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Pinda, Polius

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UNIVERSITY OF THE WITWATERSRAND



FACULTY OF HEALTH SCIENCES

MASTERS THESIS

The influence of age and distance from aquatic habitats on the pyrethroid resistance phenotypes of *Anopheles funestus* mosquitoes in south-eastern Tanzania

Student:

Polius G. Pinda

Student No: 2443845

Supervisors:

Dr Fredros O. Okumu, PhD

Prof. Lizette L. Koekemoer

A Research Report Submitted to the Faculty of Health Sciences in Partial Fulfilment of the Requirements for the Degree of Masters of Science in Epidemiology

Infectious Disease Epidemiology

May; 2022

Declaration of Authorship

I, **Polius Gerazi Pinda,** declare that this work titled "*The influence of age and distance from aquatic habitats on the pyrethroid resistance phenotypes of Anopheles funestus mosquitoes in south-eastern Tanzania*" and other materials presented in my work. I confirm that;

- This work was wholly done by the candidate for the fulfilment of the Master's Degree at the University unless otherwise the contributions by other participants are stipulated.
- Whenever the materials either submitted to the university or other platforms were concerted, the sources were credited.
- It is clearly stated whichever the role played by other colleagues on this thesis including the data ownership from the primary source.

Signed: 

Date: 27th May 2022

Dedication

This work is dedicated to my respectful parents and beloved family for their constant support without whom this work would have been impossible. They always inspired me. At the same time, special dedications go to my caring siblings whose advice worked for the achievement of this thesis. Distinctive thanks go to my mother who always made me dream and achieve big regardless of the circumstances.

Abstract

Background: Malaria transmission risk can be unevenly distributed due to various environmental factors such as the distribution of mosquito breeding habitats and human dwellings, mosquito-related factors such as blood-feeding behaviours, susceptibility to insecticides, dispersal range, age and non-random host selection thus, shaping the effectiveness of interventions used. This study investigated how the age of *Anopheles funestus* and their susceptibility to insecticides used for vector control vary by distance from their aquatic habitats, and how these correlations may inform vector control strategies in southern Tanzania.

Methods: Adult female *An. funestus* were collected in houses located at 50-100 m, 150-200 m or over 200 m from the nearest known aquatic habitats. The mosquitoes were exposed to 1× (the standard diagnostic dose), 5× (five times the diagnostic dose) and 10× (ten times the diagnostic dose) of either deltamethrin or permethrin, or to the synergist, piperonyl butoxide (PBO) followed by the pyrethroids, then monitored for 24hr mortality. Ovaries of exposed and non-exposed mosquitoes were dissected to assess parity status as a proxy for age. Emergent adults from larval collections in the same villages were exposed to the same insecticides at either 3-5 days, 8-11 days or 17-20 days old. Generalized linear mixed-effects models were used to compare the 24hr-mortality for candidate insecticides by age and distance.

Findings: Mosquitoes collected nearest to the aquatic habitats (50-100 m) had the lowest mortality compared to those from other distances (150-200 m, over 200 m); with a maximum of 51% mortality at 10× permethrin. For the age-synchronized emergent mosquitoes collected as larvae, the highest mortalities were observed at the oldest age (17-20 days) when exposed at 1x doses. At 10× permethrin and deltamethrin doses, 99% and 76% mortality was observed among

the 8-11 day-olds compared to 56% and 58% among 3-5 day old mosquitoes respectively. Pre-exposure to PBO restored the potency of insecticides on mosquitoes. The percentage of parous mosquitoes increased with distance from aquatic habitats, thus indicating the increased age of mosquitoes with distance from aquatic habitats.

Conclusion: Older *An. funestus* and those collected farthest from the aquatic habitats (near the centre of the village) were more likely to die from exposure to pyrethroids than younger ones and those caught nearest to the habitats. Additional studies should investigate whether insecticide-based interventions would remain sufficiently effective in areas of widespread pyrethroid resistance by killing the older, less resistant and potentially-infective females or if fine-scale targeting of insecticide-based interventions such as IRS (e.g. by prioritizing households farthest from habitats) would be optimal.

Keywords: Insecticide resistance, mosquito age, age grading, parity, *Anopheles funestus*, piperonyl butoxide, malaria, aquatic habitats.

Abbreviations

CDC: Centre for Disease Control and Prevention

DDT: Dichloro-diphenyl-trichloroethane

GLMM: Generalized Linear Mixed Effect Model

GPIRM: Global Plan for Insecticide Resistance Management

GPS: Global Positioning System

GSTs: Glutathione-S-transferase

IHI: Ifakara Health Institute

IRS: Indoor Residual Spraying

ITNs: Insecticide-treated Nets

IVM: Integrated Vector Management

ITS2: Internal Transcribed Spacer Region 2

IRB: Institutional Review Board

LLINs: Long-lasting Insecticidal Nets

NIMR: National Institute for Medical Research,

PBO: Piperonyl Butoxide

PCR: Polymerase-chain reaction

WHO: World Health Organization

Acknowledgements

Firstly, I would like to thank my academic supervisors, Dr Fredros O. Okumu and Prof. Lizette L. Koekemoer for their tireless support throughout this project. Your timely and effective discussions, comments and suggestions were of many contributions to the achievement of this project. I would like to extend my acknowledgement to all my mentors and peers for their dedicated time to this work. Without this, it would have been difficult to reach the complete final version of this thesis. I would like to give special thanks to Ifakara Health Institute for providing financial and material support for this project. I would like to acknowledge the statistics team at Ifakara Health Institute for the dedicated analytical advice on this project whenever consulted. Special gratitude goes to my institution mentors and peers under whose constant guidance I have completed this journey. They did not only enlightened me with the academic knowledge but also gave me valuable advice whenever I needed them the most.

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CHAPTER ONE

1. INTRODUCTION

1.1 Background

Malaria is a life-threatening disease caused by the *Plasmodium* parasites. Human parasite transmission is via the *Anopheles* mosquito vector. Over the past two decades, malaria transmissions have been dominant in the World Health Organization (WHO) regions of the Western Pacific, South-East Asia, America, Eastern Mediterranean and sub-Saharan Africa. The latter contributes to the largest proportion of malaria cases and deaths (1). In the WHO World Malaria Report (2021), sub-Saharan Africa contributed >95% of the global malaria cases and deaths, with children under the age of five counting 67% of deaths (1). From the year 2000 to 2015, a decrease of more than 27% in malaria case incidences was observed. These achievements on the malaria burden have been contributed by the vector control strategies among other control interventions. In sub-Saharan Africa, the effective use of insecticide-treated nets (ITNs) and indoor residual spraying (IRS), together with improved case management have vastly reduced the burden of the disease (2–4). In contrast, there was less than a 2% decrease in malaria cases between 2015 to 2019 and a resurgence in 2020, suggesting that the goals of malaria elimination are being derailed (1,5).

Malaria transmission can be unevenly distributed due to various environmental factors such as the distribution of mosquito breeding sites and human dwellings (6), mosquito-related factors such as blood-feeding behaviour (7), dispersal range, non-random host selection (8) or effectiveness of interventions used. However, when human settlements are close to the breeding sites, the range at which mosquitoes can disperse is limited; and mosquitoes tend to remain where human settlements are concentrated (9). A study in coastal Kenya indicated that female

Anopheles mosquitoes were able to travel over 500 m and survive for over 17 days from their point of release (10), while field observations in south-eastern Tanzania showed that biting densities were highest in areas with the greatest human densities and household occupancy was greatest (11). It has also been shown that mosquitoes found farther from their larval (aquatic) habitations in the natural population are more likely to be infected with parasites (9).

It has previously been demonstrated that the susceptibility of mosquitoes to insecticides tends to increase with age (12–14). These findings can have direct implications on the effectiveness of the insecticide-based interventions, such as ITNs and IRS, which now dominate malaria control (1,5). However, there is currently limited research on how these interactions play out in communities such as south-eastern Tanzania where the less-studied *An. funestus* dominates malaria transmission (15,16), and where the vector exhibits moderate to strong pyrethroid resistance (17,18). Moreover, it is unclear whether the variation in phenotype by age can be tracked over fine scales across landscapes and how they would inform malaria vector control.

Studies have shown that mosquito infectivity in nature depends on both the vector age (both biological age and chronological days) and the density of the parasite in the vector (19). Additionally, the distance from the larval (aquatic) habitations increases the probability of them being infected with parasites (9). This might imply a differential age distribution of human-biting mosquitoes by distance from aquatic habitats and hence different infectivity since mosquitoes require about ten days to complete the parasite incubation before they can transmit malaria (20).

The age of wild female mosquitoes can be determined using the morphology of their ovaries (21). Ovaries from wild-caught females can be removed through dissection, and specific morphological characteristics are then evaluated under a stereo or compound microscope. Mosquitoes are identified to be young or old based on the presence or absence of the coiled

tracheolar skeins (also referred to as nulliparous or parous respectively) (21). Ovaries can be further dissected for the presence of ovariole sacs/dilations allowing for the determination of the number of times a female mosquito has laid eggs. The aforementioned factors (age and parasite intensities) have a significant impact on susceptibility towards the insecticides, with the older mosquitoes infected with the parasite being much more susceptible to insecticides (12–14,22).

The WHO insecticide susceptibility procedures recommend that insecticide monitoring is done using the filial one (F_1) 3-5 days old obtained from larval collections or wild-caught female mosquitoes (23). However, in cases where larval collections are insufficient and obtaining laboratory progeny of wild-caught material is difficult, some studies have used adults directly obtained from field trapping (17). Evaluation using such wild-caught mosquitoes has several setbacks including; the variation of the physiological conditions and potential prior exposure to insecticides in the houses from where the mosquitoes were caught. Despite these drawbacks, the wild population still provides a reasonable reflection of the change in the efficacy of insecticidal based interventions in the field. Insecticide resistance in mosquitoes can be detected using specific assays and the phenotypic observation identifies the presence or absence of resistance (23). Standard concentrations of respective insecticide-impregnated filter paper are used according to WHO susceptibility test procedures (23).

When interpreting the tests, insecticide resistance is confirmed if the mosquito mortality is less than 98%, 24 hours post-exposure. The intensity of the resistance can then be determined using an intensity bioassay. This assay relies on exposure of mosquitoes to 5x and 10x the standard concentration in a sequential manner (23). The bioassay determines the intensity of the insecticide resistance whereby so doing indicate the effectiveness of the intervention on a targeted malaria vector in the community.

Once the resistance has been identified in the vector, it is important to understand the mechanism responsible for resistance as directed by the global plan for insecticide resistance management (GPIRM) (24). Metabolic resistance is the most common mechanism of insecticide resistance and is mainly due to increased metabolic enzyme detoxification (23). This can be due to the presence of a large quantity of enzyme production or alteration in the enzymatic catalytic centre, resulting in resistance (25,26).

The main enzyme families responsible for detoxification are P450s (monooxygenase), esterases, and glutathione-S-transferase (GSTs). P450s have been identified as one of the most common enzyme families showing increased activity in resistant vectors. One method to incriminate an enzyme family is through the use of synergists (23). Synergist will block the activity of these enzyme families and if it results in a susceptible phenotype, it will indicate the association of that family with the specific insecticide detoxification (27). For instance, the synergist piperonyl butoxide (PBO), inhibits the P450s. If P450s are the solo resistance enzymes responsible for the mechanism, a 100% mortality will be achieved after exposure to the PBO synergist followed by the insecticide, while the un-synergized group will show mortality below 98%. Metabolic resistance can be further evaluated through microarray assays, real-time polymerase chain reaction (PCR) or biochemical assays by measuring the differential expression of specific genes which code for detoxifying enzymes (28).

1.2 Problem statement

In south-eastern Tanzania where long-lasting insecticidal nets (LLINs) are widely used with indoor residual spraying (IRS) never imposed, the main vectors *An. funestus* is implicated in nearly 80% of malaria transmission, even when present at low abundance (15,16). Furthermore, *An. funestus* is now showing moderate to strong resistance to the commonly used insecticides,

compared to its counterpart, *An. arabiensis* in south-eastern Tanzania (17,18). Except for organophosphates, *An. funestus* mosquitoes are resistant to the other three insecticide classes; carbamate, organochlorine, and pyrethroids. The detected resistance in this vector species shows heterogeneous distribution from one location to another, with an extremely high level of resistance to pyrethroids (17). Likewise, the sympatric species, *An. arabiensis* shows the same heterogeneity by season and location of the collection which necessities the factor-based selection of insecticide based interventions (17,29).

Malaria transmission in these areas and other similar sites can be heterogeneous between and within villages and is influenced by multiple environmental factors such as the distribution of mosquito breeding habitats and human dwellings, mosquito-related factors such as blood-feeding behaviours, dispersal range, age and non-random host selection thus, shaping the effectiveness of interventions used. However, there has not been any in-depth investigation as to how these variabilities would influence the fine-scale allocation of insecticide-based interventions. Likewise, it is unclear whether and how the age of individual mosquitoes and their susceptibility to insecticides used for vector control varies by distance from aquatic habitats, and how these correlations may inform fine-scale interventions in the target villages.

1.3 Justification and relevance to public health

This study was an attempt to complete a more in-depth analysis of the interplay of the above factors and therefore aimed to assess how the age of *An. funestus* mosquitoes and their susceptibility to common public health pyrethroid insecticides vary by distance from the aquatic habitats of these mosquitoes. These investigations allowed us to hypothesize how any such correlations may inform vector control strategies in the specific study areas in south-eastern Tanzania as well as in the other similar sites. For instance, for older, more susceptible

mosquitoes and mostly in the middle of the villages, a ringed control approach involving IRS at the centre and larval source management at the edge of the village may be considered thus interrupting malaria transmission. Since the coverage of pyrethroid-treated nets in the areas is high and P450s are implicated in pyrethroid resistance (30), this study investigated the potency of PBO synergists in enhancing susceptibility to insecticides. The data was collected as part of a larger ongoing project "*Funestus PopBiol (INV-002138)*", and was retrospectively utilized to achieve the objectives of this study.

1.4 Research questions

The main research questions were as follows:

1. What is the influence of age and distance from aquatic habitats on pyrethroid resistance profiles of *An. funestus*, a dominant malaria vector in south-eastern Tanzania?
2. How would the correlation between these factors inform fine-scale targeting of malaria interventions in and around human settlements?

1.5 Aim of the study

To assess the influence of age and distance from aquatic habitats on the pyrethroid resistance status of *An. funestus* mosquitoes in rural south-eastern Tanzania.

1.6 Objectives

1. To assess the influence of age (number of days lived) on the level and intensity of pyrethroid insecticide resistance in *An. funestus*.
2. To assess the influence of distance from aquatic habitats on the level and intensity of pyrethroid resistance in *An. funestus* mosquitoes collected in households.

3. To assess the relationship between distance from the aquatic habitats and mosquito age and how this influences susceptibility.

CHAPTER TWO

2. LITERATURE REVIEW AND SYNTHESIS

This chapter synthesizes the literature and knowledge relevant to this study, covering the distribution of key malaria vectors, the dispersal range of *Anopheles* mosquitoes, the mode of action of insecticides, the role played by insecticides on malaria control and how the age of mosquitoes play a part in the potency of insecticides. It also explores the impact of insecticide resistance of mosquitoes to public health insecticides and how insecticide resistance impacts insecticidal-based interventions. The synthesis is mostly focused on pyrethroid insecticide since our study evaluated permethrin and deltamethrin, the pyrethroid class of insecticide.

2.1 Distribution of malaria vectors

Global and regional malaria elimination and eradication targets are more realistic if the ecology, distribution of malaria vectors and other malaria indicators are well understood. Over 460 *Anopheles* mosquito species are known (31), about 70 of which are capable of transmitting *Plasmodium* parasites to humans (32). Of these, only 41 species are considered to transmit malaria at the public health concern level (33). Currently, in the African region where most of the transmission occurs (1), the malaria vector population is dominated by *An. gambiae s.s.*, *An. arabiensis*, and *An. funestus*, all of which have distributions dependent on climate (34). These vector species have adapted to different feeding preferences and resting locations, the latter preferably feeding on humans (anthropophilic) (7,35) and resting in the house (endophilic) (36), potentially targeted by indoor interventions namely, IRS and ITNs.

The different vector species respond differently to the interventions. For instance, ITNs mostly target mosquitoes that bite indoors and rest indoors. ITNs have been associated with the crash of

An. gambiae s.s populations but not *An. funestus* in some parts of Tanzania and Kenya (37–39). However, in Southern Africa, *An. funestus* population was crashed by IRS, before they became resistant to pyrethroids in the late 1990s (40). Evidence from south-eastern Tanzania indicates that *An. funestus* mosquitoes are now driving malaria transmission even when they occur in low fractions (15,16,18), and are highly resistant to public health insecticides (17,18). This necessitates a thorough understanding of inter and intraspecific disparities of malaria vectors at a local scale to interrupt the transmission.

2.2 Dispersal range of *Anopheles* mosquitoes

Understanding the range at which malaria vectors can disperse is essential for the evidence-based distribution and evaluation of limited vector control interventions. Malaria vector species have varied ranges at which they can fly from their habitats, sometimes cross-boundaries from one village to another. For instance, *An. gambiae s.s.* mosquitoes can fly up to 1.5 km from their aquatic habitats (41–43). Another species, *An. arabiensis* from the *An. gambiae* complex can be recaptured between 300 m and 700 m from the point of release (44). Although the evidence is limited on *An. funestus*, some studies have indicated that this vector species can fly to 500 m from the point of release (10,45). Nonetheless, these findings need to be cautionary inferred by considering the confounding conditions like landscapes, physical barriers, wind, distribution of human settlement and vector density which influence the dispersal range of mosquitoes (45). The flight range appears to be no more than 1.5 km for the main malaria vectors, but it is unclear if this may be extended throughout their adult life stages.

The range at which mosquitoes can fly is often determined by their age, human biting rate, mosquito density and infectivity where the high density of mosquitoes tend to aggregate where human settlements are concentrated (9). Furthermore, the older mosquitoes, mostly infective are

found at the farthest distance from the aquatic habitat (9). Therefore, understanding how the dispersal range and age of individual mosquitoes correlate to insecticide resistance is crucial.

2.3 Mode of action of pyrethroid insecticides

It is vital to understand the mode of action of public health insecticide for better evaluation of the insecticidal interventions and monitoring of insecticide resistance in vector species. Pyrethroids as other available insecticide classes for malaria control (carbamates, organophosphates, neonicotinoids and organochlorines), are a neurotoxin, affecting the central nervous systems delivered from the natural pyrethrin (46,47). Pyrethroid neuro-toxicological effect targets sodium-voltage gated channels by interrupting the opening and closing of the channels resulting in the continual firing of the impulses (**Fig. 1**) (46,48). These channels possess the domain for the attachment of pyrethroid insecticides. When binding occurs, interrupts the closing of the gates and permits continual depolarization, synaptic disturbance, conductance blocking, knockdown and eventually death (49–51). The voltage-gated channels are held at the “closed” state during the resting (hyperpolarization) state and open during excitement (depolarization) allowing specific ions to diffuse across the membrane for impulse generation (52). Upon resistance, mosquitoes avert the process by either producing a large quantity of detoxifying enzymes thus metabolizing the insecticide before reaching the target site or altering the enzymatic catalytic centre to prevent the binding of the insecticide.

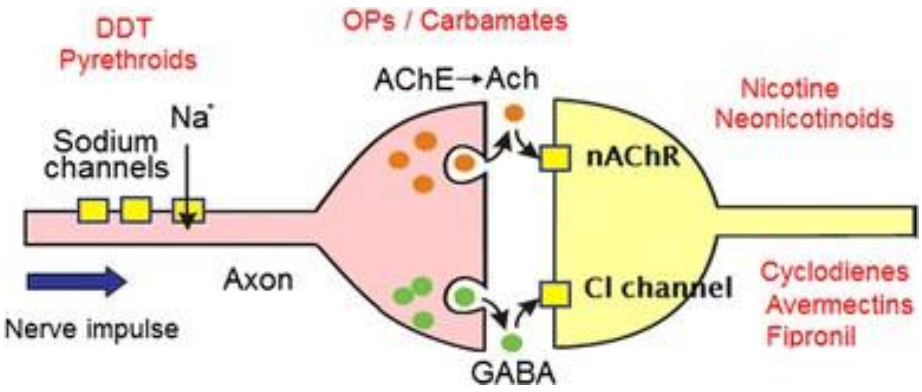

Figure 1. Representation of insecticide site of action at the neurons and synapses. Sodium-ion gate channels (*dichloro-diphenyl-trichloroethane (DDT) and pyrethroid*), Acetylcholinesterase enzymes (AChE) (*organophosphates (OP) and carbamates*), nicotinic acetylcholine receptor (nAChR) (*nicotine and neonicotinoids*), chlorine (Cl) ion channel (*ivermectin, fipronil and cyclodienes*). © Field L.M. *et al*., 2017. https://link.springer.com/article/10.1007/s00249-016-1195-1

2.4 The role of pyrethroid insecticides in malaria elimination

Malaria elimination and eradication efforts depend on a) early diagnosis, case management and treatment and b) control of malaria vector species by reducing the population of the vector in a complementary manner, the latter primarily reliant on insecticides (53). There are only four classes of insecticides that are mainly used for malaria vector control namely organochlorides, organophosphates, carbamates and pyrethroids with the additional currently approved neonicotinoids by the World Health Organization (WHO) for indoor residue spraying (54). Pyrethroids, the only approved insecticide for ITNs, has gained pronounced use in the vector control sector due to their non-lethality to non-targeted species and short persistence in the environment making them environmentally friendly (47,55). A large proportion, over 20% of the

insecticide market currently is being dominated by pyrethroids, to which most malaria vector species have become resistant (56,57).

Considering effectiveness, environmentally friendly and non-lethality to non-targeted organisms of pyrethroid insecticides, pyrethroid treated bet-nets have been widely distributed (58). This significantly interrupts malaria transmission through chemical effects and physical barriers affecting the vectorial capacity parameters like survival, human biting rates, abundance and infection rates (58). Although insecticidal malaria vector control has achieved much in malaria control, insecticide resistance to commonly used insecticides specifically pyrethroids poses a noticeable threat (56). For better and early mitigation of pyrethroid insecticide resistance challenge, a thorough understanding of the scope of a problem, and the epidemiological impact of insecticide resistance on malaria elimination and eradication efforts are essential.

2.5 The situation of pyrethroid resistance in Africa

Insecticide resistance in malaria vectors has become a challenge to malaria elimination efforts, thus exhausting the available vector control opportunities largely depending on insecticide based intervention. Pyrethroid resistance in Africa was already detected from the early 1970s to the late 1990s which was linked with exposure to mosquitoes in agricultural activities when pyrethroids were used for crop protection (59,60). The situation worsened in the 20th century, with the majority of malaria vectors currently being resistant to pyrethroid insecticides (3,61). These vectors are reported to have developed cross-resistance with multiple resistance mechanisms especially the insecticides with shared target sites for instant pyrethroid and DDT (62,63). This is worsening the situation and poses a question of whether new insecticide formulations with a different mode of action, the combination of insecticides or even insecticide-free interventions or integrated vector management (IVM) is best.

In the eastern parts of Africa, Tanzania's mainland, pyrethroid based ITNs programmes have been implemented since the 1990s and malaria vectors are reported to be resistant to the common insecticides, including key pyrethroids (64). In the past two decades, the resistance of malaria vector species to pyrethroids has drastically increased from 8-33% proportion of study sites reporting resistance in 2006 to 27-39% in 2017 (65). However, the delayed strengthening of resistance monitoring must be considered when interpreting this evidence as it might be influencing the observed trend.

2.6 The age of mosquitoes and insecticide resistance

Malaria vectors require at least ten days for parasite incubation before they can transmit the disease to the next host (20). Likewise, the susceptibility of mosquitoes to insecticide is age-dependent with the younger ones being less susceptible compared to the older ones, able to transmit the parasites (12–14,66). Unfortunately, insecticide susceptibility test procedures by WHO preferably direct the use of young mosquitoes (3-5 days old) for insecticide resistance monitoring (23). Moreover, mosquitoes of the recommended age group are younger and unable to transmit the parasite. This poses the dispute of if LLINs are more effective in the areas where the vector species are still susceptible to the insecticide and only marginal value is added when the vectors become resistant (67). However, insecticidal interventions, for instance, LLNs, which are pyrethroid-impregnated might still effectively kill the older mosquitoes and also provide a physical barrier to the young resistant population, thus interrupting malaria transmission.

2.7 The epidemiologic link between insecticide resistance and operational failure

The epidemiological link between insecticide resistance and malaria vector control failure is still a debatable subject, especially for ITNs, but there is clear evidence of linkage in the case of

purely insecticidal tools such as IRS (68). A study in the late 1990s demonstrated the correlation between malaria resurgence and the change in insecticide use from dichloro-diphenyl-trichloroethane (DDT) to pyrethroid in South Africa (**Fig. 2 & 3**) (40,69,70). DDT was promptly reintroduced shortly after the resurgence of malaria cases (40) and the detection of insecticide resistance in malaria vectors (71), hence the deceleration of malaria outbreaks (70) (**Fig. 3**). However, this should be interpreted with care, as the study was not specifically designed to assess how the scenarios correlate. Moreover, the detection of once-eliminated *An. funestus* (69,72) in the area and the resurge of the cases (40) could be concomitant to the competitive replacement of *An. gambiae* (*s.l.*) (73), however, this was not reported in South Africa. Another study in Malawi revealed the operation failure of IRS in areas with high pyrethroid resistance levels (68). Contrary to the widespread insecticide resistance of mosquitoes to commonly used insecticides, the evidence on the link between insecticide resistance to operation failure is inadequate.

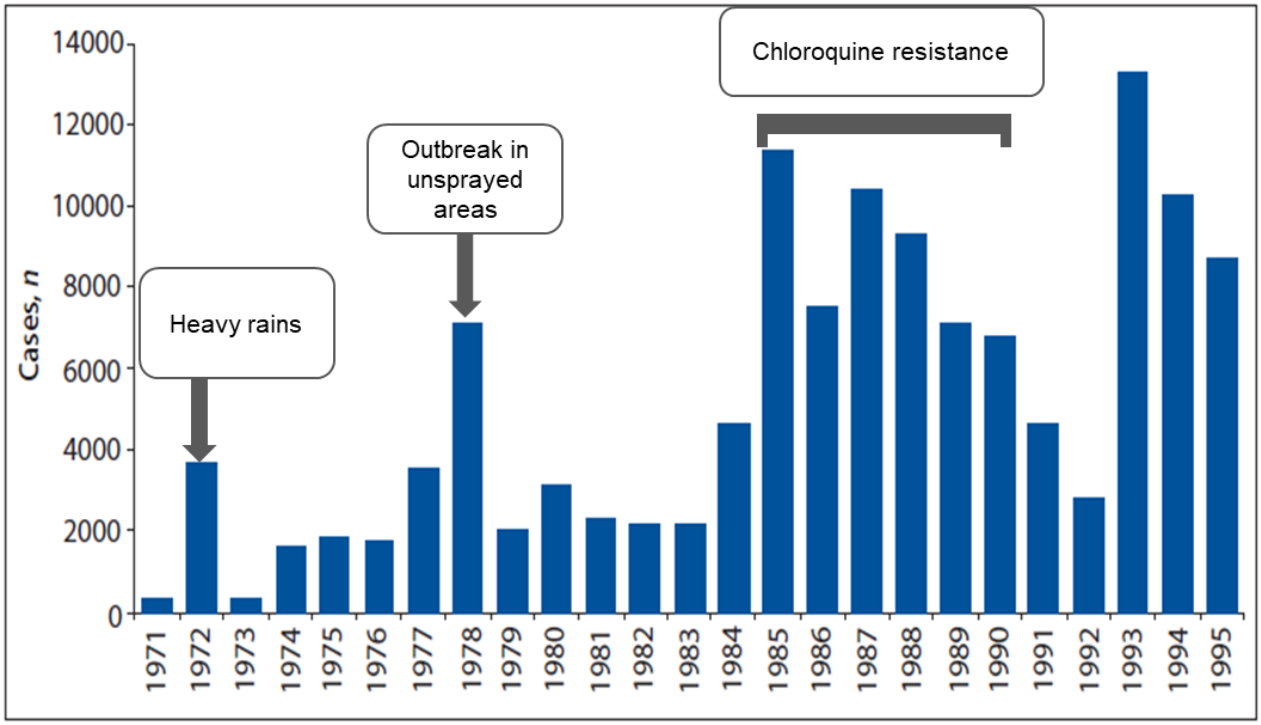

Figure 2. The maintained low number of malaria cases by the introduction of DDT (dichloro-diphenyl-trichloroethane) and the elimination of *An. funestus* in South Africa since 1945. Modified from © Coetzee M. *et al*., 2013.

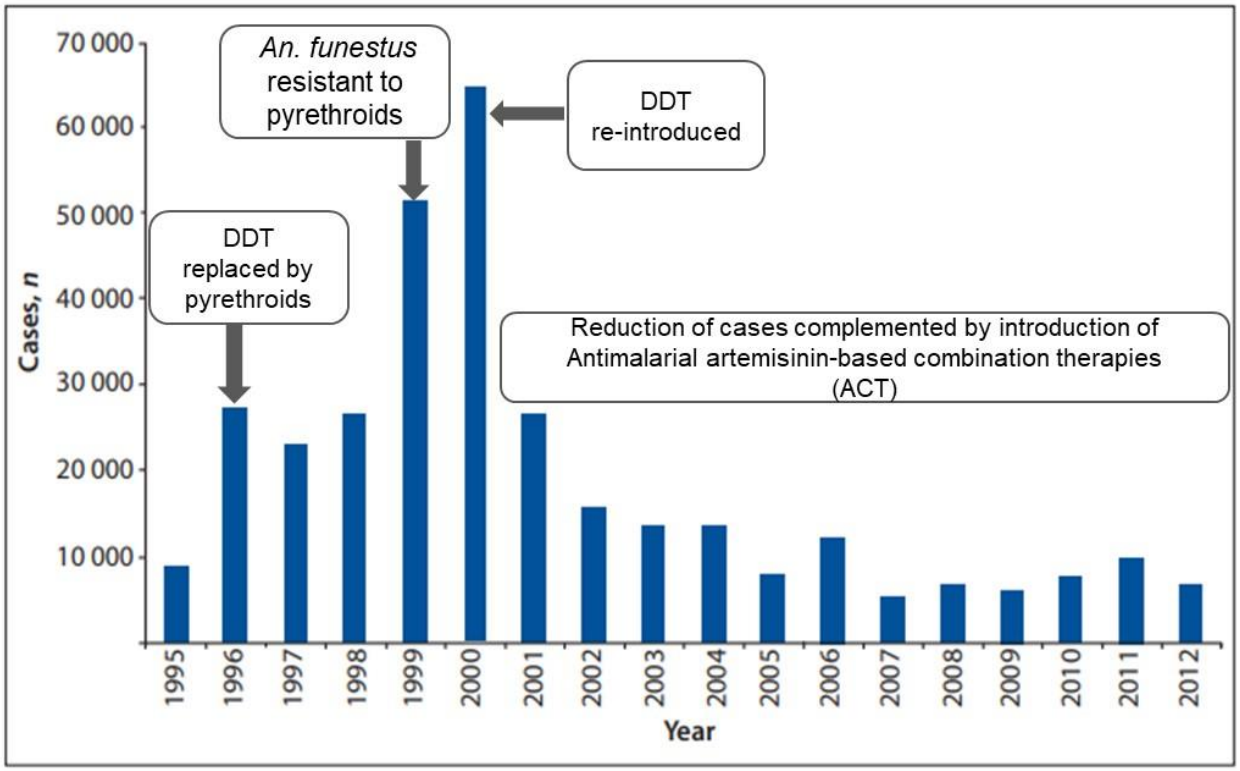

Figure 3. The link between malaria cases doubling in 1995 and pyrethroid introduction, *An. funestus* re-invasion and resistance and the decline in 2001 by DDT (dichloro-diphenyl-trichloroethane) reintroduction in South Africa. Modified from © Coetzee M. *et al*., 2013.

Thus, scholars are yet to agree on whether pyrethroid-based interventions, the insecticide to which malaria vectors are resistant are effectively serving the purpose (74,75). It is argued that although mosquitoes are resistant to pyrethroid, LLNs still offer physical protection to the users as they could have when untreated (74,75). Despite the hypothesis that the areas with high insecticide resistance would have high malaria prevalence and incidences, WHO multi-country studies have demonstrated that there is no correlation between insecticide resistance and malaria cases (76). Nevertheless, the evidence in South Africa might still hold as *An. funestus* elimination and re-invasion are associated with the low and high malaria transmission in the area

respectively (70–72). The debate remains as to whether insecticide resistance directly implies the operational failure of insecticidal based vector control intervention and whether these interventions should be insecticidal free and or new insecticide formulations are urgently needed.

Although multiple attempts have been investigated to incriminate the insecticide resistance and operational failure, it is still imprecise and methodologically challenging to associate this with malaria prevalence and incidences. Therefore, many studies have used the standard WHO susceptibility test procedures as a proxy for the efficacy and effectiveness of the insecticidal interventions on the natural vector population (23). This allows using both field population and the laboratory age-synchronized specimens.

When the vectors show resistance under intensity bioassays (5×, 10× the standard concentration), it is inferred to reflect the operational failure although this provides no linkage to malaria incidences. However, when this approach is used in combination with age, infectivity, distance, distribution of human settlements and mosquito larval distribution parameters it might still inform the vector control strategies and insecticide resistance at the fine scale. A modelling work by Smith revealed that a large proportion of older, parasite-infected mosquitoes is found at the farthest distance from their aquatic habitat (9). Thus, more transmission might be concentrated away from the aquatic habitats. This could have motivated the absence of a correlation between the insecticide resistance and malaria epidemiology where the samples were randomly selected considering only the resistance profile and malaria status (76,77).

CHAPTER THREE

3. MATERIALS AND METHODS

Since this study was retrospectively evaluated, section A provides methods that were conducted by others participating in this study during data collection (Section A). However, this is included to provide background on the data that were analysed. Section B explains the methods that were employed for the analysis of the data for this project and research report.

3.1 Section A

3.1.1 Study site

Adult female *An. funestus* mosquitoes were collected from the spatially isolated hamlet in Ikwambi village in Kilombero district, south-eastern Tanzania from August to December 2020. The village is located at 7.98033°S, 36.81701°E; elevated 400 m above the sea level; rainfall: 1200-1800 mm per year; temperatures: 20-33°C) (78) (**Fig. 4**). Additionally, mosquito larvae were collected from known aquatic habitats of *An. funestus* in the area as previously described by Nambunga *et al*., (79). The area had been selected based on previous surveys indicating that *An. funestus* dominated in both densities and malaria transmission activity.

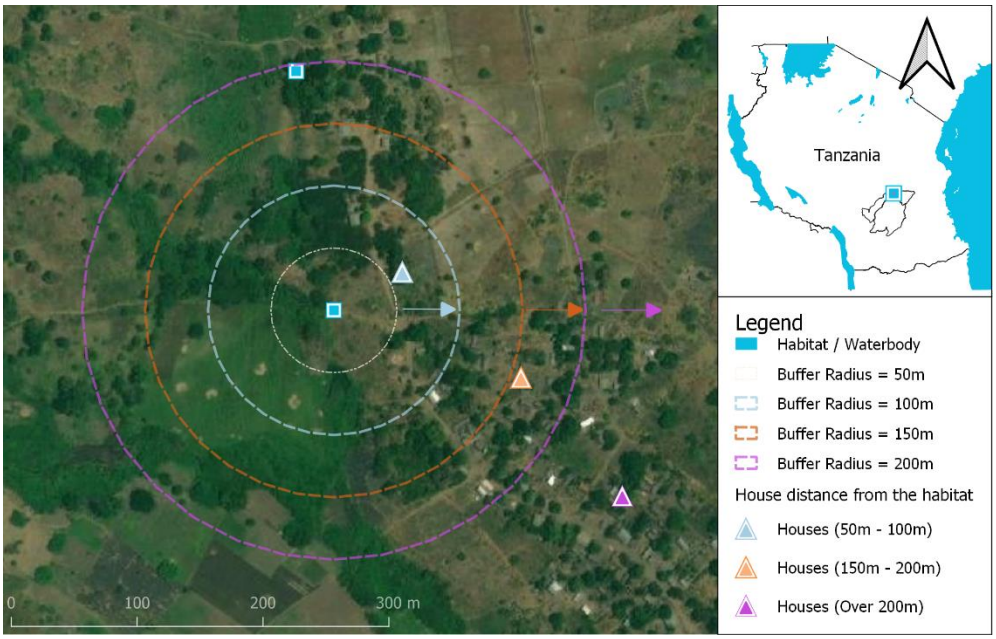

Figure 4: Map of the study area showing adult mosquito collection sites and distance from aquatic habitats.

3.1.2 Field collections of adult mosquitoes

The WHO guidelines on the collection of specimen for insecticide susceptibility bioassay was used for the experiment (23). Initial surveys were done to identify and mark all possible aquatic habitats for *An. funestus*, following the attributes previously described by Nambunga *et al* (79), using either a standard dipper or 10L bucket for larval sampling to confirm the presence of the species. The distance from confirmed habitats of *An. funestus* to human habitations were calculated based on geo-locations data obtained using a handled Global Positioning System (GPS) receiver (eTrex, Vista, Garmin, USA). Three buffer distances were created from the confirmed habitats as follows; a) 50-100 m (edge of the village), b) 150-200 m (intermediate distance), and c) over 200 m (at the centre of the village) (**Fig. 4**).

The Centre for Disease Control and Prevention (CDC) light trap placed beside the volunteer-occupied bed nets was used to collect host-seeking female mosquitoes from 18:00-07:00 hours

between August and December 2020. The collections, separated by distance from the nearest aquatic habitat were acclimatized for 24 hours in an insectary and supplied with 10% glucose solution and dead ones were discarded before the insecticide susceptibility tests were started. A separate cohort of mosquitoes was collected at the same distance using a volunteer-occupied miniaturized double net trap (DN-Mini) placed beside human dwellings (80). These additional collections were dissected and used for estimating mosquito age distribution by distance. The identification of the collected mosquitoes was done using dichotomous keys (81).

3.1.3 Larvae sampling and rearing

Anopheles funestus larvae were collected from different sites. Similar larval sampling methods and tools were used as aforementioned above, using procedures previously described by Nambunga *et al* (79). The collected larvae were transported with their natural water and reared in an insectary at 80 ± 10% relative humidity and 27 ± 2°C. Tetramin® fish food (Tetra GmbH, Melle, Germany) was provided to supplement the aquatic diet, and the pupae collected were placed in separate cages and supplied with 10% glucose solution for the subsequently emerging adults. For this round of insecticide susceptibility tests, the mosquitoes were used in three separate age groups post-emergence; **a)** (3-5 days), **b)** (8-11 days) and **c)** (17-20 days).

3.1.4 Insecticide resistance bioassays

Two candidate pyrethroids (deltamethrin and permethrin) were evaluated using WHO guidelines (23), in a step-wise fashion, starting with standard concentrations (1×) for phenotypic resistance evaluation, followed by 5× and 10× the standard concentrations to assess the intensity of resistance. The synergist bioassay using PBO was also conducted to assess the possible involvement of the cytochrome P450's enzyme-based metabolic resistance.

Each test had six replicates, 20 individual mosquitoes per replicate, per concentration (3×) per insecticide (2×), per distance (3×)/age class(3×), totalling 4,320 mosquitoes. The first round of bioassays (using adults collected from houses) was done based on the different distance ranges from the aquatic habitats, while the second round of bioassays (using emergent adults collected as larvae) at different age classes as described above. Mosquitoes were exposed to the insecticides for one hour and moved to non-insecticidal holding tubes with 10% glucose solution, then monitored 24 hours post-exposure for mortality. Since the mosquitoes were resistant to standard concentrations of both pyrethroids, intensity bioassays were completed to assess the intensity of the resistance, following procedures described in the WHO guidelines (23).

Tests with synergist (4% PBO) had three replicates, comprising four cohorts as follows: a) mosquitoes exposed to either deltamethrin or permethrin only at standard concentration, b) mosquitoes exposed to 4% PBO followed by pyrethroids, c) mosquitoes exposed to 4% PBO only, and d) mosquitoes exposed to the silicone oil-coated papers (control group). This totalling the sample size of 8,640 mosquitoes. The exposed mosquitoes were all provided with 10% glucose solution and mortality was recorded 24 hours post-exposure. Tests were discarded if the control group's mortality exceeded 20%.

3.1.5 Mosquito age grading

All mosquitoes that were exposed to insecticides were dissected, whenever alive or freshly dead after 24 hours of mortality monitoring of the insecticides (44%, n=1,577). The rest were dry and unable to be dissected (56%, n=2,023). The dissection of the ovaries followed Detinova's method described in the WHO guidelines for practical entomology (21) using a stereo-microscope. The dissected ovaries were observed under a compound microscope for the presence or absence of the coiled tracheolar skeins, ie. nulliparous (young) or parous (old) respectively.

Parous mosquito is the one which has at least laid eggs once while nulliparous are the ones which have not laid eggs (82–84).

An additional cohort of mosquitoes collected using the CDC-LT (n=560) and the miniaturized double net trap (DN-Mini) (n=78), and not exposed to the insecticides were dissected for identification of the ovariole dilations to determine how many times the mosquito had laid eggs (i.e. the number of gonotrophic cycles). The results of the dissected mosquitoes were recorded by the distance from the aquatic habitat(s) and the method of collection. In this additional cohort data, mosquitoes collected on the same day were referred to as a replicate, different from what a replicate is referred to for the WHO bioassay.

3.1.6 Molecular identification of the mosquitoes

To identify *An. funestus* sibling species for confirmation of the studied vector species, a sub-sample of the tested mosquitoes (at least two from each replicate i.e. 10%) were separately packed in 1.5ml micro-centrifuge tubes containing silica gel and transferred to the laboratory for molecular species identification. The information on a specific sample was recorded before subjection for molecular identification. The identification of the sibling species of *An. funestus* group was done using the DNA extracted from the legs as previously described by Scott et al, (85). Two legs of individual mosquitoes were placed separately in a 1.5 ml Eppendorf tube followed by the addition of 20µl of Tris-EDTA (TE) buffer then incubated at 95ºC for 15 minutes in the heating block. Afterwards, the tubes were vortexed for 2 minutes and the DNA containing supernatant was separated by centrifuging at 12,000 rpm at room temperature for 2 minutes.

The non-coding internal transcribed spacer 2 region (ITS2), a species-specific nucleotide sequence of the ribosomal DNA (rDNA), was used for identification (86). One negative control was included which contained all substances in the PCR mixture except DNA. Five positive controls were also included; *An. funestus, An. rivulorum, An. vanedeeni, An. parensis and An. leesoni* from the previous successful amplified samples. The PCR conditions included an initial denaturation step at 94ºC for 5 minutes followed by 30 cycles at 94ºC for 30 seconds, 50ºC for 30 seconds and 72ºC for 30 seconds with a final extension at 72ºC for 3 minute. After amplification, 13 µl of the PCR products were analyzed by electrophoresis on 2.5% agarose gel stained with 0.5µg/ml ethidium bromide and a 100bp DNA ladder was included in the gel. The Kodak Gel Logic 100 system of imaging was used for DNA amplicons photographed under ultraviolet light (87).

3.2 Section B

3.2.1 Data analysis

The percentage mortality of mosquitoes was calculated according to WHO insecticide resistance test procedures as a fraction of the total numbers exposed (23). Observed mortality was reported when the control mortality was less or equal to 5%. Abbott's formula was used to calculate the corrected mortality when the observed mortality in control was greater than 5% but less than 20%, but any bioassay with control mortality exceeding 20% was discarded (88). The mosquitoes were inferred to be a) susceptible, if mortality equalled or was greater than 98%, b) resistant, if mortality was less than 90% or c) possibly resistant if mortality was 90% to 97%.

$$\boldsymbol{Observed\ mortality} = \left|\frac{Total\ number\ of\ mosquitoes\ dead}{Total\ number\ of\ mosquitoes\ exposed}\right| * 100$$

$$\boldsymbol{Corrected\ mortality} = \left|\frac{\%\ Observed\ mortality - \%\ Control\ mortality}{100 - \ \%\ Control\ mortality}\right| * 100$$

Further data analysis was done using statistical software, R-Software version 3.6.0 (89). Descriptive analysis, with the graphical presentation, was used to illustrate the percentage mortality and level of resistance in mosquitoes at different distances and ages. A generalized linear mixed effect model (GLMM) assuming binomial distribution and logit link function was used to compare the response of mosquitoes (dead or alive) when exposed to insecticides at different age groups and distances since the data were pooled (not individual). Age (objective 1) and distance (objective 2) individually were added as fixed factors whereas replicate was added as a random factor. The nearest distance to the habitats (50-100 m) and youngest age class of mosquitoes (3-5 days) were individually (per objective) considered as the reference for these analyses. The odds ratios were reported with a 5% level of significance with biological relevance were reported for the association of distance or age to insecticide resistance of *An. funestus* mosquitoes. Since the outcome variable (dead or alive) was uniform for objectives 1 and 2 with differing exposure variables (age, distance, insecticide, concentration, synergist), the analysis approach was used on both objectives. The motivation for using GLMM is because it generalises the linear model used in the analysis of variance (ANOVA) by allowing any other type of distribution other than Gaussian distribution.

3.3 Ethics consideration

The main project, "Funestus PopBiol (INV-002138)" was permitted by the Institute Review Board of Ifakara Health Institute, IHI/IRB/No: 26-2020 (**Appendix 1**) and Medical Research Coordinated Committee of the National Institute for Medical Research of the United Republic of Tanzania, NIMR/HQ/R.8a/Vol. IX/3495 (**Appendix 2**). In addition, although this study was

nested within a certified major project with no human participants rather invertebrates, ethical approval was attained from the University of the Witwatersrand Animal Research ethics Committee, Waiver 11-10-2021-O (**Appendix 3**) before the commencement of the study.

CHAPTER FOUR

4. RESULTS

This chapter is the presentation of the results of the study research questions. The results respond to our research questions on what is the influence of *An. funestus* age and distance from aquatic habitats on pyrethroid insecticide resistance in south-eastern Tanzania, how do these two factors (i.e. age of individual mosquitoes and their susceptibility to insecticides used for vector control) vary by distance from the aquatic habitats and how would the correlation between these factors inform fine-scale targeting of malaria interventions? For the model results presented in this section, the nearest distance (50-100 m) and the young age (2-5 days old) mosquitoes were used as the reference groups to which other distances (150-200 m, over 200 m) and ages (8-11 days, 17-20 days) were compared.

4.1 Variation of insecticide susceptibility by distance from habitats

Adult mosquitoes collected at different distances (50-100 m, 150-200 m, and over 200 m) from the aquatic habitats were used to evaluate whether pyrethroid insecticide susceptibility varies by distance. Mosquitoes collected inside houses (n=720) were resistant to both deltamethrin and permethrin at the standard doses. The percentage of mortality varied by the distance from the aquatic habitats (**Fig. 5 & Table. 1**).

The mean percentage mortality of mosquitoes exposed to the standard dose (1×) of deltamethrin increased from 11% at the nearest distance (50-100 m) to 38% at the farthest distance (over 200 m). When exposed to permethrin, mortality ranged from the means of 23% at the nearest distance to 41% at the farthest distance. Overall, at a standard dose of deltamethrin and permethrin, mosquitoes collected farthest (Over 200 m) from the aquatic habitats showed higher

mortality (41%) than those collected nearest (50-100 m) to the habitats (11%) (**Fig. 5 & Table. 1**).

The odds of dying for mosquitoes collected beyond 200 m from the aquatic habitats, exposed to deltamethrin standard dose was 5.54 (CI, 2.43-12.63, p<0.001) times compared to the odds of dying when collected at the nearest distances (50-100 m) (**Table. 1**). Although there was no statistical difference, the odds of dying for mosquitoes collected at 150-200 m from the aquatic habitat was 1.26 (CI, 0.49-3.22, p=0.633) times compared to the odds of dying when collected at the nearest distance (50-100 m) from the aquatic habitats (**Table. 1**). For mosquitoes collected beyond 200 m and exposed to permethrin standard dose, the odds of dying was 2.4 (CI, 1.06-3.26, p=0.033) times compared to the odds of dying when collected from the nearest distance (50-100 m) (**Table. 1**).

Intensity bioassays were performed on the observed varied resistance (mortality <98%) by distance at the standard dose of insecticides to evaluate the level of resistance of *An. funestus*. A similar trend was observed when the mosquitoes were exposed to five times the standard doses of deltamethrin, with mosquitoes from the farthest distance showing the highest mortality (54%) compared to the nearest distances (43%) (**Fig. 5 & Table. 1**). At five times the standard dose of permethrin, when the odds of dying for mosquitoes collected at the farthest distance (over 200 m) was 3.53 (CI, 1.76-7.04, p=0.007) times compared to the odds of dying when collected at the nearest distance (50-100 m) (**Table. 1**).

Since the mosquito species showed resistance to five times the standard dose of deltamethrin and permethrin, the maximum dose (ten times) was evaluated. It was similarly observed that the odds of dying when exposed to insecticide significantly increased with the distance from the aquatic habitats (p<0.05) (**Fig. 5 & Table.1**).

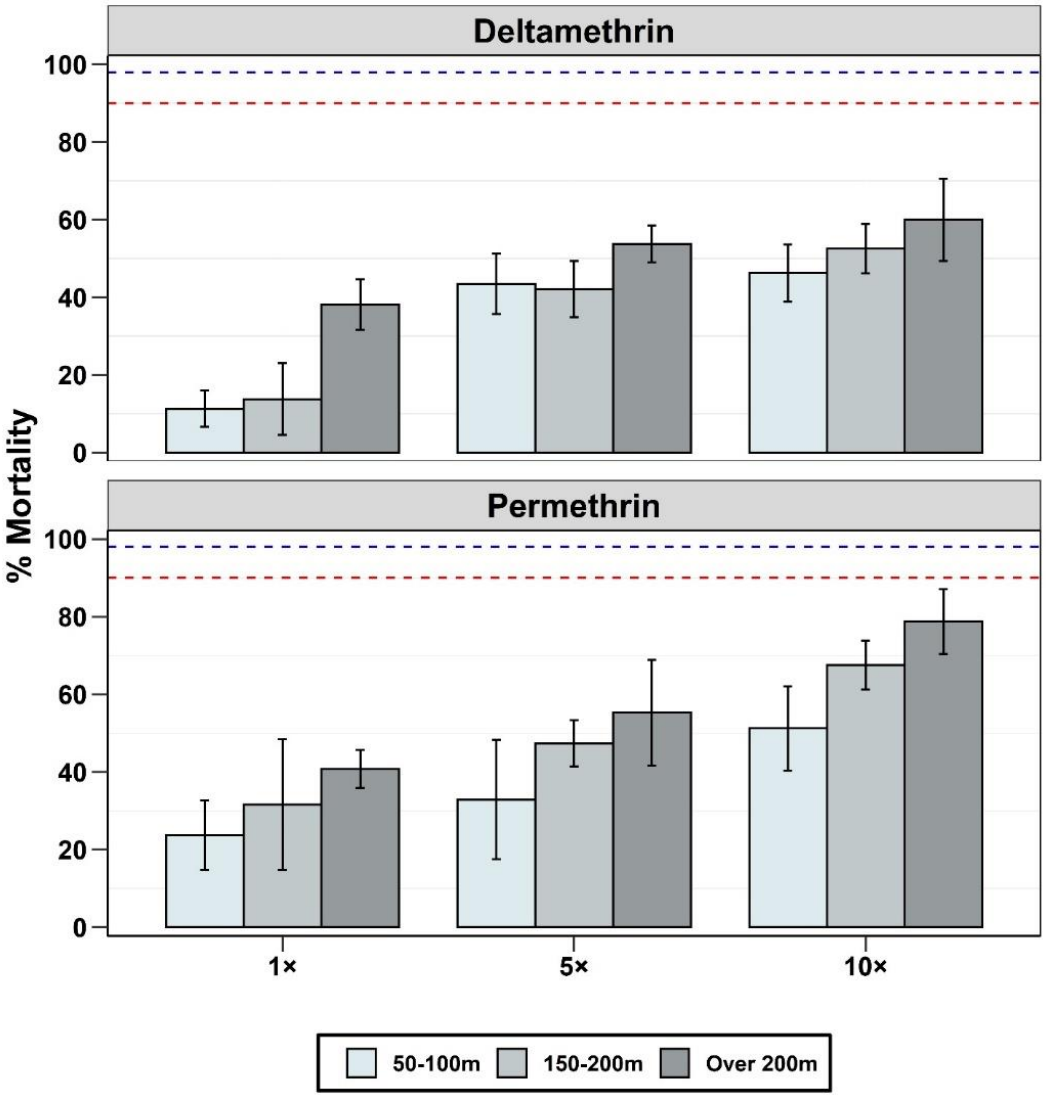

Figure 5: Percentage mortality in *An. funestus* mosquitoes that were collected at different distances from the aquatic habitats and exposed to deltamethrin or permethrin. The error bar represents the standard error, the red-dotted lines represent 90% mortality and the blue-dotted lines represent 98% mortality.

Table 1. The odds of dying of adult female *An. funestus* mosquitoes that were collected at different distances and exposed to pyrethroid insecticides (Deltamethrin, Permethrin) and synergist (PBO). **Note:** The nearest distance (50-100 m) was used as the reference distance for other distances.

Field adult-collected mosquitoes					
Insecticide	**Fold**	**Distance (m)**	**Mean mortality (%)**	**OR (95% CI)**	**p-value**
Deltamethrin	1×	50-100	11	-	-
		150-200	14	1.26 (0.49-3.22)	0.633
		>200	38	5.54 (2.43-12.63)	<0.001
	5×	50-100	43	-	-
		150-200	42	0.95 (0.51-1.77)	0.873
		>200	54	1.35 (0.73-2.51)	0.343
	10×	50-100	46	-	-
		150-200	53	1.28 (0.69-2.39)	0.429
		>200	60	1.74 (0.93-3.26)	0.082
Permethrin	1×	50-100	23	-	-
		150-200	32	1.42 (0.72-2.78)	0.307
		>200	41	2.05 (1.06-3.97)	0.033
	5×	50-100	33	-	-
		150-200	47	1.77 (0.94-3.34)	0.080
		>200	55	2.40 (1.27-4.54)	0.007
	10×	50-100	51	-	-
		150-200	67	1.98 (1.04-3.75)	0.037
		>200	79	3.53 (1.76-7.04)	<0.001
Deltamethrin + PBO	PBO	50-100	81	-	-
		150-200	93	10.41 (1.28-85.00)	0.029
		>200	91	3.35 (0.86-13.07)	0.081
Permethrin + PBO	PBO	50-100	85	-	-
		150-200	98	3.14 (0.94-10.51)	0.063
		>200	95	2.47 (0.80-7.61)	0.115

Key: OR; Odds ratio, CI; Confidence interval, 1×; Standard dose, 5×; Five times standard dose, 10×; Ten times standard dose, Fold; Dose, 50-100 m; Reference distance.

This study further investigated the potency of PBO synergists in enhancing susceptibility of insecticide and the implication of P450's enzymes in observed pyrethroid resistance. Pre-exposure to the synergist, PBO, significantly increased the potency of the candidate pyrethroids against the resistant mosquitoes, achieving more than 80% mortality at all distances (**Fig. 6**).

There were no clear trends between the final mortality outcomes post-exposure and the distance from which the mosquitoes were collected (**Fig. 6 & Table. 1**). However, mosquitoes collected from the nearest distance (50-100 m) had increased mortality but were lower compared to the ones collected from the farthest distance (over 200 m) (**Fig. 6**). The odds of dying for mosquitoes collected beyond 200 m from the aquatic habitats, exposed to synergist before deltamethrin insecticide was 3.53 (CI, 0.86-13.07, p=0.08) times the odds of dying when collected at the nearest distance (50-100 m) (**Table. 1**).

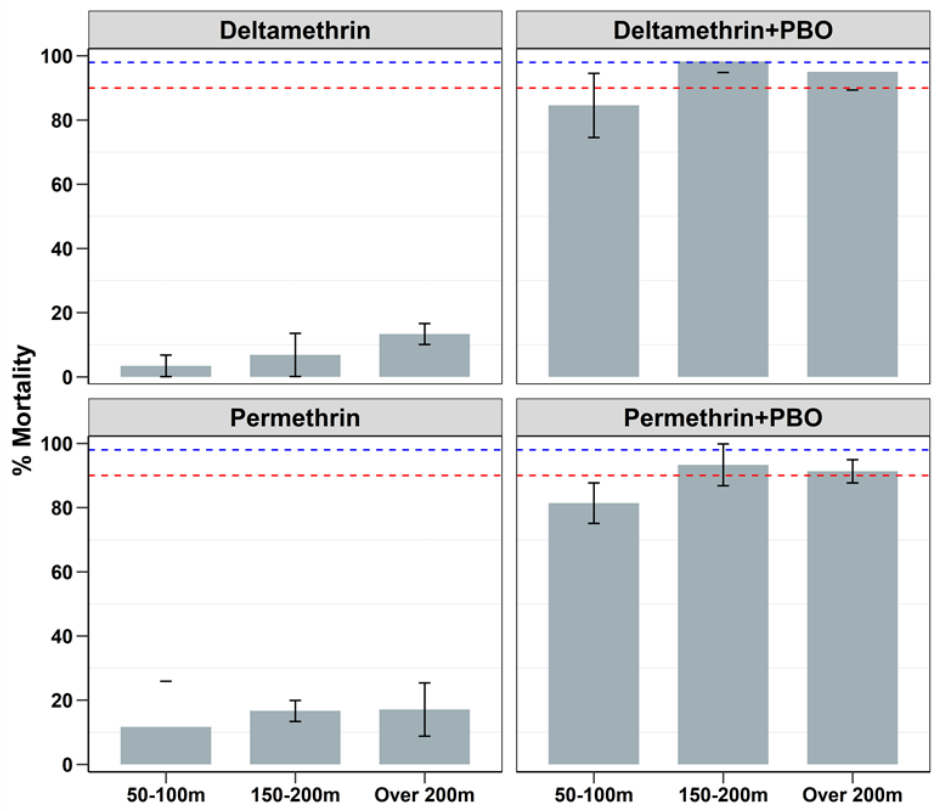

Figure 6: Percentage mortality in *An. funestus* mosquitoes that were collected at different distances from the aquatic habitats and exposed to either the candidate pyrethroids alone or PBO followed by pyrethroids. The error bar represents the standard error, the red-dotted lines represent 90% mortality and the blue-dotted lines represent 98% mortality.

4.2 Relationship between distance and biological age of mosquitoes

Mosquito dissection was used to evaluate whether the biological age of collected mosquitoes (prior exposure to insecticide) (n=1,577) varies by distance from the aquatic habitats, how it affects their response to insecticides and whether the distance can be used to estimate the age distribution of *An. funestus* in the field population (**Fig. 7**). Although the difference was marginal, mosquitoes collected CDC-LT farthest from aquatic habitats and exposed to

insecticide had greater proportions of parous mosquitoes compared to the ones collected from the nearest distance (50-100 m) (**Fig. 7**). Some replicates of mosquitoes collected beyond 200 m were 100% parous (**Fig. 7**). Furthermore, this correlation was extended without insecticide exposure and the number of gonotrophic cycles was determined (**Fig. 8**). It was observed that mosquitoes collected using the CDC-LT were mostly nulliparous (gonotrophic cycles = 0) and those with multiple gonotrophic cycles (1-3) were equally distributed over the distances (50-100 m, 150-200 m, over 200 m) (**Fig. 8 top panel**). However, when the DN-Mini trap was used, the proportion of nulliparous mosquitoes dropped while the proportion of those with multiple gonotrophic cycles increased by the distance (**Fig. 8 bottom panel**).

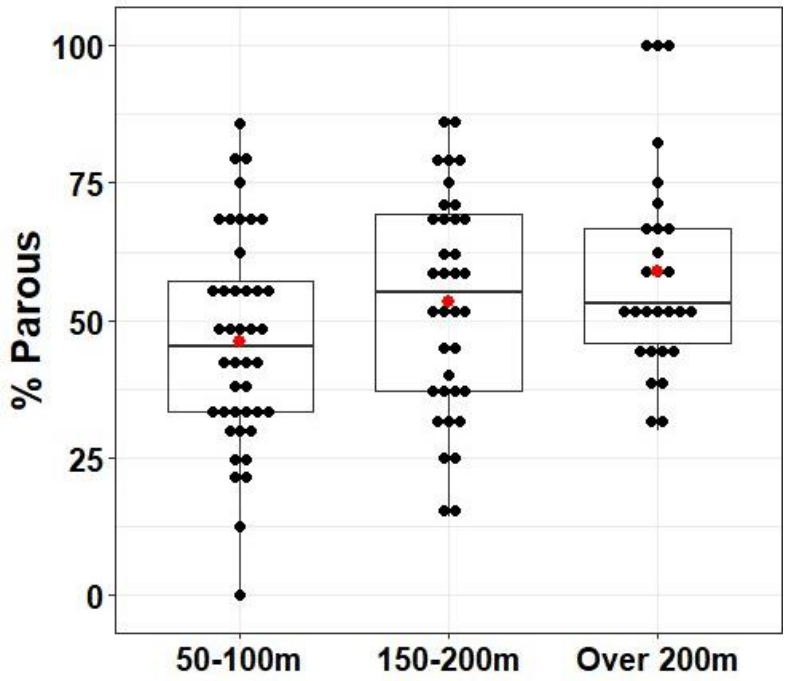

Figure 7: The parity status of *An. funestus* mosquitoes at different distances post insecticide exposure. Red dots indicate the means, the horizontal line represents the median, each horizontal black dot represents a replicate and whiskers (low and high) represent minimum and maximum values.

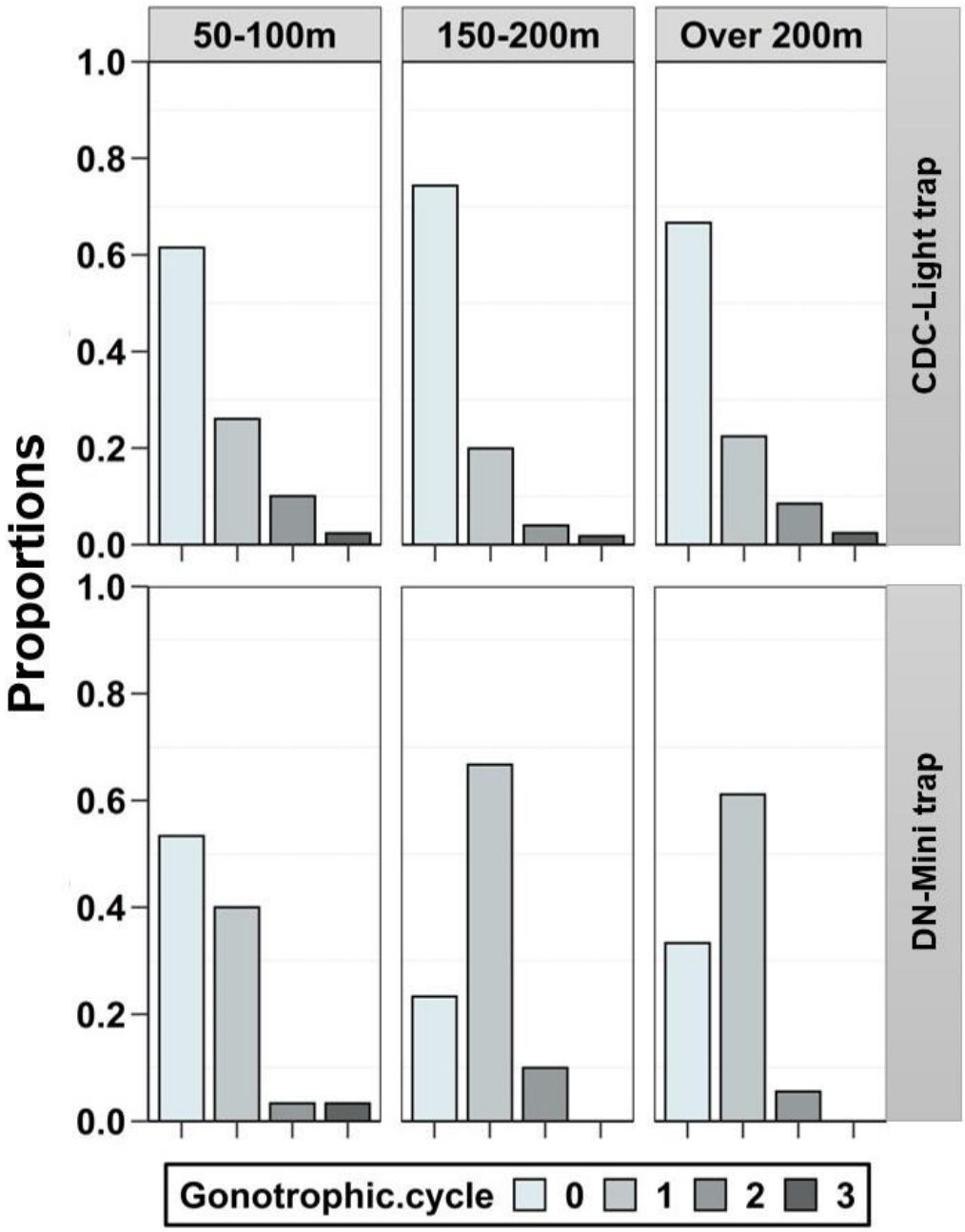

Figure 8: Proportion of mosquitoes with different numbers of gonotrophic cycles, among those collected by either CDC-LT (top panel) or DN-Mini (bottom panel) over the different distances.

4.3 Variation of insecticide susceptibility by the chronological age of mosquitoes

The mosquitoes collected as larvae were used to assess variations of insecticide susceptibility in the different chronological age classes. Overall, the percentage of mortality increased with age, suggesting that the older *An. funestus* were more susceptible than younger ones from the same area (**Fig. 9 & Table 2**). However, when exposed to deltamethrin, no age group at any concentration (1×, 5×, 10×) attained the recommended susceptibility status (≥98%). When mosquitoes in the age group of 17-20 days old were exposed to a standard dose of deltamethrin, the odds of dying was 2.00 (CI, 1.02-3.93, p=0.044) times compared to the odds of dying when exposed at 3-5 days old (**Table. 2**).

Similar to a standard dose of permethrin, the odds of dying increased with age. For example, the odds of dying for 17-20 days old mosquitoes was 14.82 (CI, 6.89-31.89, p<0.001) times compared to the odds of dying when exposed at 3-5 days old (**Table. 2**).

The intensity bioassays were conducted on the observed resistance to determine the levels of resistance and how these levels varied by age. As it was observed at the standard concentration (1×), at five times (5×) dose of deltamethrin mortality ranged from 45% to 70% and 84% to 97% for permethrin (**Fig. 9 & Table 2**). At both five times and ten times dose of deltamethrin and permethrin, the odds of dying for mosquitoes exposed at 17-20 days old were significantly higher (>2.8) compare to the odds of dying when exposed at 3-5 days old (p<0.05) (**Table. 2**).

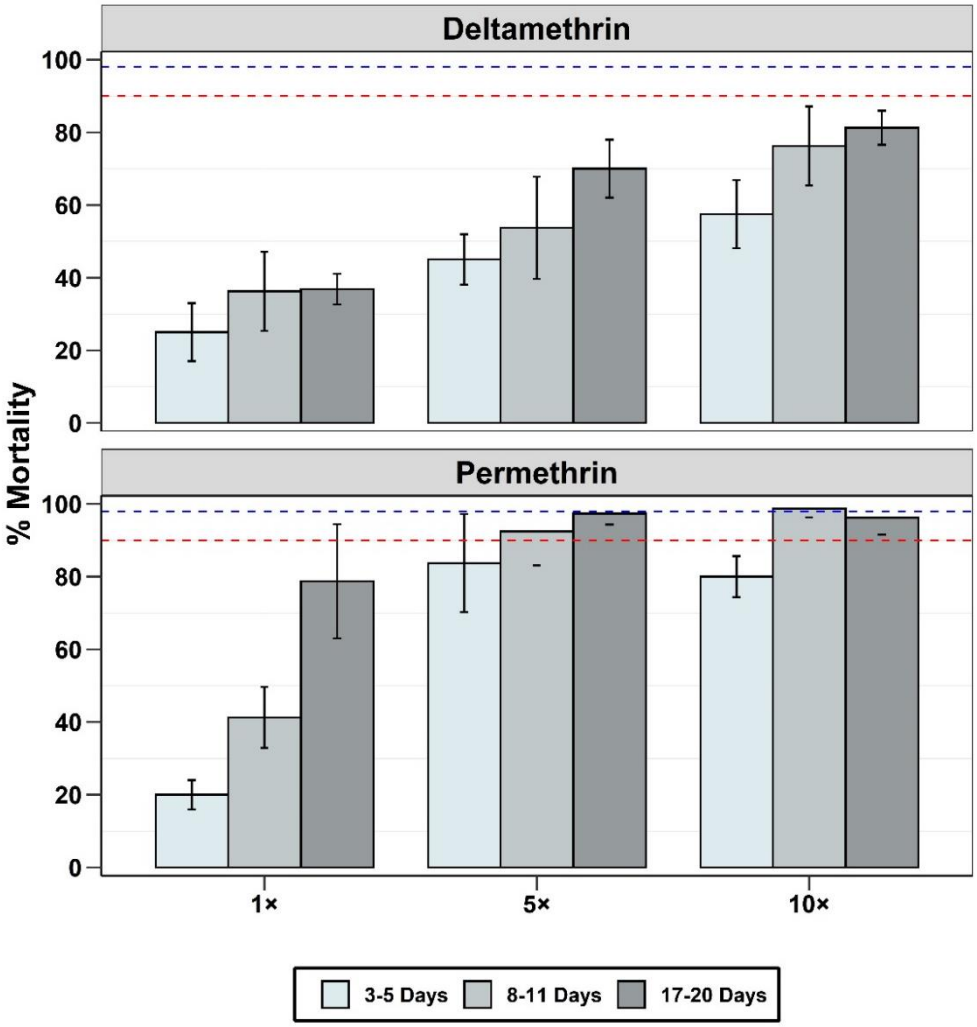

Figure 9: Percentage mortality in the different age cohorts of emergent *An. funestus* mosquitoes that were collected as larvae and exposed to deltamethrin or permethrin. The error bar represents the standard error, the red-dotted line represents 90% mortality, and the blue-dotted line represents 98% mortality.

Table 2. The odds of dying of female *An. funestus* mosquitoes collected as larvae and exposed to pyrethroid insecticides (Deltamethrin, Permethrin) when adults. **Note:** The young age (3-5 days) was used as the reference age of other ages.

Field larval collected mosquitoes					
Insecticide	**Fold**	**Age (Days)**	**Mean mortality (%)**	**OR (95% CI)**	**p-value**
Deltamethrin	1×	3-5	25	-	-
		8-11	36	1.67 (0.85-3.30)	0.138
		17-20	37	2.00 (1.02-3.93)	0.044
	5×	3-5	45	-	-
		8-11	54	1.42 (0.76-2.65)	0.269
		17-20	70	2.85 (1.49-5.46)	0.002
	10×	3-5	58	-	-
		8-11	76	2.37 (1.20-4.68)	0.013
		17-20	81	3.20 (1.57-6.55)	0.001
Permethrin	1×	3-5	20	-	-
		8-11	41	2.81 (1.39-5.69)	0.004
		17-20	79	14.82 (6.89-31.89)	<0.001
	5×	3-5	84	-	-
		8-11	93	2.39 (0.86-6.65)	0.094
		17-20	97	7.57 (1.65-34.74)	0.009
	10×	3-5	56	-	-
		8-11	99	19.75 (2.55-25.95)	0.004
		17-20	96	6.42 (1.79-23.01)	0.004

Key: OR; Odds ratio, CI; Confidence interval, 1×; Standard dose, 5×; Five times standard dose, 10×; Ten times standard dose, Fold; Dose, 3-5 Days; Reference age.

4.4 Molecular identification

A total of 409 *An. funestus* mosquitoes were tested by polymerase-chain-reaction (PCR) to identify *An. funestus* sibling species for confirmation of the studied vector species. Of those, 60% (n=245) were from adult collections and 40% (n=164) from the adults emerging from larval collections. The amplification rate for the adult collected mosquitoes was 95% (n=123), and all of them (100%) were identified as *An. funestus sensu stricto* (*s.s.*). On the other hand, the amplification rate for those larvae collected was 90% (n=148) and was amplified with 99% (n=147) being *An. funestus s.s.* and 1% being *An. rivulorum*.

CHAPTER FIVE

5.1 DISCUSSION

From the findings in chapter four above, this chapter discusses how the age of *An. funestus* and distance from aquatic habitats influence pyrethroid insecticide resistance in south-eastern Tanzania. Furthermore, it discusses how the correlation between these factors would inform the fine-scale targeting of malaria interventions. Unlike the previous findings in the area of *An. funestus* (17) and its counterpart *An. arabiensis* (29) on the fine-scale insecticide resistance variations, our findings showed that the age and distance from their aquatic habitats play a crucial role in *An. funestus* resistance. The last paragraph of this chapter explains some of the limitations and how better were mitigated to achieve the objectives of the study.

The distance from *An. funestus* aquatic habitats to the houses had a negative correlation with pyrethroids' insecticide resistance. Mosquitoes collected at the farthest distance (over 200 m), exposed to the standard dose of deltamethrin or permethrin had higher mortality (less resistant) compared to the ones collected at the nearest distance (50-100 m). A similar trend was observed when the intensity bioassays were done. When the mosquitoes collected at over 200 m were exposed to ten times the standard concentration of permethrin and deltamethrin, only 79% and 60% mortality were attained respectively. Although mosquitoes collected at the farthest distance showed increased mortality, neither deltamethrin nor permethrin insecticide attained the recommended susceptible mortality (≥98%) not even at ten times the standard dose. This study confirms the high level of pyrethroid resistance of *An. funestus* in south-eastern Tanzania.

When ovarian dissections were used as a proxy for the age of wild mosquitoes, it was revealed that mosquitoes collected at the farthest distance (over 200 m) which were less resistant had a

large proportion of parous mosquitoes relative to (50-100 m) and (150-200 m) distances. These findings align with the previous study which showed that mosquitoes which are resistant to insecticide are more likely to be nulliparous (have not laid eggs) (90). Therefore, insecticidal-interventions against malaria vectors can effectively impact the older population, more likely found at the farthest distance from the aquatic habitats which drives the transmission even when the vectors are resistant as per WHO guidelines (23). This is because the mosquitoes need at least 10 days to be able to transmit the parasite as the extrinsic incubation period takes not less than nine days depending on the parasite species and temperature (20).

Previous studies have indicated that older mosquitoes are more likely to be found farther away from their larval habitats and more likely to be infected with parasites (9). Midega *et al*., using the mark-release and recapture method in coastal Kenya, revealed that female *Anopheles* mosquitoes can survive over 17 days in the wild, likely to be collected at the farthest distance (over 500 m) from a point of release and (10). This suggests that the age distribution of mosquitoes in the field might not be random, but is instead rather distance-dependent from their aquatic habitats. Such non-random age distribution of mosquito populations could have a direct impact on the heterogeneity of risk of mosquito-borne diseases (9).

In addition to the parity dissections, the ovaries were further analysed to determine the number of times the mosquito has laid eggs (gonotrophic cycles). Overall, when CDC-LT was used as the method of collection a small proportion of mosquitoes (<30%, n=52) had at least two gonotrophic cycles with three maximum cycles (**Fig. 8 top panel**). This might be explained by work done previously by Charlwood *et al*., who showed that when CDC-LT is used for mosquito collections, parous mosquitoes are three times more likely to die and thus, be discarded

during collection (91). Furthermore, a large proportion of mosquitoes collected from the farthest distance died post-exposure and could therefore not be dissected for parity evaluation.

These observations also align with the previous study indicating that the parity and gonotrophic cycles of mosquitoes are better evaluated when mosquitoes are freshly dead (91). Thus, another explanation of a few mosquitoes which had at least two gonotrophic cycles post insecticide exposure, but still obeyed the WHO susceptibility test procedures (23).

When the DN-Min was used as the trapping method, the proportion of nulliparous mosquitoes decreased, however, the number of mosquitoes that have laid eggs twice increased throughout all the distances (**Fig. 8 bottom panel**). This shift might be attributed to the CDC-LT mostly collecting young host-seeking mosquitoes and DN-Mini collecting mostly the parous *An. funestus* indoor (80,92). This indicates the variability of mosquito age by trapping method, therefore, further investigations are needed to evaluate the effect of trapping methods on the age grading of wild mosquitoes.

PBO synergists were used to evaluate the enhancement of insecticide susceptibility and the implication of P450's enzymes in observed pyrethroid resistance. Mortality of mosquitoes significantly increased with PBO pre-exposure indicating potential involvement of metabolic mechanism of resistance. These findings align with the previous study done in the same study area where PBO pre-exposure increased the pyrethroid insecticide potency to *An. funestus* (17). However, there was no clear correlation between distance and insecticide potency restoration post PBO exposure. (**Fig. 6 &Table 1**). These results harmonise with the previous findings in which age-related pyrethroid resistance was not a function of metabolic (P450's) gene expression in *An. funestus* (66). Furthermore, the potency of pyrethroids was not fully restored (100%) with

a minimum of 80%, and a maximum of 98% which might be suggesting the involvement of alternative resistance mechanisms other than P450s metabolic resistance.

The age synchronized mosquitoes were used to evaluate how their age correlates to pyrethroid insecticide resistance. A positive correlation was observed where younger mosquitoes (3-5 days) were resistant to pyrethroid insecticides compared to older mosquitoes (17-20 days) (**Fig. 9 & Table 2**). Mortality was much lower when the young mosquitoes were exposed to deltamethrin compared to permethrin, attaining no susceptibility level even at ten-time the standard dose. These findings align with the previous studies which detected the increased insecticide susceptibility with the increased age of mosquitoes (12–14,66). However, more evidence is essential to demonstrate how insecticide resistance affects malaria control and elimination. Few studies have successfully demonstrated the epidemiological link between insecticide resistance and malaria epidemic(s) (40,68–70). The inadequate literature on the subject might be an indication of the absence of reliable surveillance systems to link entomological to epidemiological data. However, WHO multi-country studies have demonstrated that there is no correlation between insecticide resistance and malaria cases (76). Therefore, the outcome of this study might be vital to malaria interventions to consider the age distribution and the distance from the aquatic habitats thus, the fine-scale evidence-based rather than a random distribution of limited resources to interrupt malaria transmission.

Provided with achievements on the hypothesis of this study, there were some limitations. Firstly, as previously described by Charlwood *et al*., the older mosquitoes were more likely to die during collection when the CDC-LT is used during specimen collection (91). This might have marginally confounded the age evaluation of both insecticide pre-exposed and non-exposed cohorts. However, the larval collected mosquitoes showed the same trend as the mortality of

mosquitoes increased with the increased age and concentration of insecticide, the same case as the adult field collected. Therefore, these results could be considered conditional but still precise. Secondary, with the insecticide pre-exposed cohort, few numbers of mosquitoes from the far distance (over 200 m) were dissected compared to the other distances as most of them were susceptible to the insecticide, dead and dry, and unable to be dissected. Insecticide pre-exposure effect on dissection was minimized by dissecting the insecticide non-exposed mosquitoes under a similar methodology. Lastly, due to the rarity of *An. funestus* at the larval stage, synergist pre-exposure on the field larval collected mosquitoes was not evaluated.

5.2 CONCLUSION AND RECOMMENDATIONS

This study aimed to assess the influence of age and distance from aquatic habitats on the pyrethroid resistance status of *An. funestus* mosquitoes in rural south-eastern Tanzania. In this study area where *Anopheles funestus* has been dominating malaria transmission, this study showed that older mosquitoes are more likely to be found at a farther (centre of the village) distance from their larval habitats and are more likely to die after exposure to pyrethroid insecticides. Using an age synchronized sample of mosquitoes, it was found that older mosquitoes (17-20 days) were more susceptible to insecticides compared to the young ones (3-5 days). For the older mosquitoes, the susceptibility threshold was attained at five times the standard concentration of permethrin however this was not evident in deltamethrin exposure even at ten times the standard concentration. These findings provide insight into how the insecticidal based intervention including synergist (PBO) could still provide a potential impact on malaria vector control in the community regardless of the spread of insecticide resistance currently being reported. Moreover, to achieve maximum outcomes, the interventions might not necessarily need to be randomly distributed, rather targeting the specific older population which

drives the transmission and is more likely to be impacted by the insecticides. Since older, more susceptible mosquitoes are mostly in the middle of the villages, a ringed control approach involving indoor residual spraying at the centre and larval source management at the edge of the village could have much potential. Future studies might consider the linkage of epidemiological data to entomological data by considering the age distribution of mosquitoes and the distance from the aquatic habitats for effective malaria control.

Availability of data and materials

The data will be shared under the Ifakara Health Institute data sharing policy upon request and agreement.

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Appendices

1. Ethical clearance from the Institute Review Board of Ifakara Health Institute; IHI/IRB/No: 26-2020.
2. Ethical clearance from the Medical Research Coordinated Committee of the National Institute for Medical Research of the United Republic of Tanzania; NIMR/HQ/R.8a/Vol. IX/3495.
3. Ethical clearance from the University of the Witwatersrand Animal Research ethics Committee; Waiver 11-10-2021-O.

Plot 463, Kiko Avenue, Mikocheni | P.o. Box 78,373 Dar es Salaam. Tanzania | Phone: +255222774756 Email: irb@ihi.or.tz

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May 18, 2020

National Institute for Medical Research
P O Box 9653
Dar Es Salaam
Email; headquarters@nimr.or.tz

Najat F. Kahamba

Ifakara Health Institute

P O Box 53

Ifakara

IHI/IRB/No: 26-2020

INSTITUTIONAL CLEARANCE CERTIFICATE FOR CONDUCTING HEALTH RESEARCH

On 15th May 2020, the Ifakara Health Institute Review Board (IHI-IRB) reviewed from study titled: ***"Population biology and genomics studies on anopheles funestus in Tanzania"*** submitted by the Principal Investigator Najat F. Kahamba.

The following documents were reviewed:

1. Protocol
2. Informed Consent Forms
3. Budget
4. Tools
5. CVs

The study has been approved for implementation after IRB consensus. This certificate thus indicates that; the above-mentioned study has been granted an Institutional Ethics Clearance to conduct this study in Kilombero and Ulanga districts.

The Principal Investigator of the study must ensure that, the following conditions are fulfilled during or after the implementation of the study:

1. PI should submit a six-month progress report and the final report at the end of the project
2. Any amendment, which will be done after the approval of the protocol, must be communicated as soon as possible to the IRB for another approval
3. All research must stop after the project expiration date, unless there is prior information and justification to the IRB.
4. There should be plans to give feedback to the community on the findings
5. Any publication needs to pass through the IRB and NIMR
6. The approval is valid until 15th May 2021

The IRB reserves the right to undertake field inspections to check on the protocol compliance

Deputy Chairperson
Dr Ahmed Abdullah

IRB Secretary
Dr Mwifadhi Mrisho

THE UNITED REPUBLIC OF TANZANIA

National Institute for Medical Research
3 Barack Obama Drive
P.O. Box 9653
11101 Dar es Salaam
Tel: 255 22 2121400
Fax: 255 22 2121360
E-mail: nimrethics@gmail.com

Ministry of Health, Community Development, Gender, Elderly & Children
University of Dodoma, College of Business Studies and Law
Building No. 11
P.O. Box 743
40478 Dodoma

NIMR/HQ/R.8a/Vol. IX/3495

05th August 2020

Najat F. Kahamba
Research Scientist
Ifakara Health Institute
P O Box 53
Morogoro

RE: ETHICAL CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA

This is to certify that the research entitled: Population biology and genomics studies on *Anopheles funestus* in Tanzania (Kahamba N F. et al), has been granted ethical clearance to be conducted in Tanzania.

The Principal Investigator of the study must ensure that the following conditions are fulfilled:

1. Progress report is submitted to the Ministry of Health, Community Development, Gender, Elderly & Children and the National Institute for Medical Research, Regional and District Medical Officers after every six months.
2. Permission to publish the results is obtained from National Institute for Medical Research.
3. Copies of final publications are made available to the Ministry of Health, Community Development, Gender, Elderly & Children and the National Institute for Medical Research.
4. Any researcher, who contravenes or fails to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine as per NIMR Act No. 23 of 1979, PART III Section 10(2).
5. Sites: Dodoma, Kagera, Katavi, Lindi, Manyara, Mbeya, Morogoro, Mwanza, Rukwa, Songea and Tanga regions.

Approval is valid for one year: 05th August 2020 to 04th August 2021.

Name: Prof. Yunus Daud Mgaya

Signature
CHAIRPERSON
MEDICAL RESEARCH
COORDINATING COMMITTEE

Name: Prof. Abel Nkono Makubi

Signature
CHIEF MEDICAL OFFICER
MINISTRY OF HEALTH, COMMUNITY DEVELOPMENT, GENDER, ELDERLY & CHILDREN

CC: Director, Health Services-TAMISEMI, Dodoma.
RMO of Dodoma, Kagera, Katavi, Lindi, Manyara, Mbeya, Morogoro, Mwanza, Rukwa, Songea and Tanga regions.
DMO/DED of respective districts.

ANIMAL RESEARCH ETHICS COMMITTEE
Registration number: AREC-101210-002

Date:11/10/2021

Certificate reference: Waiver 11-10-2021-O
Category: O
Applicant: Polius Gerazi Pinda
Department: School of Public Health – Epidemiology and Biostatistics
Tel: 0812725453; **Email**: 2443845@students.wits.ac.za; ppinda@ihi.or.tz

Re: Waiver from the Animal Ethics Research Committee of the University of the Witwatersrand

This letter is to confirm that Polius Gerazi Pinda, MSc student (#2443845) at the School of Public Health under the supervision of Prof Koekemoer and Okumu (Wits), does not require full Animal Ethics Research Committee clearance to undertake the work titled "**The influence of age and distance from aquatic habitats on the pyrethroid resistance phenotypes of *Anopheles funestus* mosquitoes in south-eastern Tanzania**".

Reason for waiver
The study will be conducted on invertebrates (*Anopheles funestus*) – 6500 mosquitoes from Ifakara Health Institute (Morogoro, Tanzania.).

Details of the study
The aim of the study is to assess the influence of age (number of days lived) on the level and intensity of pyrethroid insecticides in *An. funestus* and to assess the influence of distance from aquatic habitats on the level and intensity of pyrethroid insecticides in *An. funestus* mosquitoes collected in households.
World Health Organization (WHO) insecticide resistance test procedure guidelines will be followed (WHO, 2018). Twenty (20)-25 mosquitoes per replicate will be exposed to insecticide or control group for 60 minutes. The knockdown number will be recorded. Thereafter, the specimens will be transferred to the clean holding tubes provided with 10% glucose solution where the mortality will be recorded after 24 hours. The treatment group will constitute four replicates and two replicates for control per test as per guideline.

The individuals covered by the waiver are Polius Gerazi Pinda, Prof Koekemoer and Okumu.

Please contact me should you require further information.

Yours sincerely

Prof Frederic Michel
Chair: Animal Research Ethics Committee
University of the Witwatersrand