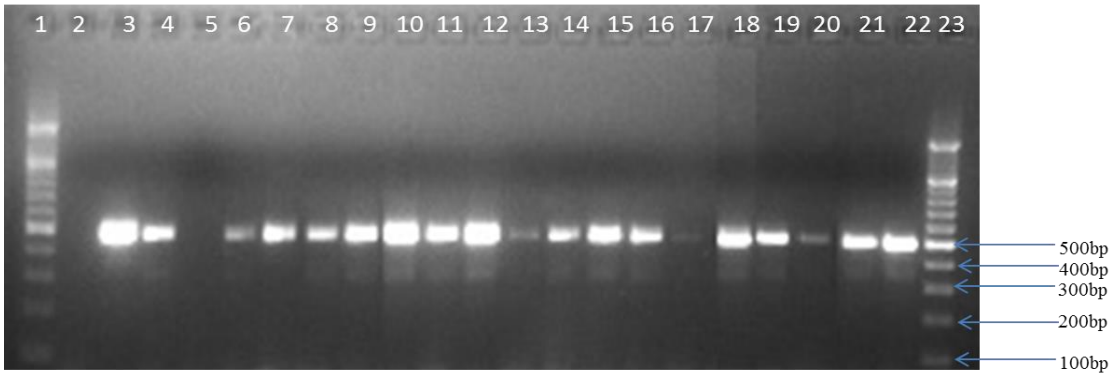


Figure 3.1: *Anopheles funestus* species-specifc PCR for samples collected in Honde Valley, Zimbabwe. Lanes 1 and 23 are molecular weight markers. Lanes 2 and 5 are negative controls. Lane 3 (500 bp) is *An. funestus* positive control. All the amplicons were identified as *An. funestus*.

Table 3.1: *Anopheles funestus* clades identified from collections made in Zambia and Zimbabwe (February-March 2014).

		Anopheles funestus clade families raised	
Locality	n	Clade I	Clade II
Zambia	332	265 (79.5)	67 (20.5%)
Zimbabwe	139	139 (100)	-

3.2 Knockdown trends over 8 hour exposure on deltamethrin and bendiocarb

Deltamethrin and bendiocarb knockdown trends are shown in Figures 3.2 and 3.3 for the *An. funestus* strains and Figures 3.4 and 3.5 for the *An. arabiensis* strains. The susceptible strains used as controls (FANG, SENN-BASE and MBN-BASE) showed similar knockdown trends and all reached a 100% knockdown on both insecticides within 1 hour of exposure. FUMOZ-R presented the lowest knockdowns of all *An. funestus* strains for both deltamethrin and bendiocarb (Figures 3.2 and 3.3 respectively). FUMOZ-BASE on the other hand had higher knockdowns on deltamethrin than all wild caught strains and FUMOZ-R but fewer knockdowns than wild-caught strains on bendiocarb (Figures 3.2 and 3.3 respectively). Difference in knockdown percentage amongst wild caught strains was observed on deltamethrin but not on bendiocarb.

The *An. arabiensis* DDT resistant strains showed varying knockdown trends for deltamethrin. SENN-DDT showed higher knockdowns than MBN-DDT on deltamethrin resistance intensity exposures (Figure 3.4). As for bendiocarb resistance intensity exposures, both SENN-DDT and MBN-DDT showed no difference to the susceptible SENN-BASE and MBN-BASE (Figure 3.5).

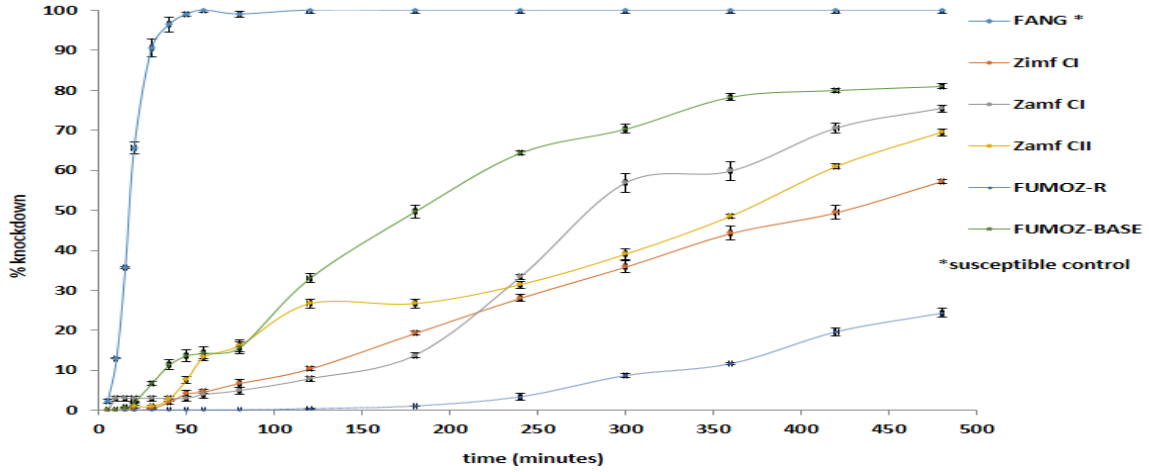


Figure 3.2: Deltamethrin induced knockdown trends in wild caught and laboratory *An. funestus* over an 8 hour exposure period. The susceptible FANG strain reached 100% knockdown within 1 hour, while none of the other (i.e. resistant strains) test strains were fully knocked down by 8 hours. The FUMOZ-R strain showed the lowest knockdowns over time. Wild-caught strains (Zimf CI, Zamf CI, Zamf CII) displayed knockdown rates lower than the unselected FUMOZ-BASE strain.

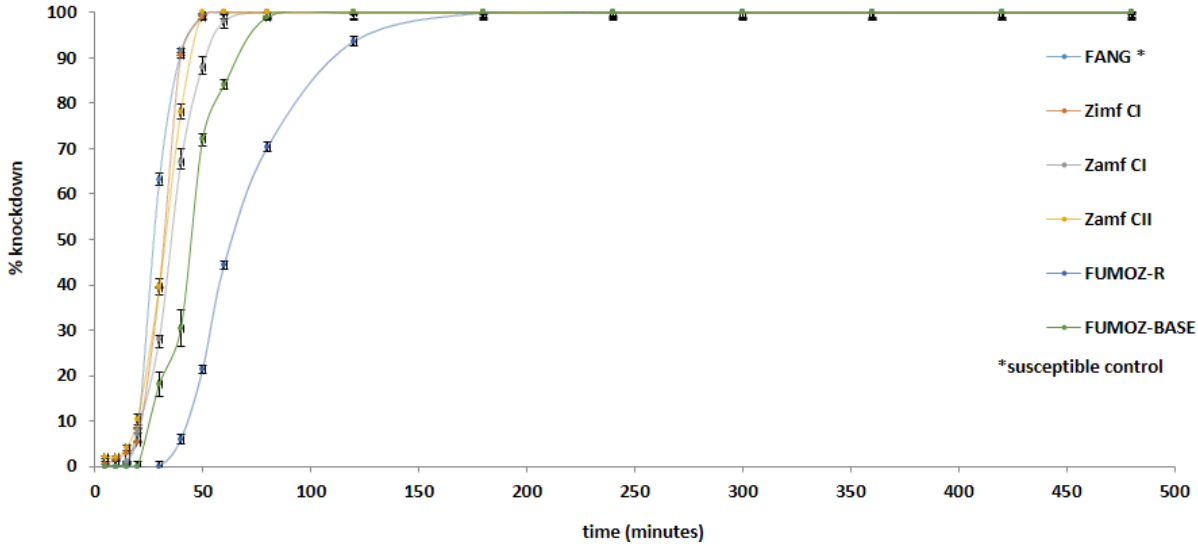


Figure 3.3: Bendiocarb induced knockdown trends in wild caught and laboratory *Anopheles funestus* over an 8 hour exposure period. All the strains reached 100% knockdown by 3.33 hours. The FUMOZ-R strain displayed the slowest knockdown rates, followed by FUMOZ-BASE, while the wild strains (Zimf CI, Zamf CI, Zamf CII) displayed knockdown rates similar to the susceptible FANG strain.

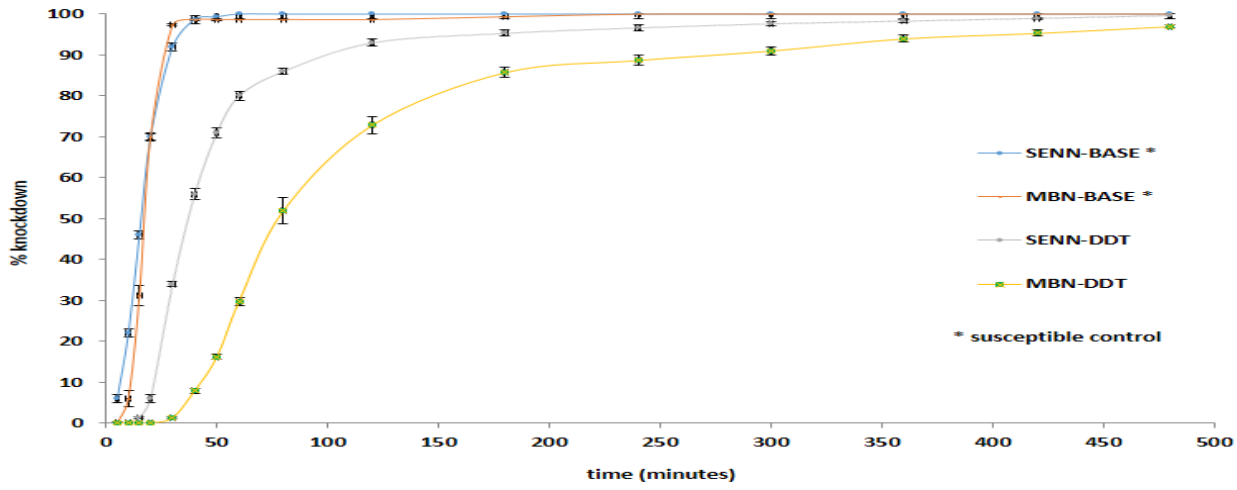


Figure 3.4: Deltamethrin induced knockdown trends in laboratory *An. arabiensis* over 8 hour exposure period. Susceptible laboratory strains (SENN-BASE and MBN-BASE) portrayed similar knockdown trends and 100% knockdown was reached in l hour. SENN-DDT displayed higher deltamethrin knockdown trend than MBN-DDT. However, the two resistant strains showed no difference to sucsceptible strains post the 6th hour of exposure.

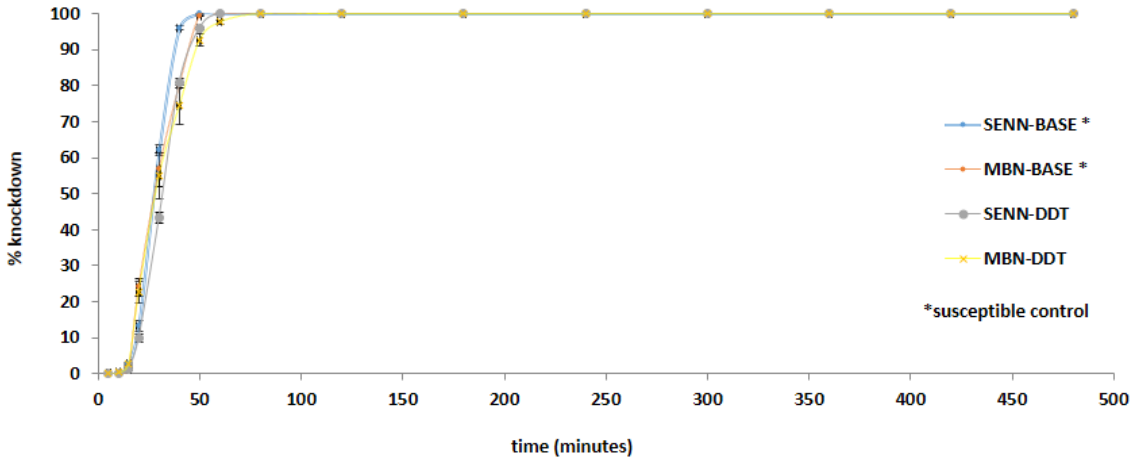


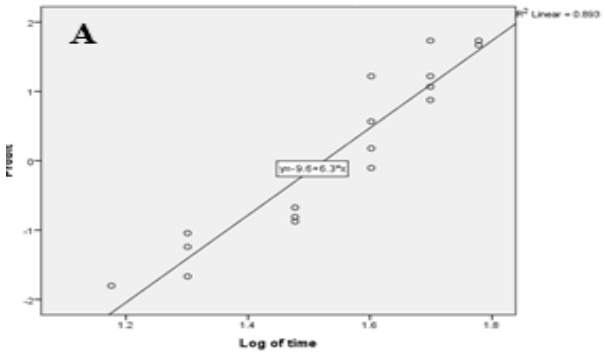
Figure 3.5: Bendiocarb induced knockdown trends in laboratory *An. arabiensis* over 8 hour exposure period. Pyrethroid susceptible laboratory strains (SENN-BASE and MBN-BASE) showed similar knockdown trends. Even though MBN-DDT has cross resistance to bendiocarb (Nardini *et al*., 2012), it's resistance intensity knockdown trends do not show difference to bendiocarb susceptible strains.

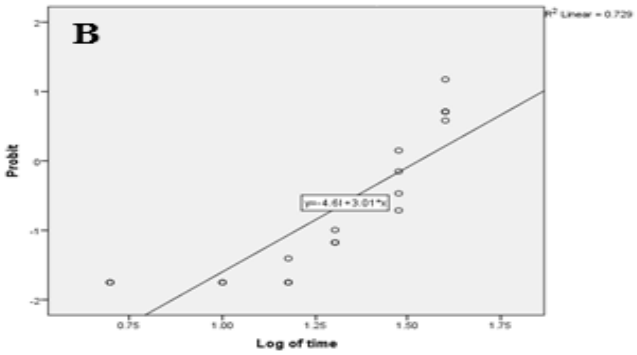
3.3 Probit analysis for measure of resistance intensity.

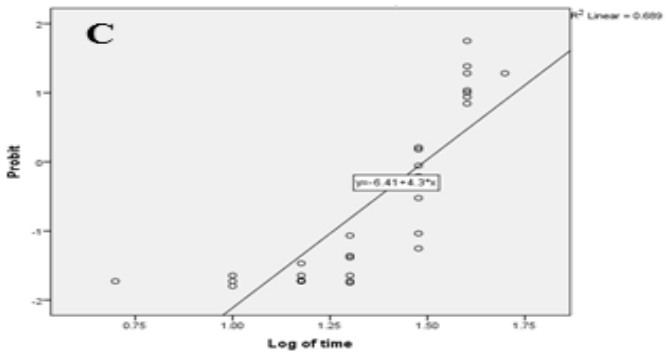
Log-probit graphs were generated with equations for lines of best fit using SPSS regression analysis. The same program was used to compute LT_{50}s and R-square (R^2) values. Figure 3.5 and 3.6 are the log-probit graphs for all the laboratory and wild strains tested for resistance intensities on bendiocarb and deltamethrin respectively. The probit transformed graphs show the strength of correlation between time and knockdown (R^2) for strains exposed on bendiocarb and deltamethrin discriminating dosages. An arbitrary scale was set denoting R-square values below 0.5 as illustrative of weak correlation; 0.5 to 0.7 as strong correlation and $\geq$ 0.7 to 1.0 as very strong correlation.

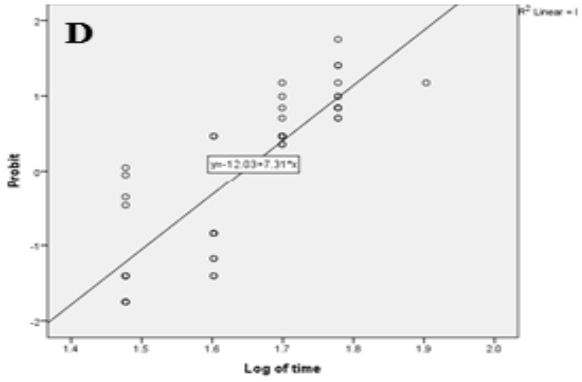
All laboratory and wild strains showed a very strong correlation between time and knockdown on bendiocarb exposures except for Zimf clade I and MBN-DDT that showed strong correlation. The highest R-square value was 0.93 on SENN-DDT and the least was 0.568 on MBN-DDT (Figure 3.6).

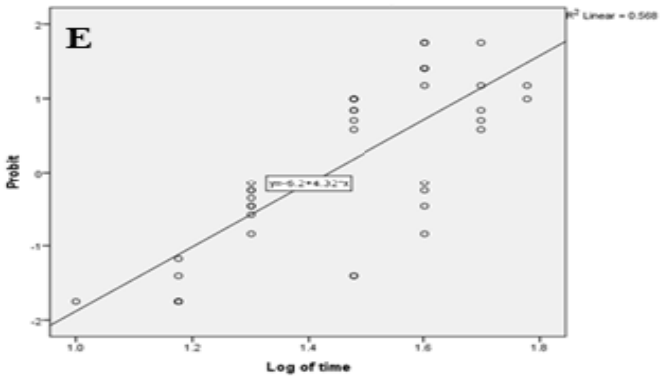
Figure 3.6: **Probit transformed bendiocarb induced knockdowns as a function of time.** Probit transformed mortality was plotted as a function of time for wild caught and laboratory strains. A) Zamf clade I, B) Zamf clade II, C) Zimf clade I, D) FUMOZ BASE, E) FUMOZ-R, F) MBN-DDT and G) SENN-DDT. SENN-DDT displayed the strongest linear relationship between time and bendiocarb-induced mortality, while FUMOZ-R displayed the weakest linear relationship between time and bendiocarb-induced mortality.

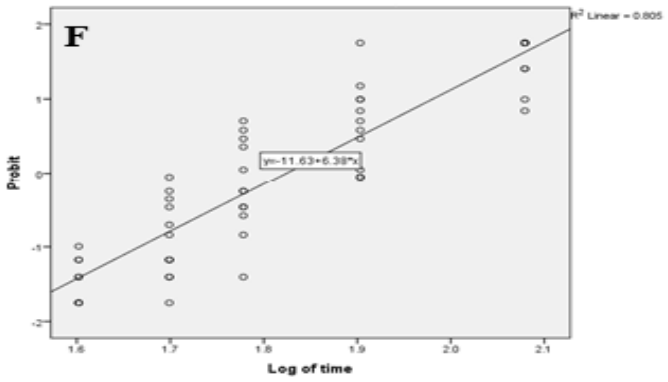

A
y=-9.6+6.3*x
Log of time


B
Probit
y=-4.61+3.01*x
Log of time


C
Probit
y=-6.41+4.3*x
Log of time


D
Probit
y=-12.03+7.31*x
Log of time


E
Probit
y=-6.2+4.32*x
Log of time


F
Probit
y=-11.63+6.38*x
Log of time

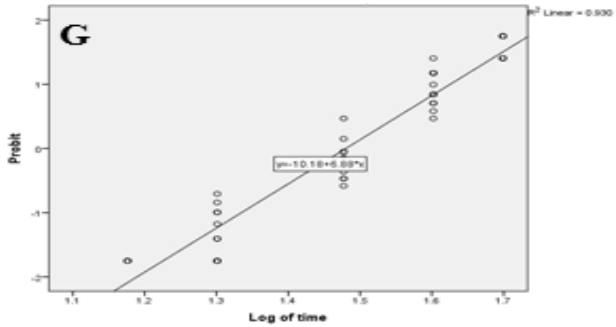
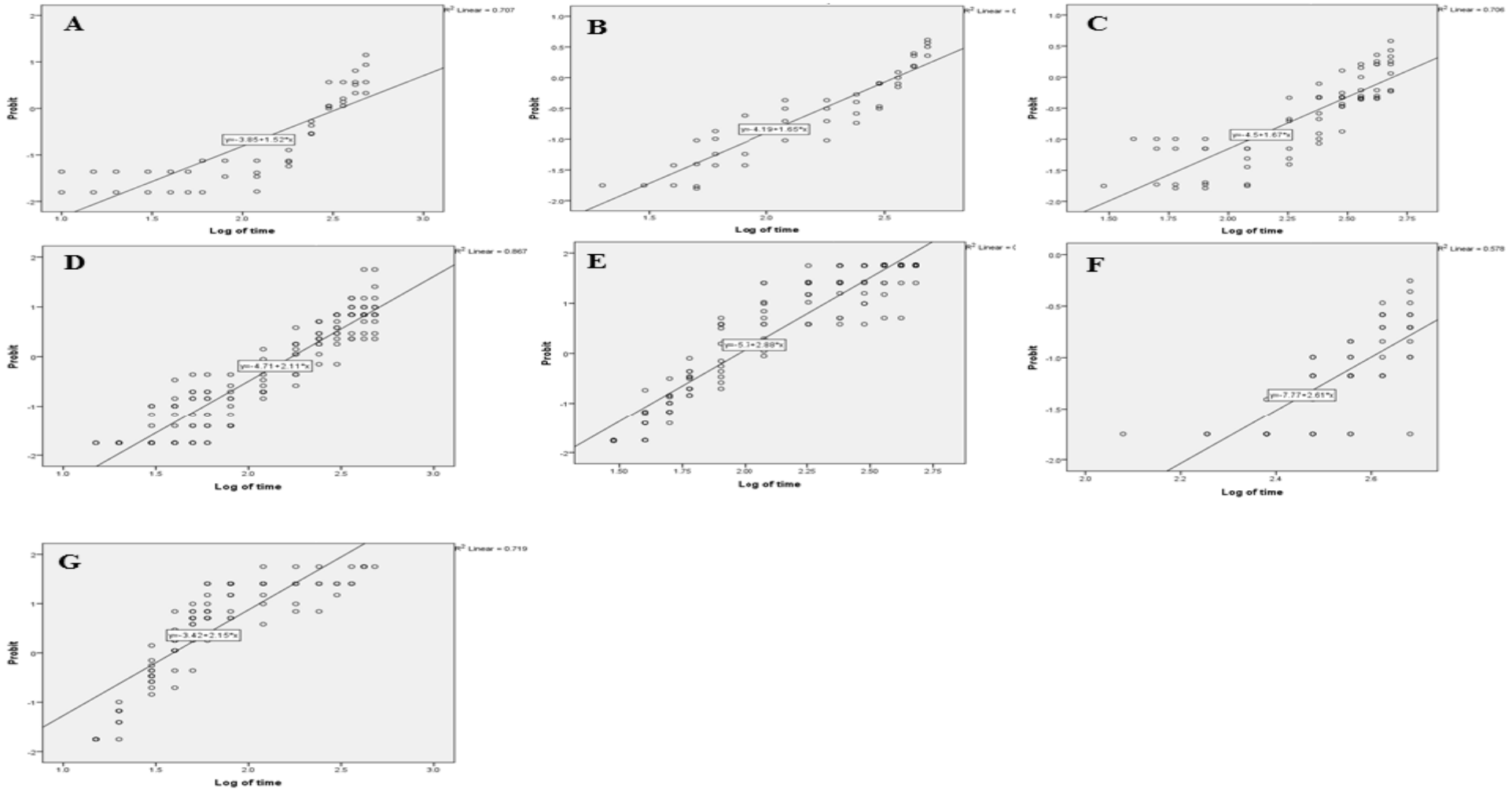

G
Probit
y=-10.18+6.69*x
Log of time

Figure 3.7: Probit transformed deltamethrin induced knockdowns as a function of time. Probit transformed mortality was plotted as a function of time for wild and laboratory strains. A) Zamf clade I, B) Zamf clade II, C) Zimf clade I, D) FUMOZ BASE, E) FUMOZ-R, F) MBN-DDT and G) SENN-DDT. MBN-DDT displayed the weakest linear relationship between time and deltamethrin-induced mortality, while FUMOZ-R and FUMOZ-BASE had strong linear time-mortality relationships.


A
B
C
D
E
F
G
Probit
Log of time

Only FUMOZ-R showed a strong correlation between time and knockdown (R-square = 0.578) on deltamethrin exposure. All the other strains had R-square values above 0.70 which indicated a very strong correlation between time and deltamethrin induced knockdown rates (Figure 3.7).

3.4 Deltamethrin and bendiocarb resistance intensities for different strains

The time taken to achieve 50% mortality (LT_{50}) (as a measure of resistance intensity) was calculated from log-probit graphs equations. The replicate LT_{50s} were used to test for level of significance between different strains. The bar graphs (Figure 3.8 and 3.9) show difference in resistance intensities on wild and strain strains.

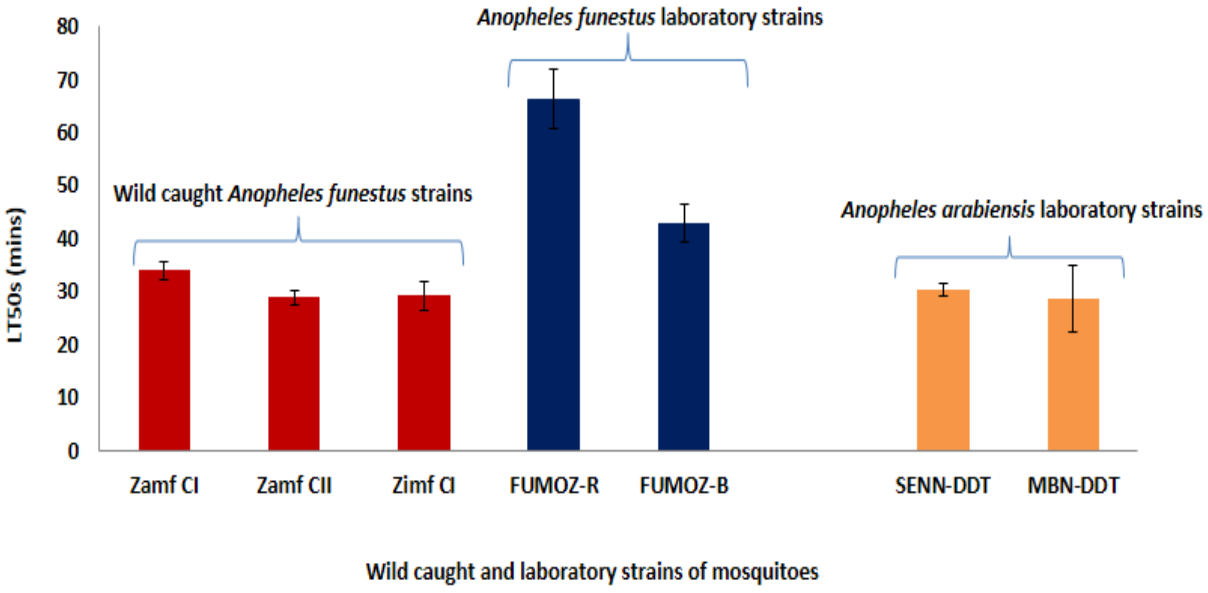


Figure 3.8: Bendiocarb lethal times of wild caught and laboratory *Anopheles* strains. Wild caught *An. funestus* strains dark red were compared to pyrethroid resistant laboratory strains dark blue and DDT resistant *An. arabiensis* laboratory strains, represented with orange bars. There were no differences in bendiocarb lethal times amongst wild *An. funestus* strains and *An. arabiensis* strains. Laboratory *An. funestus* strains had higher lethal times than wild strains and *An. arabiensis* strains. FUMOZ-R had higher lethal times than FUMOZ-BASE.

There were no differences in bendiocarb resistance intensities between wild caught *An. funestus* strains (ANOVA: P=0.070; DF= 2; F=3.68). FUMOZ-R was significantly higher than FUMOZ-BASE (ANOVA: P=0.013; DF=1; F=17.9). Laboratory *An. funestus* strains were significantly higher than wild strains (ANOVA: P= 0.0002; DF=4; F=25.4) and laboratory *An. arabiensis* strains (ANOVA: P= 0.0012; DF= 3; F= 15.0). There was no difference between wild caught *An. funestus* strains and laboratory *An. arabiensis* (ANOVA: P= 0.5; DF= 4; F= 0.89). There was also no difference between laboratory *An. arabiensis* strains (ANOVA: P= 0.95; DF= 1; F= 0) (Figure 3.8).

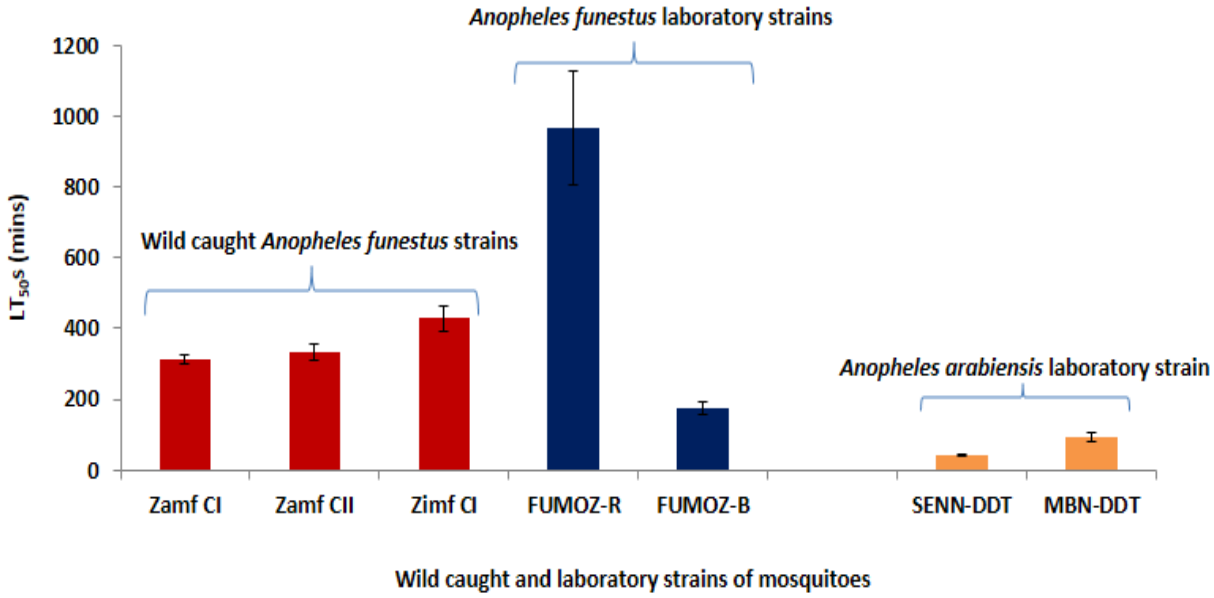


Figure 3.9: Deltamethrin lethal times of wild caught and laboratory *Anopheles* strains. Wild caught *An. funestus* strains dark red are compared to laboratory *An. funestus* strains dark blue and laboratory *An. arabiensis* strains represented with orange bars**.** The Zimbabwe wild *An. funestus* strain (Zimf CI) had higher deltamethrin induced lethal times than Zambia strains (Zamf CI and Zamf CII). There was no difference in deltamethrin induced lethal times between the Zambia strains (Zamf CI and Zamf CII). FUMOZ-R had higher deltamethrin induced lethal time than FUMOZ-BASE and *An. funestus* wild caught

strains. All *An. funestus* wild caught strains had higher deltamethrin induced lethal times than FUMOZ-BASE. Laboratory *An. arabiensis* strains had lower deltamethrin induced lethal times than all *An. funestus* strain. MBN-DDT had higher deltamethrin induced lethal time than SENN-DDT.

There were no differences in deltamethrin resistance intensities between Zambia *An. funestus* clades I and II (ANOVA: P=0.35; DF = 1; F=1.05). There was a significant difference in deltamethrin resistance intensity between Zambia and Zimbabwe *An. funestus* clade I (ANOVA: P=0.025; DF=2; F=5.75). FUMOZ-R presented higher deltamethrin resistance intensities than FUMOZ-BASE (ANOVA: P=0.002; DF=1; F=51.90) and wild strains (ANOVA: P=0.001; DF; = 2; F= 26.30). FUMOZ-R's calculated deltamethrin resistance intensity (LT_{50}= 966.67 minutes; SE= 160.24) is extrapolated since only 24% knockdown was achieved by the end of eight hour exposure period. FUMOZ-BASE had lower deltamethrin resistance intensities than *An. funestus* wild strains (ANOVA: P= 0.12; DF= 3; F= 2.81).

MBN-DDT presented significantly higher deltamethrin resistance intensity than SENN-DDT (P=0.018; DF= 1; F= 15) The *An. arabiensis* DDT resistant laboratory strains (SENN-DDT and MBN-DDT) portrayed significantly lower deltamethrin resistance intensities than pyrethroid resistant *An. funestus* laboratory strains (ANOVA: P<0; DF=3; F= 44.8) and wild caught *An. funestus* strains (P< 0; DF= 4; F= 57.2) (Figure 3.9).

3.5 Deltamethrin and bendiocarb resistance intensity ratios

Resistance Intensity Ratios (RIRs) were calculated for each resistant strain by computing quotients of resistance intensities for the resistant strain and the reference susceptible strain used as control. FUMOZ-R had the highest deltamethrin resistance intensity ratio, more than double the strain that follows it (i.e. Zimf clade I). FUMOZ-BASE had the lowest

deltamethrin resistance ratio amongst resistant *An. funestus* strains. Laboratory *An. arabiensis* strains had lower deltamethrin resistance intensity ratios than *An. funestus* strains with MBN-DDT displaying a higher RIR than SENN DDT. Bendiocarb resistance intensity ratios were much lower than deltamethrin´s. FUMOZ-R was the only strain with a bendiocarb RIR that was more than twice that of susceptible reference strain (Table 3.2).

Table 3.2: Deltamethrin and bendiocarb RIRs

Strain	Resistance Intensity Ratios (RIRs)	
	deltamethrin	bendiocarb
Zamf Clade I	15.36	1.2
Zamf Clade II	16.41	1.02
Zimf Clade I	21.08	1.03
FUMOZ-BASE	8.63	1.51
FUMOZ-R	47.50	2.35
SENN-DDT*	2.80	1.13
MBN-DDT*	5.49	1.04

FUMOZ-R showed the highest RIR for both deltamethrin and bendiocarb (Table 3.2). Zimbabwe wild strain (Zimf clade I) had the highest deltamethrin resistance intensity ratio amongst wild strains. Zambia wild strains resistance intensity ratios were almost double that of *An. funestus* resistance reference strain, FUMOZ-BASE.

Bendiocarb resistance intensities for all laboratory and wild strains were not as pronounced as that of deltamethrin. FUMOZ-R presented bendiocarb RIR that was more than double the reference susceptible strain named FANG. FUMOZ-BASE and the wild strains resistance intensity ratios ranged between 1 and 1.5 times compared to the reference susceptible strain (Table 3.2).

With respect to the *An. arabiensis* strains, MBN-DDT was double SENN-DDT in RIRs but there was a very small difference (i.e. 0.09) in bendiocarb resistance intensity ratios. The *An. arabiensis* strains had very low deltamethrin resistance intensity ratios compared to *An. funestus* strains. FUMOZ-R was the only *An. funestus* strain with significantly higher bendiocarb resistance intensity ratios than *An. arabiensis* strains.

3.6 Comparisons of resistance intensity assays and resistance monitoring assays

The need to elucidate the distinguishing importance of resistance intensities necessitated comparisons between resistance intensity studies and standard WHO bioassays. The results of standard WHO bioassays conducted on FUMOZ-BASE and FUMOZ-R are shown in Figure 3.10 and were subsequently discussed in comparison with those of resistance intensity studies. FUMOZ-R showed significantly higher deltamethrin resistance levels than FUMOZ- BASE.

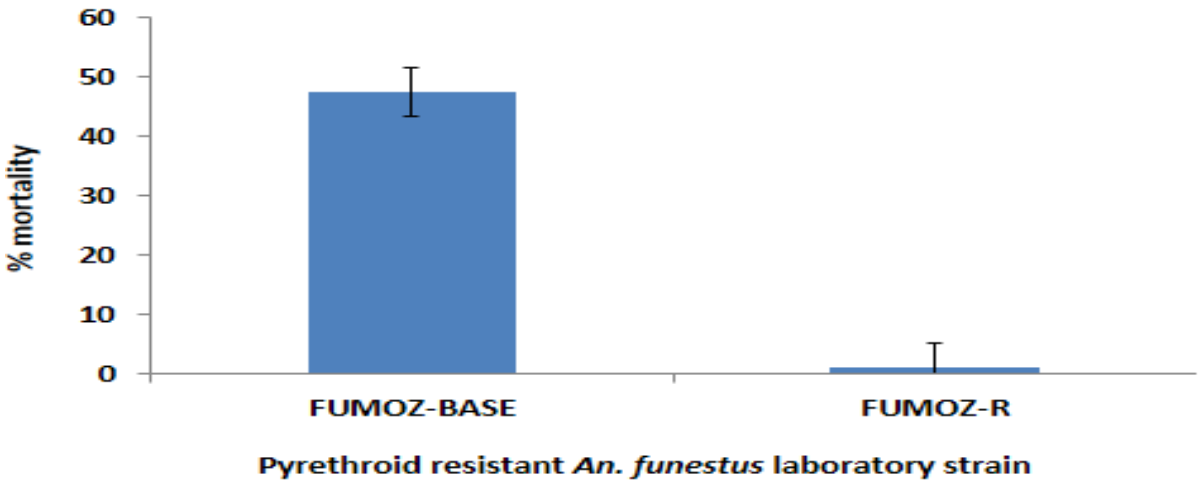


Figure 3.10: Standard WHO bioassay results for deltamethrin exposure in pyrethroid resistant laboratory *An. funestus* strains. The 24-hour mortality post 1 hour exposure on standard WHO bioassays showed 1.33% mortality for FUMOZ-R and 49.5% for FUMOZ-BASE.

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 Important findings

Anopheles funestus has been incriminated as the dominant vector species in the two regions encompassing the sampling sites (Henning *et al*., 2014). Sample sizes from Zimbabwe were low and might have contributed to failure to detect clade II. However, these findings are in agreement with Choi *et al*. (2014) on the absence on *An. funestus* clade II in Zimbabwe. More studies have to be conducted in future to ascertain the presence, relative abundance and coexistence of the two *An. funestus* clades in Mutasa district.

This study demonstrated established the importance of resistance intensity assays. High resistance levels in *An. funestus* populations based on the WHO discriminating dose do not necessarily translate to high resistance intensities. The March, 2014 bendiocarb resistance on wild *An. funestus* strains from Zambia is reported as high as 45.2% mortality (Choi *et al*., 2014). However, all wild strains portrayed similar knockdown trends to the susceptible FANG on bendiocarb resistance intensity exposures.

FUMOZ-R had a very high extrapolated resistance intensity (LT_{50}= 966.67) which is attributed to sustained laboratory selection for high pyrethroid resistance. This strain showed 1.33% mortality on deltamethrin in the present study. Pyrethroid resistance mediated by cytochrome P450 activity has been demonstrated not to incur a fitness cost on FUMOZ-R and proved to be fitter than the susceptible FANG counterpart (Okoye *et al*. 2007). Choi *et al*., (2014) evidenced resistance in Nchelenge and Honde Valley *An. funestus* populations to be mediated by cytochrome P450 monooxygenases. This study showed the same populations to have conserved high levels of pyrethroid resistance intensities in the presence of pyrethroid

selection pressure. Therefore, pyrethroid resistance mediated by P450 monooxygenase enzymes would also likely not incur a fitness cost in presence of pyrethroid selection pressure.

Another important finding from this study is the ability by pyrethroid resistant laboratory *An. funestus* strains to confer higher levels of deltamethrin resistance intensities than *An. arabiensis* selected for DDT resistance but with cross resistance to deltamethrin. This finding is not surprising since pyrethroid resistance in the *An. arabiensis* laboratory strains is not by pyrethroid selection pressure but as a secondary consequence of DDT selection.

4.2 Insecticide resistance and fitness costs in *An. funestus*

Insecticide resistance has been associated with a number of fitness costs in *Culex* species. These include lower fecundity and longer larval development time (Berticat *et al*., 2002; Bourguet *et al*., 2004). Inbreeding depression as a result of colonization has also been demonstrated to reduce fitness in mosquitoes (Munstermann, 1994). The baseline resistant strain (FUMOZ-BASE) has maintained significant levels of pyrethroid resistance intensity since colonization despite no exposure to insecticides for the past 13 years. This shows *An. funestus* ability to conserve high levels of resistance intensities for extended periods of time even in the absence of insecticide selection pressure. A previous study has demonstrated that the resistant phenotype in this laboratory strain does not incur a fitness cost (Okoye *et al*., 2007). It is important to note that due to optimal insectary conditions and lack of natural environmental stresses, it is possible that metabolically demanding fitness traits such as resistance can be maintained without selection pressure.

Longitudinal studies by Brooke *et al*., (2001), Casimiro *et al*., 2007 and Cuamba *et al* (2010) show increasing levels of resistance among wild *An. funestus* populations in Mozambique. These findings, coupled with the high resistance intensities observed in Zimbabwe and

Zambia, suggest that resistance might not incur a fitness cost even in wild *An. funestus* populations (Casimiro *et al*., 2007; Chanda *et al*., 2011; Choi *et al*., 2014).

The pyrethroid resistance phenotype in FUMOZ-R and FUMOZ-BASE is primarily a product of elevated activity of monooxygenases and glutathione s-transferases (Brooke et al., 2001; Amenya et al., 2005, Hunt *et al*., (2005). Choi *et al*. (2014) associated *An. funestus* resistance in the two study sampling sites with elevated levels of metabolic monooxygenases. Therefore, metabolic resistance is implicated for the high pyrethroid resistance intensities and cross resistance to carbamates observed in the Zambia and Zimbabwe wild *An. funestus* populations.

4.3 Implications of existing selection pressure in Nchelenge and Honde Valley

Insecticide resistance management strategies, such as rotations of different classes of insecticides with different modes of action, will be able to remove the selection pressure (WHO, 2012). Resistance is probably easier to reverse if the resistance incurs a fitness cost (Bonning and Hemmingway, 1991). Comparisons made between the baseline resistant strain, FUMOZ-BASE, and wild strains showed that resistance intensities observed in Nchelenge and Honde Valley are excessively high and selection has probably been on-going for a while. This suggests the need for robust implementation of resistance management in order to reverse pyrethroid resistance in these regions which may take several years.

There is no doubt that strong pyrethroid resistance selection pressure occurs in the sampling areas. ICEMR (2014 unpublished report) reported the on-going usage of pyrethroid-based IRS and ITNs in both Nchelenge and Honde Valley. *Anopheles funestus* has the potential for rapid selection for pyrethroid and carbamate resistance in the wild. This was first detected in South Africa in 1999 (Hargreaves *et al.,* 2000) and the following year in southern Mozambique (Brooke et al., 2001). Another survey showed a significant increase in both pyrethroid and

carbamate resistance in the same Mozambique *An. funestus* populations (Casimiro *et al*., 2007). The continuation of pyrethroid based vector control against the same populations eventually resulted in 100% resistance (Cuamba *et al*., 2010). *Anopheles funestus* has also been shown to have potential for rapid selection to pyrethroid resistance under colonisation (Hunt *et al*., 2005). Failure to withdraw the selection pressure by managing resistance has potential for control programme failure. *Anopheles funestus* is a very efficient vector and high resistance intensities are a good indicator of potential for continued transmission even under the current pyrethroid based control interventions (Choi *et al*., 2014)

Pyrethroids are the only chemicals allowed by the WHO for use on ITN impregnation due to their low mammalian toxicity (WHO, 2012). To manage resistance in this setting, IRS must be changed from pyrethroids to either organophosphates or DDT. This might not give prompt outcomes as pyrethroid treated bed nets will still be in the system thus continuing selection. There are reports of DDT being banned in the two sampling sites owing to the close proximity of tea and tobacco plantations. This further narrows the range of available options since there is no DDT resistance in the study areas' *An. funestus* populations and DDT would manage the pyrethroid resistance. (Hargreaves *et al*., 2000) and reduce transmission significantly (Coetzee and Koekemoer, 2013).

Choi et al., (2014) reported Zambia *An. funestus* deltamethrin resistance levels of 47.2% and 45.5% mortality for 2013 and 2014 respectively. Pyrethroid treated bed net usage was 55 % and 95 % for children under the age of 5 years and adults respectively (ICEMR, 2014 unpublished report). Prolonged and extensive usage of pyrethroid based interventions in the study areas might have contributed to the high resistance intensities observed. ICEMR (2014 unpublished report) reported 100% susceptibility to bendiocarb in the Nchelenge *An. funestus* population in March 2012. Choi *et al*. (2014) detected bendiocarb resistance in the same population in March 2013, standing at 75.5% mortality and it increased to 45.5% mortality in

March 2014. This shows another ability by *An. funestus* wild populations to be selected for rapid cross resistance to bendiocarb from selection by pyrethroids. Cross resistance is also a problem as it narrows the choice of available insecticides.

4.4 Resistance intensities on *An. arabiensis* laboratory strains

Resistance intensity comparisons between the two *An. arabiensis* DDT resistant laboratory strains, MBN-DDT and SENN-DDT, showed that the former's deltamethrin resistance intensity was double the latter's. Deltamethrin resistance intensity was more pronounced than bendiocarb in the two strains, both of which have been artificially selected for high levels of DDT resistance. Mouatcho *et al*. (2009) reported cross-resistance to deltamethrin as result of overexpression of P450 genes in MBN-DDT. Nardini *et al*. (2012) reported the up-regulation of ten P450 monooyxgenase genes, 8 glutathione S-transferase and 2 estarases genes in MBN-DDT against only one P450 gene in SENN-DDT. This explains MBN-DDT's deltamethrin resistance intensity superiority over SENN-DDT.

4.5 Importance of insecticide resistance intensity studies

The resistance intensity studies carried out here give an informed estimate of lethal time (e.g. LT_{50}) of WHO discriminating dosages of a particular insecticide on a resistant population compared with a fully susceptible strain. It is important to note that several factors are excluded when testing for resistance intensities, thus making it a weak tool for field inference (Choi *et al*., 2014). However, resistant *An. funestus* wild populations have effectively nullified the insecticide-based malaria control interventions in both study sites with both areas remaining holoendemic for parasite transmission. Pyrethroid resistant *An. funestus* populations were implicated in malaria epidemics in South Africa and Mozambique (Hargreaves *et al*., 2000; Brooke et al., 2001; Casimiro et al., 2007).

The WHO resistance monitoring tests establishes the proportion of individuals with resistant genes in a population whereas intensity tests estimates the level of expression of the resistance phenotype in the resistant population. Hemingway *et al*. (1995) showed 91.5% mortality in Gambian *An. gambiae, An. arabiensis* and *An. melas* using the standard WHO protocol, but 100% mortality on a 2-hour exposure to the same diagnostic dosages. Prolonged exposure gives a separate indicator from resistance monitoring. Exposures for periods more than one hour are able to tell the extent to which the resistant population can withstand the WHO discriminating dosages.

The Zambia and Zimbabwe *An. funestus* strains have previously been shown to be resistant on standard WHO bioassays (WHO, 2013b). However, the bendiocarb resistance intensity knockdown trends observed on these populations compared with the susceptible strain FANG, suggests susceptibility status. How this could be used in a resistance management strategy remains to be determined.

4.6 Conclusion

FUMOZ-R had the highest deltamethrin and bendiocarb resistance intensities amongst laboratory and wild caught *An. funestus* strains when compared with the FANG insecticide susceptible strain. The wild caught *An. funestus* strains from Zimbabwe and Zambia presented higher deltamethrin resistance intensities than the reference base-line pyrethroid resistant strain, FUMOZ-BASE. The two Zambian *An. funestus* clades showed no significant difference in either bendiocarb or deltamethrin resistance intensities. FUMOZ-BASE, however, showed higher bendiocarb resistance intensity than all wild strains.

The *An. funestus* wild caught strains' deltamethrin resistance intensity superiority over the reference pyrethroid resistance strain, FUMOZ-BASE, shows the seriousness of pyrethroid resistance in *An. funestus* populations in Honde Valley and Nchelenge. It underlines the

urgent need for insecticide resistance management in the two study areas so that vector control interventions have the desired impact of reducing malaria transmission.

Anopheles arabiensis laboratory strains showed significantly lower deltamethrin resistance intensities compared to their *An. funestus* counterparts but there was no difference observed for bendiocarb resistance intensities. MBN-DDT had higher deltamethrin resistance intensities than SENN-DDT but there was no difference for bendiocarb resistance intensities.

There is a need to conduct resistance intensity studies, in the event that resistance is detected in a population, to augment the information collected on standard WHO bioassays. The resistance intensity studies quantify the expression of the resistance phenotype in the resistant population that would have been detected through standard WHO bioassays. WHO, (2013b) recommends that molecular tests, synergist and biochemical enzyme assays be conducted once resistance is detected to identify the underlying resistance mechanisms. Based on the findings of this study, resistance intensity assays are recommended for inclusion in WHO guidelines for resistance monitoring and management. The addition of resistance intensity data would give more understanding on the resistant population and subsequently assist in informing operational resistance management strategies.

REFERENCES

Amenya DA, Naguran R, Lo TC, Ranson H, Spillings BL, Wood OR, Brooke BD, Coetzee M and Koekemoer LL. (2008) Over expression of a cytochrome P450 (CYP6P9) in a major African malaria vector, *Anopheles funestus*, resistant to pyrethroids. *Insect Mol. Biol*., **17**, 19–25

Antinori S, Galimberti L, Milazzo L and Corbellino M. (2013) *Plasmodium knowlesi*: The emerging zoonitic malaria parasite. *Acta Trop.*, **125**, 191-201

Antonio-Nkondjio C, Awono-Ambene P, Toto JC, Meunier JY, ZebazeKemleu S, Nyambam R, Wondji CS, Tchuinkam T and Fontenille D. (2002) High malaria transmission intensity in a village close yo y in a village close to Yaounde, the capital city of Cameroon. *J. Med. Entomol.*, **39**, 350-355

Athanassiou CG, Vayias BJ, Dimizas CB, Kavallieratos NG, Papagregoriou AS and Buchelos CT. (2005) Insecticidal efficacy of diatomaceous earth against *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and *Tribolium confusum* du Val (Coleoptera: Tenebrionidae) on stored wheat: influence of dose rate, temperature and exposure interval. *J. Stored Prod. Res.*, **41**, 47–55

Berticat C, Boquien G, Raymond M and Chevillon C. (2002) Insecticide resistance genes induce a mating competition cost in *Culex pipiens* mosquitoes. *Genet. Res.*, **79**, 41–47.

Bonning BC and Hemmingway J. (1991) Identification of reduced fitness associated with an insecticide resistance gene in *Culex pipiens* by microtitre plate tests. *Med. Vet. Entomol*., **5**, 377-379

Bourguet D, Guillemand T, Chevillon C and Raymond M. (2004) Fitness costs of insecticide resistance in natural breeding sites of the mosquito *Culex pipiens. Evol.*, **58**, 128-135

Bousema T and Drakeley T. (2011) Epidemiology and infectivity of *Plasmodium falciparum* and *Plasmodium vivax* gametocytes in relation to malaria control and elimination. *J. Clin. Microbio.*, **24**, 377-410

Brooke BD, Kloke G, Hunt RH, Koekemoer LL, Temu EA, Taylor M.E, Small G, Hemingway J and Coetzee M. (2001) Bioassay and biochemical analyses of insecticide resistance in southern African *Anopheles funestus* (Diptera: Culicidae). *Bull. Entomol. Res.*, **91**, 265–272

Casimiro S, Coleman M, Hemingway J and Sharp B. (2006) Insecticide resistance in *Anopheles arabiensis* and *Anopheles gambiae* from Mozambique. *J. Med. Entomol.*, **43**, 267-275

Casimiro SL, Hemingway J, Sharp BL and Coleman M. (2007) Monitoring the operational impact of insecticide usage for malaria control on *Anopheles funestus* from Mozambique. *Malar. J.*, **6**, 142

Chanda P, Chanda E, Hawela MB and Chizema-Kawesha E. (2007) Baseline parasitological assessments and accuracy of rapid diagnostic tests in districts implementing indoor residual spraying for malaria prevention in Zambia. *Med. J. Zambia*, **34**, 48-52.

Chanda E, Hemingway J, Kleinschmidt I, Rehman AM, Ramdeen V, Phiri FN, Coetzer S, Mthembu D, Shinondo CJ, Chizema-Kawesha E, Kamuliwo M, Mukonka V, Baboo KS, Coleman M. (2011) Insecticide resistance and the future of malaria control in Zambia. *PLoS One*, **6**(9), e24336.

Charlwood JD, Smith T, Kihonda J, Heiz B, Billingsley PF and Takken W. (1995) Density-independent feeding success of malaria vectors (Diptera: Culicidae) in Tanzania. *Bull. Entomol. Res.*, **85**, 29-35.

Choi KS, Christian R, Nardini L, Wood OR, Agubuzo E, Muleba M, Munyati S, Makuwaza A, Koekemoer LL, Brooke BD, Hunt RH and Coetzee M. (2014) Insecticide Resistance and role in Malaria Transmission of *Anopheles funestus* populations from Zambia and Zimbabwe. *Parasit. Vectors*, **7**, 464

Choi KS, Coetzee M and Koekemoer LL. (2013) Dectection of clade types (clades I and II) within *Anopheles funestus sensu stricto* by the hydrolysis probe analysis (Taqman assay). *Parasit. Vectors*, **6**, 173

Choi KS, Koekemoer LL and Coetzee M. (2012) Population genetic structure of the major malaria vector *Anopheles funestus s.s* and allied species in Southern Africa. *Parasit. Vectors*, **5**, 283

Christian RN, Matambo TS, Spillings BL, Brooke BD, Coetzee M and Koekemoer LL. (2011) Age-related pyrethroid resistance is not a function of P450 gene expression in the major African malaria vector, *Anopheles funestus* (Diptera: Culicidae). *Genet. Mol. Res*., **10**, 3220-3229

Coetzee M and Koekoemoer LL. (2013) Molecular systematics and insecticide resistance in the major African malaria vector *Anopheles funestus*. *Annu. Rev. Entomol*, **58**, 393-412

Coetzee M, Hunt RH, Braack LEO and Davidson G. (1993) Distribution of mosquitoes belonging to the *Anopheles gambiae* complex, including malaria vectors, south of latitude 15°S. *S. Afr. J. Sci*., 89, 227-231

Coetzee M, Hunt RH, Wilkerson R, Torre AD, Coulibaly MB and Besansky NJ. (2013a) *Anopheles coluzzii* and *Anopheles amharicus*, new members of the *Anopheles gambiae* complex. *Zootaxa*, **3**, 246–274

Coetzee M, Kruger P, Hunt RH, Durrheim DN, Urbach J and Hansford CF. (2013b) Malaria in South Africa: 110 years of learning to control the disease. *Sth Afr. Med. J*., **103**, 770-778

Cohuet A, Simard F, Toto JC, Kegne P, Coetzee M, Fontenille D. (2003) Species identification within the *Anopheles funestus* group of malaria vectors in Cameroon and evidence for a new species. *Am. J. Trop. Med. Hyg*., **69**, 200-205

Costantini C, Sagnon N'F, Ilboudo-Sanogo E, Coluzzi M and Boccolini D. (1999) Chromosomal and bionomic heterogeneities suggest incipient speciation in *Anopheles funestus* from Burkina Faso. *Parassitologia*, **41**, 595-611

Cuamba N, Morgan JC, Irving H, Steven A and Wondji CS. (2010) High level of pyrethroid resistance in an *Anopheles funestus* population of the Chokwe District in Mozambique. *PLoS ONE 5e*, **6**, 11010

Das S, Muleba M, Eng S, Kessler M and Norris DE. (2014) *Plasmodium falciparum* infection and *Anopheles* multiple blood feeding rate during the wet and dry seasons in Nchelenge District, Zambia. World Malaria Day

Davidson G. (1956) Insecticide resistance in *Anopheles gambiae* Giles: a case of simple Mendelian inheritance. *Nature*, **178**. 863-864

Finney DJ. (1952) *Probit Analysis*. Cambridge University Press, Cambridge.

Gatton ML, Chitnis N, Churcher T, Donnelly MJ, Ghani AC, Godfray CJ, Gould F, Hastings I, Marshall J, Ranson H, Rowland M, Shaman J, Lindsay WS. (2013) The importance of mosquito behavioral adaptations to malaria control in Africa. *Evol*., **67**, 1218-1230

Gillies MT and Coetzee M. (1987) *A supplement to the Anophelinae of Africa south of the Sahara.* Publications of the South African Institute for Medical Research, Johannesburg, No. 55

Gillies MT and De Meillon B. (1968) *The Anophelinae of Africa south of the Sahara*. Publications of the South African Institute for Medical Research, Johannesburg, No.54

Gimonneau G, Pombi M, Choisy M, Morand S, Dabire RK and Simard F. (2012) Larval habitat segregation between molecular forms of the mosquito *Anopheles gambiae* in a rice field area of Burkina Faso, West Africa. *Med. Vet. Entomol.,* **26**, 9-17

Githeko AK, Adungo NI, Karanja DM, Hawley WA, Vulule JM, Seroney IK, Ofulla AV, Atieli FK, Ondijo SO, Genga IO, Odada PK, Situbi PA and Oloo JA. (1996) Some observations on the biting behavior of *Anopheles gambiae s.s.*, *Anopheles arabiensis*, and *Anopheles funestus* and their implications for malaria control. *Exp. Parasitol.*, **82**, 306-315

Green CA. (1981) Malaria epidemiology and anopheline cytogenetics. In: Pal R, Kitzmiller JB and Kanda TK (Eds) Cytogenetics and Genetics of Vectors. 21-29. Elsievier Biomedical Press, Amsterdam

Harbach RE. (2004) The classification of genus Anopheles (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. *Bull. Entomol. Res*. **94**, 537–53

Hargreaves K, Hunt RH, Brooke BD, Mthembu J, Weeto MM, Awolola TS and Coetzee M. (2003) *Anopheles arabiensis* and *An. quadriannulatus* resistance to DDT in South Africa. *Med. Vet. Entomol.,* **17**, 417-422

Hargreaves K, Koekemoer LL, Brooke BD, Hunt RH, Mthembu J and Coetzee M. (2000) *Anopheles funestus* resistant to pyrethroid insecticides in South Africa. *Med. Vet. Entomol.,* **2**, 181-189.

Hemingway J, Lindsay SW, Small GJ, Jawara M and Collins FH. (1995) Insecticide susceptibility status in individual species of the *Anopheles gambiae* complex *(*Diptera: Culicidae*)* in an area of The Gambia where pyrethroid impregnated bed nets are used extensively for malaria control. *Bull. Entomol. Res.,* **85,** 229-234

Hemingway J and Ranson H. (2000) Insecticide resistance in insect vectors of human disease. *Annu. Rev. Entomol.*, **45**, 371–391

Henning T, Lukwa N, Gwanzura L and Norris DE. (2014) Vector bionomics of *Anopheles funestus* complex in Mutasa District, Zimbabwe. World Malaria Day

Hunt RH and Coetzee M. (1989) A parasitological, cytogenetic and biochemical study of *Anopheles gambiae* (Diptera: Culicidae) from the People's Republic of Congo. *Sth. Afr. Med. J.*, **76**, 362-364

Hunt RH, Brooke BD, Pillay C, Koekemoer LL and Coetzee M. (2005) Laboratory selection for and characteristics of pyrethroid resistance in the malaria vector *Anopheles funestus*. *Med. Vet. Entomol.*, **19**, 271–75

Hunt RH, Coetzee M and Fettene M. (1998) The *Anopheles gambiae* complex: A new species from Ethiopia. *Trans. R. Soc. Trop. Med. Hyg.*, **92**, 231-235

Hunt RH, Edwardes M and Coetzee M. (2010) Pyrethroid resistance in southern African *Anopheles funestus* extends to Likoma Island in Lake Malawi. *Parasit. Vectors*, **3**, 122

Jawara M, Pinder M, Drakeley CJ, Nwakanma CD, Jallow CD, Jallow E, Bog C, Lindsay SW and Conway DJ. (2008) Dry season ecology of *An. gambiae* complex mosquitoes in the Gambia. *Malar. J.*, **7**, 156

Kloke RG, Nhamahanga E, Hunt RH and Coetzee M. (2011) Vectorial status and insecticide resistance of *Anopheles funestus* from a sugar estate in southern Mozambique. *Parasit. Vectors*, **4**, 16

Koekemoer LL, Kamau L, Hunt RH and Coetzee M. (2002) A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (Diptera Culicidae) group. *Am. J. Trop. Med. Hyg.*, **66**, 804–811

la Grange JJP. (1995) Survey of anopheline mosquitoes (Diptera: Culicidae) in malarious area of Swaziland. *J. Entomol. Soc. South Afr*., **32**, 217-219

Leeson HL. (1937) The mosquitoes of the *An. funestus* series in the East Africa. *Bull. Entomol. Res.*, **28**, 387-682

Lukwa N, Sande S, Makuwaza A, Chiwade T, Netsa M, Asamoa K, Vazquez-Prokopec G, Reithinger R and Williams J. (2014) Nationwide assessment of insecticide susceptibility in *Anopheles gambiae* populations from Zimbabwe, *Malar. J.*, **13**, 408

Masaninga F, Chanda E, Chanda-Kapata P, Hamainza B, Masendu H, Kamuliwo M, Kapelwa W, Chimumbwa J, Govere J, Otten M, Fall IS and Babaniyi O. (2013) Review of the malaria epidemiology and trends in Zambia. *Asian Pac. J. Trop. Biomed.*, **3**, 89-94

Masendu HT, Hunt RH, Koekemoer LL and Brooke BD. (2005) Spatial and Temporal distributions and insecticide susceptibility of malaria vectors in Zimbabwe. *Afr. Entomol.*, **13**, 25-34

Mharakurwa S, Mutambu SL, Mberikunashe J, Thuma PE, Moss WJ, Mason PR and Southern Africa ICEMR Team. (2013) Changes in the burden of malaria following scale up of malaria control interventions in Mutasa District, Zimbabwe. *Malar. J.*, **12**, 223

Ministry of Health. (2000) Malaria situation analysis. National Malarial Control Center, Lusaka, Zambia

Michel AP, Guelbeogo WM, Grushko O, Schemerhorn BJ, Kern M, Willard MB, Sagnon N'F, Costantini C and Besansky NJ. (2005) Molecular differentiation between chromosomally defined incipient species of *Anopheles funestus*. Insect *Mol. Biol.,* **14,** 375-387

Michel AP, Ingrasci MJ, Schemerhorn BJ, Kern M, Le Goff G, Coetzee M, Elissa N, Fontenille D, Vulule J, Lehmann T, Sagnon N, Costantini C and Besansky NJ. (2005) Rangewide population genetic structure of the African malaria vector *Anopheles funestus*. *Mol. Ecol.*, **14**, 4235-4248

Midzi S, Teveredzi V, Mudyiradima R, Chihanga S, Netsa M, Mugove AT, Charimari L, Pasipamire J, Mutambu S, Kibassa C, Ngwenya N, Gausi K, Banda J, Mukelabai K, O'Connell T and Root G. (2004) *Zimbabwe Roll Back Consultative Mission (Reaping): Essential actions to support the attainment of the Abuja Targets*. Zimbabwe RBM Country Consultative Mission

Moreno M, Vincente JL, Cano J, Bersoza PJ, Nzambo S, Bobuakasi L, Buatiche JN, Ondo M, Micha F, DoRosario VE, Pinto J and Benito A (2008) Knockdown resistance mutations (kdr) and insecticide susceptibility to DDT and pyrethroids in *Anopheles gambiae* from Equatorial Guinea. *Tropical Medicine and International Health*. **13**, 430-433

Mouatcho JC, Munhenga G, Hargreaves K, Brooke BD, Coetzee M and Koekemoer LL. (2009) Pyrethroid resistance in a major African malaria vector *Anopheles arabiensis* from Mamfene, northern KwaZulu-Natal, South Africa. *S Afr. J. Sci*., **105**,127–131

Munhenga G, Masendu HT, Brooke BD, Hunt RH, Koekemoer LL. (2008) Pyrethroid resistance in the major malaria vector *Anopheles arabiensis* from Gwave, a malaria-endemic area in Zimbabwe. *Malar. J*., **7**, 247

Munhenga G and Koekemoer LL. (2011) Differential expression of cytochrome P450 genes in a laboratory selected *Anopheles arabiensis* colony. *Afr. J. Biotechnol.*, **10**, 12711-12716

Munstermann LE. (1994) Unexpected genetic consequences of colonization and inbreeding: allozyme tracking in Culicidae (Diptera), *Ann. Entomol. Soc. Am*., **87**, 157-164

Nardini L, Christian RN, Coetzer N, Ranson H, Coetzee M, Koekemoer LL. (2012) Detoxification enzymes associated with insecticide resistance in laboratory strains of *Anopheles arabiensis* of different geographic origin, *Parasit. Vectors*, **5**,113

Oaks SC, Mitchell VS, Pearson GW, Pearson GW and Carpenter CCJ. (1991) *Malaria; Obstacles and Opportunities*. National Academy Press, Washington D.C.

Okoye PN, Brooke BD, Hunt RH and Coetzee M. (2007) Relative developmental and reproductive fitness assosciated with pyrethroid resistance in the major southern African malaria vector, *Anopheles, funestus. Bull. Entomol. Res.,* **97**, 599-605

Ranson H, Guessan NR, Lines J, Moiroux N, Nkuni Z and Corbel V. (2011) Pyrethroid resistance in African anopheline mosquitoes: What are the implications for malaria control*? Trends Parasitol*., **27**, 91-98

Rozendaal JA. (1997) *Vector Control: Methods for use by Individuals and Communities*. World Health Organization, Geneva

Service MW. (1970) Ecological notes on species A and B of the *Anopheles gambiae* in the Kisumu area of Kenya. *Bull. Entomol. Res.*, **60**, 105-108

Sharp BL and Le Sueur D. (1991) Behavioral variation of *Anopheles arabiensis* (Diptera: Culicidae) populations in Natal, South Africa. *Bull. Entomol. Res.,* **81**, 107-110

Shiff CJ, Minjas JN, Hall T, Hunt RH, Lyimo S and Davis JR. (1995) Malaria infection potential of anopheline mosquitoes sampled by light trapping indoors in coastal Tanzanian villages. *Med. Vet. Entomol.,* **9,** 256-262

Singh B, Sung LK, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J. (2004) A large focus of naturally acquired *Plasmodium knowlesi* infection in human beings. *Lancet.* **363**, 1017-1024

Sinka ME, Bangs MJ, Manguin S, Coetzee M, Mbogo CM, Hemingway J, Patil AP, Temperly WH, Gething PW, Kabaria CW, Okara RM, Boeckel TV, Godfray HC, Harbach RE and Hay SI. (2010) The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis. *Parasit. Vectors,* **3**, 117

Spillings BL, Brooke BD, Koekemoer LL, Chiphwanya J, Coetzee M and Hunt RH. (2009) A new species concealed by *An. funestus* Giles, a major malaria vector in Africa. *Am. J. Trop. Med. Hyg*., **81**, 510-515

Taylor P and Mutambu SL. (1986) A review of the malaria situation in Zimbabwe with special reference to the period 1972–1981. *Trans. R. Soc. Trop. Med. Hyg.,* **80**, 12-19

Temu, EA, Minjas JN, Coetzee M, Hunt RH and Shiff CJ. (1998) The role of four anopheline species (Diptera: Culicidae) in malaria transmission in coastal Tanzania. *Trans. R. Soc. Trop. Med. Hyg.*, 92, 152–158

White GB. (1985) *Anopheles bwambae* spp., a malaria vector in the Semliki Valley, Uganda, and its relationships with other sibling species of the *Anopheles gambiae* complex (Diptera: Culicidae). *Syst. Entomol.,* **10**, 510-522

White GB. (1974) *Anopheles gambiae* complex and disease transmission in Africa. *Trans. R. Soc. Trop. Med. Hyg.*, **68**, 278-301

WHO. (2006) *Indoor residual spraying: Use of indoor residual spraying for scaling up global malaria control and elimination*. World Health Organization, Geneva

WHO. (2012) *Global plan for insecticide resistance management in malaria vectors*. World Health Organization. Geneva

WHO. (2013a) *World Malaria Report*. World Health Organization, Geneva

WHO. (2013b) *Test procedures for insecticide resistance monitoring in malaria vector mosquitoes*. World Health Organization, Geneva

Wood OR, Hanrahan S, Coetzee M, Koekemoer LL and Brooke BD. (2010) Cuticle thickening associated with pyrethroid resistance in the major malaria vector *Anopheles funestus*. *Parasit. Vectors*, **3**, 67

Zimbabwe Malaria Operational Plan, PMI Publication, 2012, "http://www.pmi.gov" (accessed April, 2015)

APPENDIX I

Table 1: Zambia *An. funest us* clade I resistance intensity knockdown rates on deltamethrin (0.05 %) exposures.

Test	Strain	Sex	Age (Days)	5 min	10 min	15 min	20 min	30 min	40 min	50 min	60 min	80 min	120 min	3 hrs	4 hrs	5 hrs	6 hrs	7 hrs	8 hrs
Negative Control	Zamf CI	Males	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Treatment	Zamf CI	Females	3	0	0	0	0	0	0	0	0	0	2	3	7	12	14	19	21
Treatment	Zamf CI	Females	3	0	0	0	0	0	0	0	0	0	1	5	8	14	15	17	17
Treatment	Zamf CI	Females	3	0	2	2	2	2	2	2	3	3	3	3	9	12	12	16	19
Treatment	Zamf CI	Females	3	0	1	1	1	1	1	1	1	2	2	3	10	20	20	20	20
Treatment	Zamf CI	Mix	3	0	0	0	0	0	0	1	3	4	6	11	16	19	22	22	22
Positive Control	FANG	Females	4	1	1	5	7	23	23	27	27	27	27	27	27	27	27	27	27
Positive Control	FANG	Females	4	0	1	4	9	22	24	23	28	28	28	28	28	28	28	28	28
Total treatment mortality	Zamf CI			0	3	3	3	3	3	3	4	5	8	14	34	58	61	72	77
% positive control mortality	FANG			1.82	3.64	16.4	29.1	81.8	85	91	100	100	100	100	100	100	100	100	100
% treatment mortality	Zamf CI			0	2.94	2.94	2.94	2.94	2.9	2.9	3.922	4.9	7.8	14	33	57	60	71	75

Table 2: Zambia *An. funestus* clade I resistance intensity knockdown rates on bendiocarb (0.1 %) exposures

Test	Strain	Age (Days)	Sex	5 min	10 min	15 min	20 min	30 min	40 min	50 min	60 min	80 min	120 min	3 hrs	4hrs	5 hrs	6 hrs	7 hrs	8 hrs
Negative Control	Zamf CI	2 to 5	Males	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Treatment	Zamf CI	2 to 5	Females	0	0	0	1	4	12	17	20	21	21	21	21	21	21	21	21
Treatment	Zamf CI	2 to 5	Females	0	0	0	0	5	11	23	23	24	24	24	24	24	24	24	24
Treatment	Zamf CI	2 to 5	Females	0	0	1	3	7	20	24	28	28	28	28	28	28	28	28	28
Treatment	Zamf CI	2 to 5	Females	0	0	0	4	12	24	24	27	27	27	27	27	27	27	27	27
Positive Control	FANG	3	Females	0	0	2	6	20	23	23	23	23	23	23	23	23	23	23	23
Positive Control	FANG	3	Females	0	0	2	10	21	25	25	25	25	25	25	25	25	25	25	25
total test mort	Zamf CI			0	0	1	8	28	67	88	98	100	100	100	100	100	100	100	100
% treatment mortality	Zamf CI			0	0	1	8	28	67	88	98	100	100	100	100	100	100	100	100
% positive control mortality	FANG			0	0	8.333	33.33	85.42	100	100	100	100	100	100	100	100	100	100	100

Table 3: Zambia *An. funestus* clade II resistance intensity knockdown rates on deltamethrin (0.05 %) exposures.

Test	Strain	Age (Days)	Sex	5 min	10 min	15 min	20 min	30 min	40 min	50 min	60 min	80 min	120 min	3 hrs	4hrs	5 hrs	6 hrs	7 hrs	8 hrs
Negative Control	Zamf CII	3	males	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Treatment	Zamf CII	2	Females	0	0	0	0	0	2	4	5	7	8	8	9	12	13	15	19
Treatment	Zamf CII	2	Females	0	0	0	1	1	1	2	4	5	6	6	7	8	11	16	16
Treatment	Zamf CII	2	Females	0	0	0	0	0	0	1	2	2	4	4	6	8	12	17	18
Treatment	Zamf CII	2	Females	0	0	0	0	0	0	1	3	3	10	10	11	13	15	16	20
Positive Control	FANG	4	Females	0	0	5	12	13	21	26	27	27	27	27	27	27	27	27	27
Positive Control	FANG	4	Females	0	0	6	11	13	24	26	26	26	26	26	26	26	26	26	26
total test mort	Zamf CII			0	0	0	1	1	3	8	14	17	28	28	33	41	51	64	73
% treatment mortality	Zamf CII			0	0	0	0.95	0.95	2.86	7.62	13.33	16.2	26.7	26.7	31.4	39.05	48.57	60.95	69.5
% positive control mortality	FANG			0	0	20.8	43.4	49.06	84.91	98.11	100	100	100	100	100	100	100	100	100

Table 4: Zambia *An. funestus* clade II resistance intensity knockdown rates on bendiocarb (0.1 %) exposures

Test	Strain	Age (Days)	Sex	5 min	10 min	15 min	20 min	30 min	40 min	50 min	60 min	80 min	120 min	3 hrs	4hrs	5 hrs	6 hrs	7 hrs	8 hrs
Negative Control	Zamf CII	3	Males & Females	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Treatment	Zamf CII	3	Females	0	0	1	4	8	22	25	25	25	25	25	25	25	25	25	25
Treatment	Zamf CII	3	Females	0	0	0	0	5	16	21	21	21	21	21	21	21	21	21	21
Treatment	Zamf CII	3	Females	1	1	2	3	11	19	25	25	25	25	25	25	25	25	25	25
Treatment	Zamf CII	3	Females	1	1	1	3	14	18	25	25	25	25	25	25	25	25	25	25
Positive Control	FANG	4	Females	1	1	3	5	15	18	23	23	23	23	23	23	23	23	23	23
Positive Control	FANG	4	Females	0	0	3	6	20	21	25	25	25	25	25	25	25	25	25	25
Total test mort	Zamf CII			2	2	4	10	38	75	96	96	96	96	96	96	96	96	96	96
% mortality	Zamf CII			2.08	2.08	4.16	10.41	39.58	78.12	100	100	100	100	100	100	100	100	100	100
% positive control mortality	FANG			2.08	2.08	12.5	22.91	72.92	81.25	100	100	100	100	100	100	100	100	100	100

Table 5: FUMOZ-R resistance intensity knockdown rates on deltamethrin (0.05 %) exposures

Test	Strain	Age (Days)	Sex	5 min	10 min	15 min	20 min	30 min	40 min	50 min	60 min	80 min	120 min	3 hrs	4 hrs	5 hrs	6 hrs	7 hrs	8 hrs
Negative Control	FUMOZ-R	4	Females	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Negative Control	FANG	2	Females	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Treatment	FUMOZ-R	4	Females	0	0	0	0	0	0	0	0	0	1	2	3	8	9	16	21
Treatment	FUMOZ-R	4	Females	0	0	0	0	0	0	0	0	0	0	0	3	9	12	20	23
Treatment	FUMOZ-R	4	Females	0	0	0	0	0	0	0	0	0	0	1	2	6	9	13	16
Treatment	FUMOZ-R	4	Females	0	0	0	0	0	0	0	0	0	0	0	2	3	5	10	13
Positive Control	FANG	2	Females	0	14	76	120	148	150	150	150	150	150	150	150	150	150	150	150
% positive control mortality	FANG			0	9.3	50.6	80	98.67	100	100	100	100	100	100	100	100	100	100	100
Total test mortality	FUMOZ-R			0	0	0	0	0	0	0	0	0	1	3	10	26	35	59	73
% test mortality	FANG			0	0	0	0	0	0	0	0	0	0.33	1	3.33	8.67	11.6	19.6	24.3

Table 6: FUMOZ-R resistance intensity knockdown rates on bendiocarb (0.1 %) exposures

Test	Strain	Age (Days)	Sex	5 min	10 min	15 min	20 min	30 min	40 min	50 min	60 min	80 min	120 min	3 hrs	4 hrs	5 hrs	6 hrs	7 hrs	8 hrs
Negative Control	FUMOZ-R	4	Females	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Negative Control	FANG	2	Females	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Treatment	FUMOZ-R	4	Females	0	0	0	0	0	5	16	40	60	73	75	75	75	75	75	75
Treatment	FUMOZ-R	4	Females	0	0	0	0	0	8	20	36	56	69	75	75	75	75	75	75
Treatment	FUMOZ-R	4	Females	0	0	0	0	0	2	14	34	51	72	75	75	75	75	75	75
Treatment	FUMOZ-R	4	Females	0	0	0	0	0	3	14	23	44	67	75	75	75	75	75	75
Positive Control	FANG	2	Females	0	0	0	15	99	143	150	150	150	150	150	150	150	150	150	150
% positive control mortality	FANG			0	0	0	10	66	95.33	100	100	100	100	100	100	100	100	100	100
Total test mortality	FUMOZ-R			0	0	0	0	0	18	64	133	211	281	300	300	300	300	300	300
% test mortality	FANG			0	0	0	0	0	6	21	44.3	70.33	93.67	100	100	100	100	100	100

Table 7: FUMOZ-BASE resistance intensity knockdown rates on deltamethrin (0.05 %) exposures

Test	Strain	Age (Days)	Sex	5 min	10 min	15 min	20 min	30 min	40 min	50 min	60 min	80 min	120 min	3 hrs	4 hrs	5 hrs	6 hrs	7 hrs	8 hrs
Negative Control	FUMOZ-R	4	Females	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Negative Control	FANG	2	Females	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Treatment	FUMOZ-R	4	Females	0	0	0	0	0	0	0	0	0	1	2	3	8	9	16	21
Treatment	FUMOZ-R	4	Females	0	0	0	0	0	0	0	0	0	0	0	3	9	12	20	23
Treatment	FUMOZ-R	4	Females	0	0	0	0	0	0	0	0	0	0	1	2	6	9	13	16
Treatment	FUMOZ-R	4	Females	0	0	0	0	0	0	0	0	0	0	0	2	3	5	10	13
Positive Control	FANG	2	Females	0	14	76	120	148	150	150	150	150	150	150	150	150	150	150	150
Total test mortality	FUMOZ-R			0	0	0	0	0	0	0	0	0	1	3	10	26	35	59	73
% test mortality	FUMOZ-R			0	0	0	0	0	0	0	0	0	0.33	1	3.3	8.6	12	20	24
% positive Control mortality	FANG			0	9.3	50.67	80	98.67	100	100	100	100	100	100	100	100	100	100	100

Table 8: FUMOZ-BASE resistance intensity knockdown rates on bendiocarb (0.1 %) exposures

Test	Strain	Age (Days)	Sex	5 min	10 min	15 min	20 min	30 min	40 min	50 min	60 min	80 min	120 min	3 hrs	4 hrs	5 hrs	6 hrs	7 hrs	8 hrs
Negative Control	FUMOZ-BASE	4	mix	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Negative Control	FANG	4	Females	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Treatment	FUMOZ-BASE	4	Females	0	0	0	0	21	35	58	64	72	75	75	75	75	75	75	75
Treatment	FUMOZ-BASE	4	Females	0	0	0	0	19	36	57	65	75	75	75	75	75	75	75	75
Treatment	FUMOZ-BASE	4	Females	0	0	0	0	16	33	57	67	75	75	75	75	75	75	75	75
Treatment	FUMOZ-BASE	4	Females	0	0	0	0	20	37	62	69	75	75	75	75	75	75	75	75
Positive Control	FANG	4	Females	0	0	5	13	105	135	148	150	150	150	150	150	150	150	150	150
Total test mortality	FUMOZ-BASE			0	0	0	0	76	141	234	265	297	300	300	300	300	300	300	300
% test mortality	FUMOZ-BASE			0	0	0	0	25.3	47	78	88.3	99	100	100	100	100	100	100	100
% positive Control mortality	FANG			0	0	3.33	8.67	70	90	98.6	100	100	100	100	100	100	100	100	100

Table 9: MBN-DDT resistance intensity knockdown rates on deltamethrin (0.05 %) exposures

Test	Strain	Age (Days)	Sex	5 min	10 min	15 min	20 min	30 min	40 min	50 min	60 min	80 min	120 min	3 hrs	4 hrs	5 hrs	6 hrs	7 hrs	8 hrs
Negative Control	MBN-DDT	2	Females	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Negative Control	MBN-BASE	2	Females	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Treatment	MBN-DDT	2	Females	0	0	0	0	0	7	12	21	40	50	63	65	68	71	73	74
Treatment	MBN-DDT	2	Females	0	0	0	0	0	2	11	20	38	61	68	71	73	74	74	74
Treatment	MBN-DDT	2	Females	0	0	0	0	3	10	15	26	44	55	64	66	68	71	73	73
Treatment	MBN-DDT	2	Females	0	0	0	0	1	5	11	23	35	54	64	66	66	68	68	72
Positive Control	MBN-BASE	2	Females	0	9	47	105	146	148	148	148	148	148	149	150	150	150	150	150
Total test mortality	MBN-DDT			0	0	0	0	4	24	49	90	157	220	259	268	275	284	288	293
% test mortality	MBN-DDT			0	0	0	0	1.33	7.95	16.23	29.80	51.99	72.88	85.8	88.7	91.1	94.0	95.4	97.2
% positive control Mortality	MBN-BASE			0	6	31.3	70	97.3	98.67	98.6	98.6	98.6	98.67	99	100	100	100	100	100

Table 10: MBN-DDT resistance intensity knockdown rates on bendiocarb (0.1 %) exposures

Test	Strain	Age (Days)	Sex	5 min	10 min	15 min	20 min	30 min	40 min	50 min	60 min	80 min	120 min	3 hrs	4 hrs	5 hrs	6 hrs	7 hrs	8 hrs
Negative Control	MBN-DDT	4	Females	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Negative Control	MBN-BASE	4	Females	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Treatment	MBN-DDT	4	Females	0	0	2	17	41	56	69	75	75	75	75	75	75	75	75	75
Treatment	MBN-DDT	4	Females	0	0	1	16	44	59	72	75	75	75	75	75	75	75	75	75
Treatment	MBN-DDT	4	Females	0	0	3	19	39	53	68	71	75	75	75	75	75	75	75	75
Treatment	MBN-DDT	4	Females	0	1	2	16	41	55	69	72	75	75	75	75	75	75	75	75
Positive Control	MBN-BASE	4	Females	0	0	2	36	85	120	149	150	150	150	150	150	150	150	150	150
Total test mortality	MBN-DDT			0	1	8	68	165	223	278	293	300	300	300	300	300	300	300	300
% test mortality	MBN-DDT			0	0.33	2.67	22.67	55	74.33	92.67	97.67	100	100	100	100	100	100	100	100
% positive control Mortality	MBN-BASE			0	0	1.33	24	57	80	99.3	100	100	100	100	100	100	100	100	100

Table 11: SENN-DDT resistance intensity knockdown rates on deltamethrin (0. 05 %) exposures

Strain	TEST	Age (Days)	Sex	5 min	10 min	15 min	20 min	30 min	40 min	50 min	60 min	80 min	120 min	3 hrs	4 hrs	5 hrs	6 hrs	7 hrs	8 hrs
Negative Control	SENN-DDT	3	Females	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Negative Control	SENN-BASE	3	Females	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Treatment	SENN-DDT	3	Females	0	0	1	5	25	47	58	62	68	73	73	73	73	74	74	75
Treatment	SENN-DDT	3	Females	0	0	2	5	27	42	56	63	67	72	73	73	73	73	74	75
Treatment	SENN-DDT	3	Females	0	0	1	4	25	44	55	62	68	72	74	74	75	75	75	75
Treatment	SENN-DDT	3	Females	0	0	0	4	25	35	44	53	55	62	66	70	72	73	74	74
Positive Control	SENN-BASE	3	Females	4	33	69	105	138	148	149	150	150	150	150	150	150	150	150	150
Total test mortality	SENN-DDT			0	0	4	18	102	168	213	240	258	279	286	290	293	295	297	299
% test mortallity	SENN-DDT			0	0	1.33	6	34	56	71	80	86	93	95.33	96.67	97.67	98.33	99	99.67
% positive control Mortality	SENN-BASE			2.67	22	46	70	92	98.67	99.33	100	100	100	100	100	100	100	100	100

Table 12: SENN-DDT resistance intensity knockdown rates on bendiocarb (0. 1 %) exposures

Test	Strain	Age (Days)	Sex	5 min	10 min	15 min	20 min	30 min	40 min	50 min	60 min	80 min	120 min	3 hrs	4 hrs	5 hrs	6 hrs	7 hrs	8 hrs
negative control	SENN-DDT	3	Females	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
negative control	SENN-BASE	3	Females	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
treatment	SENN-DDT	3	Females	0	0	2	5	27	59	73	75	75	75	75	75	75	75	75	75
treatment	SENN-DDT	3	Females	0	0	2	9	36	62	71	75	75	75	75	75	75	75	75	75
treatment	SENN-DDT	3	Females	0	0	0	7	34	61	73	75	75	75	75	75	75	75	75	75
treatment	SENN-DDT	3	Females	0	0	0	9	33	61	71	75	75	75	75	75	75	75	75	75
positive control	SENN-BASE	3	Females	0	0	4	20	93	144	150	150	150	150	150	150	150	150	150	150
Total test mortality				0	0	4	30	130	243	288	300	300	300	300	300	300	300	300	300
% test mort				0	0	1.3	10	43.3	81	96	100	100	100	100	100	100	100	100	100
% positive control Mortality				0	0	2.7	13	62	96	100	100	100	100	100	100	100	100	100	100