BMJ Open

Implication of Anopheles gambiae and Anopheles funestus coexistence on malaria elimination efforts in an urban setting of Ndola district, Zambia.

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2024-091319
Article Type:	Original research
Date Submitted by the Author:	17-Jul-2024
Complete List of Authors:	Hamwata, Westone; Tropical Diseases Research Centre, Department of Biomedical Sciences; University of Zambia - Ridgeway Campus, Biomedical Sciences Muleba, Mbanga; Tropical Diseases Research Centre, Department of Biomedical Sciences Hazyondo, Mwendalubi; University of Zambia - Ridgeway Campus, Biomedical Sciences Shimaponda-Mataa, Nzooma ; University of Zambia - Ridgeway Campus, Biomedical Sciences Daka, Victor; The Copperbelt University School of Medicine, Public Health
Keywords:	Behavior, Cross-Sectional Studies, Malaria, Mosquito Vectors, PARASITOLOGY

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Implication of *Anopheles gambiae* and *Anopheles funestus* coexistence on malaria elimination efforts in an urban setting of Ndola district, Zambia.

Westone Hamwata[1,2]; Mbanga Muleba[1]; Mwendalubi Hazyondo[2,3]; Victor Daka[4]; Nzooma M. Shimaponda-Mataa[2];

1. Tropical Diseases Research Centre, Department of Biomedical Sciences, Vector Biology and Parasitology Unit
2. The University of Zambia, School of Health Sciences, Department of Biomedical Sciences
3. Department of National Parks and Wildlife, Chilanga, Lusaka, Zambia
4. The Copperbelt University, Michael Chilufya Sata School of Medicine, Department of Public Health

Corresponding Author:

Westone Hamwata

Tropical Diseases Research Centre, Department of Biomedical Sciences

Ndola, Zambia.

Telephone: +260972790935

Email: phamwata@gmail.com

ABSTRACT

Background: Malaria remains a public health issue in Zambia and insecticide-based vector control is the main malaria elimination strategy. Success of these strategies is dependent on a clear understanding of bionomics and susceptibility of the local vectors to insecticides used. Therefore, the objective of this study generate baseline data on vector behaviour and phenotypic resistance for effective vector control programming.

Methods: This was a cross-sectional study conducted in Ndola district in from July to October 2021 from four sites; two urban and two per-urban sites. Mosquitoes were collected using CDC-LT, PSC, Aspirations and larval collection. Mosquito identification was done using standard identification keys and Polymerase Chain Reaction (PCR). Williams's mean was used to determine mosquito densities and Kruskall Wallis H test was used to compare the distribution of mosquitoes. A negative binomial with a log link function was used to determine factors affecting mosquito counts. Susceptibility of the local vectors was determined using WHO tube and CDC bottle bioassay.

Results: The main breeding sites identified were irrigation trenches (4.67 larvae/dip) and garden ponds (2.72 larvae/dip) created from extensive urban agriculture practices. *Anopheles funestus* and *Anopheles gambiae* were found to coexist in all the four sites with *An. funestus* identified as the most dominant malaria vector. Densities of *An. gambiae s.s* seeking a blood meal (X^2=12.566, df=3, P=0.001) and resting indoors (Z=56.5, P=0.019) were found to be higher in urban than peri-urban sites compared to *An. funestus s.s* which had similar distribution across the study sites. Sprayed houses were significantly associated with reduced mosquito numbers (B = -0.956, IRR = 0.384, P = 0.001. *An. gambiae* s.s was fully susceptible to organophosphates and neonicotinoids but highly resistant to pyrethroids, carbamates and organochlorines.

Conclusions: The emergence of *An. funestus s.s* in an area previously dominated by *An. gambiae s.s* and its coexistence with *An. gambiae s.s* in the dry season pose a risk of sustaining malaria transmission all year round. Agriculture practices in urban areas resulted in highly productive mosquito breeding sites, thus the need for targeted vector control.

Key Words: *malaria vector coexistence, vector behaviour, insecticide resistance, Ndola, urban setting*

STRENGTHS AND LIMITATIONS OF THIS STUDY

- The study design and sampling strategies used allow for the determination of species composition, abundance, host-seeking and resting behaviour of malaria vectors.
- Presence of *An gambiae s.s* larval in the dry season facilitates mosquito breeding which may drive malaria transmission thus the need to plan for additional measures.
- The susceptibility of *An. gambiae s.s* the most efficient malaria vector was determined against seven different insecticides from five different classes but not for *An. funestus* s.s. due to limited numbers of adult *An. funestus* s.s due to the difficulty in finding larval habitats nor sufficient adults to conduct forced oviposition.
- This study was conducted in the dry season and the entomological indices determined may only be applicable to the dry season.

BACKGROUND

Malaria remains a public health challenge in Zambia accounting for approximately 1.4% of the global malaria disease burden. It is estimated that about four people die from malaria every day in Zambia [1,2]. High rainfall regions in northern Zambia experience the highest disease burden, while densely populated and arid regions in the south experience lower burden [3–6].

The primary malaria vectors in Zambia include *Anopheles funestus, Anopheles gambiae* and *Anopheles arabiensis* [7–9]. *Anopheles funestus,* the most abundant and widely distributed malaria vector in the country thrives during the dry season whereas *Anopheles gambiae*, the most efficient malaria vector thrives predominantly in the wet season [8]. Historically, *Anopheles gambiae* has been the dominant malaria vector on the Copperbelt Province in the past decades [9]. In contrast, *Anopheles arabiensis,* a more zoophilic mosquito, is the primary malaria vector in the southern regions and a secondary malaria vector in the eastern parts of the country – a region of moderate transmission [5,9–11].

Insecticide-treated bednets (ITNs), indoor residual spraying (IRS) and artemisinin combined therapy (ACTs) have played a vital role in reducing malaria disease burden in Zambia [12–14]. These interventions have been informed by entomological and parasite surveillance data generated from several parts of the country in the past two decades [13–15]. The Copperbelt province on the other hand has been implementing IRS since the 1950s and the early 2000s saw a scaling up of IRS, ITNs and ACTs which was associated with a significant decrease in the malaria disease burden [16,17]. However, this success also led to a decline in entomological surveillance in the province. Since 2017, the Copperbelt province has experienced a rise in the number of malaria cases indicating a change in the epidemiological landscape, necessitating renewed entomological activities for informed vector control programming.

Vector surveillance across Zambia has revealed some level of heterogeneity in the behaviour and susceptibility of malaria vectors within and between selected districts [9,18]. Most active entomological sites are located in areas of high or low malaria transmission, with limited representation in settings of moderate transmission [19]. Additionally, over 95% of all entomological surveillance activities are conducted in rural settings yet a substantial number of reported malaria cases originate from peri-urban and urban areas [20–22]. To address these gaps, we conducted entomological studies in Ndola between July 2021 and October 2021, in two ecologically distinct settings representing the peri-urban and urban areas of Ndola district a moderate malaria transmission setting to assess vector behaviour and phenotypic resistance for effective vector control programming.

METHODS

Study design and study area

This was a cross sectional study was conducted in the dry season in Ndola district, the provincial capital of the Zambia's Copperbelt Province (figure 1). The mean annual temperatures range from 12^{o}C to 25^{o}C, with mean annual rainfall ranging from 200 to 900 mm. The rainy season spans November to March, followed by a longer dry season from April to October.

Two catchment areas Chipulukusu and Kaniki were selected for their high malaria prevalence rates in 2020 [2]. Chipulukusu is an urban catchment area with houses constructed with cement blocks, burnt bricks, or mud bricks and have iron or grass roofing. Mosquito collection in Chipulukusu was conducted in two zones: Musalu (-12.9524 S, 28.66012 E), a densely populated area with limited road access and extensive vegetable gardening activities and Mapalo (-12.9374 S, 28.67564 E) an equally densely populated area but with road access and very minimal vegetable

gardening activities. Kaniki is a peri-urban catchment consisting mainly of mud houses with thatched grass or iron sheet roofs. Mosquito collection in Kaniki was conducted in Kamalasha (-12.8556955 S, 28.5311082 E), a densely populated area near the Sakania border with a swamp on the western side of the Ndola-Mufulira Road, and Pima (-12.77416 S, 28.483865 E), a farming setting with houses organized in clusters. Both catchment areas serve as low-cost residential settings.

Sample size

A total of 166 houses were selected for adult mosquito collection; 56 houses for CDC light traps, 30 houses for pyrethrum spray catches and 80 houses for aspirations. An additional 60 collection efforts were made for larval collection from potential larval habitats.

Sample size justification

This study utilized WHO guidelines on mosquito sampling and the sample size used for this study follows previous modeling studies conducted on the minimum number of houses required to estimate mosquito abundance using a precision of 20% allowable for ecological studies [23,24].

Public Involvement

The community was actively involved in problem identification and sensitization during data collection. However, they were not involved in setting of the research questions and study design.

Inclusion and exclusion criteria

The inclusion criteria for this study were twofold; firstly, only houses with an adult (16 years and above) were considered and houses were written consent was gotten. Houses were people cook using firewood from inside were excluded from the study.

House selection and adult mosquito collection

Collection of adult mosquitoes was conducted from July to September 2021 from 166 randomly selected houses; 83 houses from Chipulukusu catchment area, and the other 83 houses from Kaniki catchment area.

CDC light traps

The CDC-LT was used as a proxy for determining the biting density of mosquitoes to human hosts. The traps were set in randomly selected houses, and mosquito collection occurred from 18:00 hours to 06:00 hours the following morning. Each trap was set at a height of 1.5 metres from the ground, adjacent to a sleeping person and near their legs.

Aspirations

The live adult mosquitoes were collected using prokopack aspirator from 05:00 hours to 07:00 hours in the morning in houses where people slept. The live adult mosquitoes were then put in bugdom cages where they were supplied with 10% sugar solution and transported to the laboratory for identification.

Pyrethrum Spray Catches (PSC)

Adult mosquitoes resting indoors were collected indoors using PSC from 05:00 hours to 07:00 hours in the morning. Multiple pieces of white linen were spread over the floor, bed and furniture inside the house. Household members were asked to briefly exit the house and then the house was sprayed the house to saturation using pressurised two-in-one pyrethroid insecticide (imiprothrin 1.00 g/kg and deltamethrin 0.51 g/kg) can. After 10 minutes, all the mosquitoes that were knocked down were picked using a pair of forceps and placed into properly labelled petri dishes.

Collection of immature mosquitoes (larvae)

Larval collection was carried out in October 2021. Potential larval habitats were initially visually inspected for the presence of larvae using a 350 ml capacity standard dippers (BioQuip Products, Inc., California, USA) followed by sampling. The number of dips and number of larvae scouped were recorded. Afterwards, the collected larvae was transported to the TDRC laboratory for rearing in a controlled microenvironment (temperature of 27°C ± 2°C and a relative humidity of 75% ± 10%).

Susceptibility testing

Adults, F0 *Anopheles gambiae* reared from field collected larvae from Musalu, were exposed to five different classes of insecticides. The mosquitoes, aged two to five days, obtained from wild collected larvae, were exposed to pirimiphos-methyl (0.25%), malathion (5%), deltamethrin (0.05%), permethrin (0.75%), bendiocarb (0.01%) and dichlorodiphenyltrichloroethane (4%) standard World Health Organisation (WHO) impregnated test paper. The bioassays were conducted in accordance with the WHO guidelines [25]. For Clothianidin, CDC bottle bioassay as described by Brogdon and Chan [26].

Experimental Procedures

1. **To determine species composition and abundance of malaria vectors in Ndola:** The species composition and abundance was determined by collections from CDC - LT, PSC, and aspirations.

2. **To assess the biting and resting behaviour of mosquitoes in Ndola:** The CDC – LT was used as a proxy for assessing the biting behaviour of malaria vectors. The densities of mosquitoes collected per trap per night (mosquitoes/trap/night) was used to assess the host

seeking behaviour of the malaria vectors. The **Indoor Resting behaviour** is indicative of the mosquitoes that rest indoors. This indicator is an important one when considering whether to implement IRS or not. The mean indoor resting densities calculated in this study were determined by mosquito collections from PSC only.

3. **To determine the insecticide resistance status of primary malaria vectors:** The mosquitoes used for susceptibility testing were the first filial generation (F1) from the larvae collected.

Mosquito processing

Morphological identification

The female *Anopheles* mosquitoes collected were initially morphologically identified to the genus level using an identification key for Afrotropical Anopheles mosquitoes [27]. Thereafter, the mosquito samples were individually preserved in 1.5 mL Eppendorf tubes containing silica gel. These preserved samples were stored for molecular identification using PCR.

Molecular identification

A selected adult mosquitoes reared from field collected larvae and those collected as adults, morphologically identified as *An. funestus s.l.* and *An. gambiae s.l.*, were further subjected to molecular identification to determine sibling species. Nucleic acid extraction for this process was performed using the QIAGEN DNeasy Blood and Tissue kit for insects (QIAGEN Inc., USA). DNA amplification was performed using the Applied Biosystems GeneAmp PCR System 9700 thermocycler. For sibling species determination, the methods described by *Koekemoer. L et al; 2002* [28] and *Scott W.G et al; 1993* [29] were used for *An. funestus s.l* and *An. gambiae s.l.* respectively.

STATISTICAL ANALYSIS

The data collected was entered in Microsoft Excel and mean densities excluding larval density were derived from log transformed data using Williams mean (M_w= [$(X_1+1)(X_2+1)(X_3+1)....(X_n+1)]^{1/n}$ to account for skewed (non-normal distribution) and count data [21,30]

The data were then exported to IBM SPSS statistics version 25. The Kruskal–Wallis H test was used to compare the means (Mw) of malaria vectors seeking a host. The Mann–Whitney U test was used to compare the densities of the malaria vectors resting indoors from the two sites where PSC was conducted. Additionally, a negative binomial model with a log function was used to identify factors associated with counts of malaria vectors in the sampled housing structures. Susceptibility status of *An. gambiae s.s.* was determined using WHO mortality scoring guidelines [23], [24].

RESULTS

Species composition and abundance from adult mosquito surveys

A total of 166 houses were sampled, and a total of 744 female mosquitoes were collected. *Culex* accounted for 53% (392/744), *An. funestus s.l.* 17% (123/744), *Mansonia* 16% (106/744), *An. gambiae s.l.* 14% (106/744), and *An. gibbinsi s.l.* less than 1% (2/744) of the total mosquitoes collected (Table 1). Mosquito abundance by site showed the highest mosquito collections were from Musalu (338/744) and Mapalo (208/744), the two urban sites, followed by Pima (131/744) and Kamalasha (67/744), the two peri-urban sites. Notably, *Culex* mosquitoes comprised the largest proportion at each of the four sites (Table 1). Further, species composition by site shows that rural sites from Kaniki; Kamalasha and Pima had one more species A*n. gibbinsi* not found in

the two sites from Chipulukusu (Mapalo and Musalu). *Culex* were the most abundant mosquito collected from each site with (Kamalasha 67%, Pima 59%, Musalu 53% and Mapalo 42%). The second most abundant mosquito in Kaniki (Kamalasha 22% and Pima 30%) was *An. funestus s.l.* This was followed by *An. gambiae s.l.* (Kamalasha 7% and Pima 5%). Whilst from Chipulukusu, *Mansonia (24%)* and *An. gambiae s.s* (21%) were the second most abundant mosquito species collected from Mapalo and Musalu respectively (table 1).

Molecular identification and determination of sibling species

Eighty-nine percent (89%; 67/75) of the female *An. funestus s.l.* analysed for molecular identification amplified as *An. funestus s.s* at 505 base pairs and 85.5% (171/200) of *An. gambiae s.l* successfully amplified as *An. gambiae* s.s at 390 base pairs.

Biting and resting behaviour of malaria vectors

Mean density of malaria vectors

The mean number of *An. funestus s.s* seeking a blood meal from Mapalo was 2.42 times higher (Mw = 0.97/trap/night) than in Kamalasha (0.4/trap/night) and Pima (0.4/trap/night) and 9.7 times higher than in Musalu (0.1/trap/night) (table 1). For *An. gambiae s.s* the mean number of mosquitoes seeking a blood meal from Musalu (1.26/trap/night) were 1.5 more than in Mapalo (0.83/trap/night) and higher than in Kamalasha (0.1/trap/night) and Pima (0.14/trap/night), with differences of 12.6 and 9 times, respectively. Despite these variations in mean densities, the Kruskal–Wallis H test revealed no statistical difference in the host seeking behaviour of *An. funestus s.l.* (X^2=4.598, df=3, P=0.204) across the four sites. However, a statistical difference was observed in the host seeking behaviour of *An. gambiae s.l.* (X^2=12.566, df=3, P<0.001).

Indoor Resting Density of Malaria Vectors – PSC

The indoor resting density of *An. funestus s.l.* in Pima (1.2 mosquitoes per house) was 1.67 times higher than in Musalu (0.72 mosquitoes per house) whereas for *An. gambiae s.l.* in Musalu (1.31 mosquitoes per house), the indoor resting density was 262 times higher than in Pima (0.05 vectors per house) (Table 1). The Mann–Whitney U test indicated no statistical difference in the resting densities of *An. funestus s.l* between Pima and Musalu (Z=143.5, P=0.202), but statistical difference in the resting densities of *An. gambiae s.l* (Z=56.5, P<0.019) was observed between the two sites.

***Anopheles* mosquito larval habitats**

A total of 43 potential *anopheline* larval habitats were identified, and 55.81% (*n=24*; 95 CI: 40% – 71%) of these found to contain larvae. All the larval habitats found to contain larvae were from either Musalu (70.83%; 95% CI: 49% – 87%) or Mapalo (29.12%; 95% CI: 13% – 51%) sites. Seven different categories of potential larval habitats identified included blocked trenches, foundation trenches, garden ponds, irrigation canals (channels), shallow wells, streams and tyre marks. From the different larval habitats, 2,643 larvae were collected from a total of 914 dips. The number of larvae collected from Musalu was 2,510/2,643 (94.97%; 95% CI: 94% – 96%), whereas 133/2,643 (5.03%; 95% CI: 4.2% – 5.9%) were collected from Mapalo (Table 2). Additionally, 1,690/2,643 (63.94%; 95% CI: 62% – 66%) of the collected larvae were from irrigation canals, 553/2,643 (20.92%; 95% CI: 19% – 22%) were from garden ponds, 289/2,643 (10.93%; 95% CI: 9.7% – 12%) were from tire marks, 89/2,643 (3.37%; 95% CI: 2.7 – 4.1%) were from foundation trenches and 22/2,643 (0.83%; 95% CI: 0.52% – 1.3%) were from blocked trenches.

The larval density was highest in irrigation canals, with 4.67 larvae per dip, this was followed by garden ponds with 2.72 larvae per dip, tyre marks with 1.30 larvae per dip, foundation trenches with 1.25 larvae per dip and blocked drainages with 0.49 larvae per dip.

Factors affecting mosquito counts in housing structures

Seven predictors were utilised to identify associations with mosquito counts of *An. funestus s.s* and *An. gambiae s.s* in households, and only the spray status with Fludora® Fusion (B = -0.956, IRR = 0.384, P = 0.001) was found to be statistically significant, (Table 3). While three other predictors were associated with reduced mosquito counts, including the number of people who slept in a house the previous night (B = -0.023, IRR = 0.978, P = 0.714), housing structures with a thatched roof (B = -0.060, IRR = 0.942, P = 0.870, and the number of LLINs in a housing structure (B = -0.085, IRR = 0.918, P = 0.489, these predictors were not statistically significant. On the other hand, the other three predictors, number of animals that slept in a house the previous night (B = 0.004, IRR = 1.004, P = 0.937, housing structures plastered with mud walls or unburnt bricks (B = 0.234, IRR = 1.264, P = 0.559) and housing structures with open eaves (B = 0.203, IRR = 1.225, P = 0.557), were associated with increased mosquito counts, but they were not statistically significant.

Susceptibility status of *An. gambiae s.l*

Anopheles gambiae reared to F0 from field collected larvae from Musalu was exposed to five different classes of insecticides and the study showed full susceptibility 24 hours post exposure (100% mortality) to organophosphate (malathion 5% and pirimiphos-methyl 0.25%) and neonicotinoids (clothianidin). Conversely, resistance was confirmed to bendiocarb 0.1%,

permethrin 0.75%, deltamethrin 0.75% and Dichlorodiphenyltrichloroethane 4% (DDT) with corresponding mortalities of 23%, 14%, 18% and 4% respectively (table 4).

DISCUSSION

This study reveals that the two main malaria vectors in Zambia *An. funestus s.l.* and *An. gambiae s.l.* were found in all four sites and these were molecularly identified as *An. funestus* s.s. and *An. gambiae* s.s. respectively. In Zambia, these mosquitoes have been implicated as the main vectors responsible for malaria transmission and have been found to exist in sympatry [20,21,31,32]. Surprisingly, *Anopheles funestus* was found to be the most abundant malaria vector in Ndola. Historically, the province has been dominated by *An. gambiae s.s* but this study found *An. funestus s.s* as the dominant malaria vector in Ndola. This finding is similar to other entomological findings in other districts within the Copperbelt province were *An. funestus s.s* is the more dominant vector [9,15]. However, *An. gambiae s.s.* remained the more dominant malaria vector in urban areas, whereas *An. funestus s.s.* was more abundant in peri-urban areas, consistent with earlier studies conducted in sub-Saharan Africa [18,21]. This disparity in vector abundance could be attributed to variations in ecological habitats. *Anopheles gambiae* prefers to breed in man-made water habitats such as drainages, tire tracks, small pools and agriculture sites, whilst *An. funestus s.s.* prefers to breed in permanent and semi-permanent water habitats with some vegetative cover [33,34]. An earlier study in the northern parts of the country identified *An. funestus s.s* as the primary driver of malaria transmission in the dry season whereas *An. gambiae* s.s as the primary driver in the wet season [31]. Nonetheless, the existence of breeding grounds for *Anopheles gambiae s.s* in urban areas implies that even during the dry season, *An. gambiae s.s* will continue to be the primary driver of malaria transmission. The coexistence of these two malaria vectors pose an increased year round risk of malaria transmission in the area. The recent increase in the

incidences of malaria reported in Ndola could be attributed to the changing vector bionomics that now includes *An. funestus s.s.* not reported previously in the area.

Mosquito diversity was observed to be higher in peri-urban than urban sites with the inclusion of *An. gibbinsi,* a potential secondary malaria vector. This vector has been reported in other parts of the country as a potential secondary malaria vector [35–37]. Secondary malaria vectors have not been adequately considered in most vector control programming yet they contribute to 5% of malaria transmission in the southern African region [38]. Their contribution to transmission is significant making the need to incorporate interventions targeting secondary malaria vectors into vector control toolkits inevitable.

The host-seeking behaviours of *An. funestus s.s.* and *An. gambiae s.s.* were different. The host-seeking behaviour of *An. funestus* s.s. was found to be homogeneous across the four sites, whereas the host-seeking behaviour of *An. gambiae s.s.* was found to be much higher in urban sites with vast larval habitats. This heightened host-seeking behaviour of *An. gambiae s.s.* indicates an increased risk of disease transmission in urban sites compared to per-urban sites thus the [39]. As such the need for enhanced vector control methods in urban settings with extensive larval habitats due to the elevated risk cannot be overemphasized.

The mean densities of *An. funestus* s.s found resting indoors were generally low across the peri-urban and urban sites. However, the indoor resting density of *An. gambiae* s.s in the urban site was much higher than that in the peri-urban site. Variations in the indoor resting behaviour of *An. funestus s.s.* and *An. gambiae s.s.* could be influenced by the presence of vast *An. gambiae s.s* breeding sites in urban sites. Therefore, vector control interventions such as IRS and LLINs in such settings may need to be supplemented with larval source management [40].

The larval habitats that were active breeding sites were all from the two urban sites adjacent to a dambo. The larval habitats identified included irrigation canals (or irrigation channels), garden ponds, tire marks, foundation trenches and blocked drainages. However, irrigation channels and garden ponds were found to be the main mosquito breeding sites, similar to studies conducted in Ghana, Tanzania, Cote d'Ivoire and China [41]. However, the larval densities found in this study were higher than that found in China, possibly due to differences in the climatic conditions, variations in the bacterial diversity and physicochemical composition of the larval habitats [42]. These factors have been found to influence mosquito oviposition, survival, and development into competent malaria vectors, thereby potentially impacting malaria incidence [3,43]. Unfortunately, this study only identified the different types of larval habitats; future research is needed to fully characterize larval habitats in order to generate additional information valuable for an effective and targeted larval source management programme.

The four predictors associated with reduced counts of malaria vectors in housing structures were; the number of people who slept in a housing structure, housing structures with a thatched roof, the number of LLINs used the previous night and housing structures sprayed with *Fludora*® *Fusion*. However, only housing structures sprayed were found to be statistically associated with reduced counts of malaria vectors, similar to what was found in Sao Tome and Principe [40]. Individuals who sleep in sprayed houses experience a lower vector-to-host contact, which entails reduced exposure to infectious mosquito bites unlike those sleeping in unsprayed houses. Additionally, maximum benefit is derived when at least 85% of houses are sprayed with an efficacious insecticide to kill host seeking mosquitoes that rest indoors [44]. On the other hand, number of animals in a housing structure, housing structures with mud wall surfaces and open eaves were associated with increased counts of malaria vectors but were not statistically

significant. Elsewhere, a study conducted in Cameroon associated open eaves and holes in the walls to increased mosquito counts [45]. Another study in Gambia also found that closing the eaves reduces mosquitoes entering thatched houses but increases mosquito entry into metal-roofed houses [46].

Susceptibility tests in this study reveal that *An. gambiae* s.s. was fully susceptible to organophosphates (malathion and pirimiphos-methyl) and neonicotinoids (clothianidin). This was also observed in several other districts in Zambia, where *An. funestus s.s.* and *An. gambiae s.s.* was found to be susceptible to these two classes of insecticides [20,22]. In that regard, organophosphates and neonicotinoids could be effective at controlling mosquito populations of *An. gambiae s.s* in Ndola and several other districts in Zambia, with evidence of susceptibility. However, resistance of *An. gambiae* s.s. to pyrethroids (permethrin and deltamethrin) and carbamates (bendiocarb) was confirmed and this could be attributed to the extensive use of pesticides and insecticides for agriculture and public health purposes. These results align with previous studies that found extensive insecticide resistance to pyrethroids and carbamates in the Copperbelt province [9,18]. In the wake of widespread pyrethroid insecticide resistant dual active ingredient or piperonyl butoxide (PBO) nets must be encouraged. However, recent studies shows that use of PBO LLINs in a structure sprayed with pirimiphos-methyl reduces the efficacy of the insecticide on pyrethroid-resistant mosquitoes [47]. As a result, use of a PBO LLIN in a pirimiphos-methyl sprayed structure may not be as beneficial as using a non-PBO LLIN in a pirimiphos-methyl sprayed structure.

Limitations of this study

The study was conducted in the dry season and the entomological indices determined may only apply for the dry season. Additionally, due to the scarcity of *An. funestus s.s* larval habitats this study did not determine the susceptibility status of *An. funestus s.s*.

CONCLUSION

The two primary malaria vectors *An. funestus* s.s. and *An. gambiae* s.s. were found to coexist in the two ecologically distinct settings, with *An. funestus* s.s. being the dominant malaria vector. This coexistence has the potential of sustaining high malaria transmission throughout the year especially in urban areas. Urban agriculture practices created *An. gambiae s.s* breeding sites during the dry season, contributing to the high host seeking and indoor resting behaviour in the urban sites. Sprayed housing structures were associated with reduced counts of malaria vectors. *Anopheles gambiae* was found to be susceptible to organophosphates and neonicotinoids, but resistance to pyrethroids, carbamates and organochlorides was confirmed.

REFERENCES

1. WHO. World malaria report 2022. Geneva; 2022.
2. MOH. Health Management Information System of zambia [Internet]. Lusaka; 2020. Available from: https://www.nmec.org.zm/
3. Shimaponda-Mataa NM, Tembo-Mwase E, Gebreslasie M, Achia TNO, Mukaratirwa S. Modelling the influence of temperature and rainfall on malaria incidence in four endemic provinces of Zambia using semiparametric Poisson regression. Acta Trop. 2017 Feb 1;166:81–91.
4. Sitali L, Miller JM, Mwenda MC, Bridges DJ, Hawela MB, Hamainza B, et al. Distribution of Plasmodium species and assessment of performance of diagnostic tools used during a malaria survey in Southern and Western Provinces of Zambia. Malar J [Internet]. 2019;18(1):1–9. Available from: https://doi.org/10.1186/s12936-019-2766-2
5. MOH. Zambia National Malaria Indicator Survey 2021: MINISTRY OF HEALTH. Natl Malar Elimin Centre, Minist Heal Zambia [Internet]. 2021; Available from: papers2://publication/uuid/2E7FD35E-174F-4868-8A7F-7910082D3A11
6. Chipoya MN, Shimaponda-Mataa NM. Prevalence, characteristics and risk factors of imported and local malaria cases in North-Western Province, Zambia: a cross-sectional study. Malar J 2020 191 [Internet]. 2020 Nov 23 [cited 2021 Jul 24];19(1):1–12. Available from:

https://malariajournal.biomedcentral.com/articles/10.1186/s12936-020-03504-1

7. Cross DE, Thomas C, McKeown N, Siaziyu V, Healey A, Willis T, et al. Geographically extensive larval surveys reveal an unexpected scarcity of primary vector mosquitoes in a region of persistent malaria transmission in western Zambia. Parasites and Vectors [Internet]. 2021 Dec 1 [cited 2021 Apr 17];14(1):91. Available from: https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-020-04540-1

8. Das S, Muleba M, Stevenson JC, Norris DE. Habitat partitioning of malaria vectors in nchelenge district, Zambia. Am J Trop Med Hyg. 2016;94(6):1234–44.

9. NMEC. Insecticide Resistance Management and Monitoring Plan, Ministry of Health, Government Republic of Zambia. In 2019.

10. Fornadel CM, Norris LC, Franco V, Norris DE. Unexpected Anthropophily in the Potential Secondary Malaria Vectors An coustani s.l and An squamosus in Macha, Zambia. 2011;11(8).

11. Saili K, de Jager C, Sangoro OP, Nkya TE, Masaninga F, Mwenya M, et al. Anopheles rufipes implicated in malaria transmission both indoors and outdoors alongside Anopheles funestus and Anopheles arabiensis in rural south-east Zambia. Malar J [Internet]. 2023;22(1):1–12. Available from: https://doi.org/10.1186/s12936-023-04489-3

12. Chanda E, Masaninga F, Coleman M, Sikaala C, Katebe C, MacDonald M, et al. Integrated vector management: The Zambian experience. Malar J. 2008;7:1–8.

13. Chizema-Kawesha E, Miller JM, Steketee RW, Mukonka VM, Mukuka C, Mohamed AD, et al. Scaling Up Malaria Control in Zambia: Progress and Impact 2005–2008. Am J Trop Med Hyg [Internet]. 2010 Sep 7 [cited 2023 Apr 23];83(3):480–8. Available from: https://www.ajtmh.org/view/journals/tpmd/83/3/article-p480.xml

14. Masaninga F, Chanda E, Chanda-kapata P, Hamainza B, Masendu HT, Kamuliwo M, et al. Review of the malaria epidemiology and trends in Zambia. 2013;3(2):89–94.

15. PMI USAID. The President's Malaria Initiative (PMI) / Vectorlink; Zambia 2019-2020 Entomology Annual Report. Rockville, MD PMI VectorLink Proj Abt Assoc. 2020;

16. Barnes KI, Chanda P, Barnabas GA. Impact of the large-scale deployment of artemether / lumefantrine on the malaria disease burden in Africa : case studies of South. Malar J. 2009;7(October 2005):1–7.

17. Masaninga F, Chanda E, Chanda-kapata P, Hamainza B, Masendu HT, Kamuliwo M, et al. Review of the malaria epidemiology and trends in Zambia. 2013;3(2):89–94.

18. Thomsen EK, Strode C, Hemmings K, Hughes AJ, Chanda E, Musapa M, et al. Underpinning sustainable vector control through informed insecticide resistance management. PLoS One. 2014;9(6).

19. NMEC. National Malaria Elimination Strategic Plan 2022-2026. 2022;1(1):62.

20. PMI USAID. The President's Malaria Initiative (PMI) / Vectorlink; Zambia 2018-2019 Entomology Annual Report. Rockville, MD PMI VectorLink Proj Abt Assoc. 2019;(November 2018):37.

21. Stevenson JC, Pinchoff J, Muleba M, Lupiya J, Chilusu H, Mwelwa I, et al. Spatio-temporal heterogeneity of malaria vectors in northern Zambia : implications for vector control. Parasit Vectors [Internet]. 2016;1–15. Available from: http://dx.doi.org/10.1186/s13071-016-1786-9

22. Chanda J, Saili K, Phiri F, Stevenson JC, Mwenda M, Chishimba S, et al. Pyrethroid and Carbamate Resistance in Anopheles funestus Giles along Lake Kariba in Southern Zambia. 2020;103(Suppl 2):90–7.

23. World Health Organization(WHO). Malaria surveillance, monitoring & evaluation: a reference manual [Internet]. Licence: C. 2018. Available from: http://apps.who.int/iris.%0ASales,

24. Zhou G, Minakawa N, Githeko A, Yan G. Spatial distribution patterns of malaria vectors and sample size determination in spatially heterogeneous environments: A case study in the west Kenyan highland. J Med Entomol. 2004;41(6):1001–9.

25. World Health Organization(WHO). Test procedures for insecticide resistance monitoring in malaria vector mosquitoes Second edition. 2018;

26. Brogdon W, Chan A. Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay. 2010;1–28.

27. Coetzee M. Key to the females of Afrotropical Anopheles mosquitoes (Diptera: Culicidae). Malar J [Internet]. 2020;19(1):1–20. Available from: https://doi.org/10.1186/s12936-020-3144-9

28. Koekemoer LL, Kamau L, Hunt RH, Coetzee M. A cocktail polymerase chain reaction assay to identify members of the Anopheles funestus (Diptera: Culicidae) group. Am J Trop Med Hyg. 2002;66(6):804–11.

29. Scott JA, Brogdon WG, Collins FH. Identification of single specimens of the Anopheles gambiae complex by the polymerase chain reaction. Am J Trop Med Hyg [Internet]. 1993 [cited 2021 Apr 30];49(4):520–9. Available from: https://pubmed.ncbi.nlm.nih.gov/8214283/

30. William C. THE USE OF LOGARITHMS IN THE INTERPRETATION OF CERTAIN ENTOMOLOGICAL PROBLEMS. Ann Appl Biol [Internet]. 1937;24(March 1936):404–14. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1744-7348.1937.tb05042.x

31. Muleba M, Mbata KJ, Stevenson JC, Norris DE. Spatial–temporal vector abundance and malaria transmission dynamics in Nchelenge and Lake Mweru islands, a region with a high burden of malaria in northern Zambia. Malar J [Internet]. 2023;22(1):1–11. Available from: https://doi.org/10.1186/s12936-023-04746-5

32. Fornadel CM, Norris LC, Glass GE, Norris DE. Analysis of Anopheles arabiensis blood feeding behavior in southern zambia during the two years after introduction of insecticide-treated bed nets. Am J Trop Med Hyg. 2010;83(4):848–53.

33. Doumbe-Belisse P, Kopya E, Ngadjeu CS, Sonhafouo-Chiana N, Talipouo A, Djamouko-Djonkam L, et al. Urban malaria in sub-Saharan Africa: dynamic of the vectorial system and the entomological inoculation rate. Malar J [Internet]. 2021;20(1):1–18. Available from: https://doi.org/10.1186/s12936-021-03891-z

34. Nambunga IH, Ngowo HS, Mapua SA, Hape EE, Msugupakulya BJ, Msaky DS, et al. Aquatic habitats of the malaria vector Anopheles funestus in rural south-eastern Tanzania. Malar J [Internet]. 2020;19(1):1–11. Available from: https://doi.org/10.1186/s12936-020-03295-5

35. Lobo NF, St. Laurent B, Sikaala CH, Hamainza B, Chanda J, Chinula D, et al. Unexpected diversity of Anopheles species in Eastern Zambia: implications for evaluating vector behavior and interventions using molecular tools. Sci Rep. 2015 Dec 9;5:17952.

36. Gebhardt ME, Krizek RS, Coetzee M, Koekemoer LL, Dahan-Moss Y, Mbewe D, et al. Expanded geographic distribution and host preference of Anopheles gibbinsi (Anopheles species 6) in

northern Zambia. Malar J [Internet]. 2022;21(1):1–10. Available from: https://doi.org/10.1186/s12936-022-04231-5

37. Cooke MK, Kahindi SC, Oriango RM, Owaga C, Ayoma E, Mabuka D, et al. 'A bite before bed': exposure to malaria vectors outside the times of net use in the highlands of western Kenya. Malar J. 2015 Jun 25;14(1):259.

38. Afrane YA, Bonizzoni M, Yan G. Secondary Malaria Vectors of Sub-Saharan Africa: Threat to Malaria Elimination on the Continent? Curr Top Malar. 2016;(November).

39. Cummins B, Cortez R, Foppa IM, Walbeck J, Hyman JM. A spatial model of mosquito host-seeking behavior. PLoS Comput Biol. 2012;8(5).

40. Chen YA, Lien JC, Tseng LF, Cheng CF, Lin WY, Wang HY, et al. Effects of indoor residual spraying and outdoor larval control on Anopheles coluzzii from São Tomé and Príncipe, two islands with pre-eliminated malaria. Malar J [Internet]. 2019;18(1):1–16. Available from: https://doi.org/10.1186/s12936-019-3037-y

41. Wang Y, Cheng P, Jiao B, Song X, Wang H, Wang H, et al. Investigation of mosquito larval habitats and insecticide resistance in an area with a high incidence of mosquito-borne diseases in Jining, Shandong Province. PLoS One [Internet]. 2020;15(3):1–14. Available from: http://dx.doi.org/10.1371/journal.pone.0229764

42. Wang H, Wang Y, Cheng P, Wang H, Wang H, Liu H, et al. The Larval Density of Mosquitos (Diptera: Culicidae) in Jiaxiang County, Shandong Province, China: Influence of Bacterial Diversity, Richness, and Physicochemical Factors. Front Ecol Evol. 2021;9(February):1–12.

43. Hinne IA, Attah SK, Mensah BA, Forson AO, Afrane YA. Larval habitat diversity and Anopheles mosquito species distribution in different ecological zones in Ghana. Parasit Vectors [Internet]. 2021;1–14. Available from: https://doi.org/10.1186/s13071-021-04701-w

44. World Health Organization(WHO). an Operational Manual for Indoor Residual Spraying (Irs) for Malaria Transmission Control and Elimination Second Edition Indoor Residual Spraying. 2015;(2).

45. Ngadjeu CS, Ngadjeu CS, Doumbe-Belisse P, Doumbe-Belisse P, Talipouo A, Talipouo A, et al. Influence of house characteristics on mosquito distribution and malaria transmission in the city of Yaoundé, Cameroon. Malar J [Internet]. 2020;19(1):1–14. Available from: https://doi.org/10.1186/s12936-020-3133-z

46. Jatta E, Jawara M, Bradley J, Jeffries D, Kandeh B, Knudsen JB, et al. Articles How house design affects malaria mosquito density , temperature , and relative humidity : an experimental study in rural Gambia. Lancet Planet Heal [Internet]. 2015;2(11):e498–508. Available from: http://dx.doi.org/10.1016/S2542-5196(18)30234-1

47. Syme T, Gbegbo M, Obuobi D, Fongnikin A, Agbevo A, Todjinou D, et al. Pyrethroid-piperonyl butoxide (PBO) nets reduce the efficacy of indoor residual spraying with pirimiphos-methyl against pyrethroid-resistant malaria vectors. Sci Rep [Internet]. 2022;12(1):1–13. Available from: https://doi.org/10.1038/s41598-022-10953-y

ABBREVIATIONS

ACTs : artemisinin combined therapy; B : Regression Coefficient; CDC – LT : Centres for Disease Control light traps; CI : Confidence Interval; DDT : Dichlorodiphenyltrichloroethane; df : degrees of freedom; DNA : Deoxyribonucleic acid; IRR : Incidence rate ratio; IRS : Indoor residual spraying; ITNs : Insecticide treated bednet; LLINs : Long lasting insecticide treated nets; Mw : Williams mean; NHRA : National Health Research Authority; NMEC : National Malaria Elimination Centre; P : Probability value; PBO : piperonyl butoxide; PCR : Polymerase Chain Reaction; PSC : Pyrethrum Spray Catches; *s.l : sensu lato; s.s : sentu stricto;* SPSS : Statistical Package for Social Sciences; TDRC : Tropical Diseases Research Centre; WHO : World Health Organisation; X^2 : Chi sqaure

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study involved collecting mosquitoes from households with no direct contact with humans. As such, ethics clearance to undertake this study was obtained from the Tropical Diseases Research Centre Ethics Committee (**Reference No. TRC/C4/06/2021**) and the National Health Research Authority (**Ref No: NHRA000016/29/06/2021**). Written consent was obtained from the head of the house prior to mosquito and larvae collection from their houses and their gardens, respectively. All data that was collected was restricted to the investigators and confidentiality was strictly maintained.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

FUNDING

This work was not a funded project but leveraged on supplies from Tropical Diseases Research Centre (TDRC) and a Pan African Mosquito Control Association (PAMCA) funded project.

CONFLICTS OF INTEREST / COMPETING INTERESTS

Financial interests: The authors as well as the funders declare that they have no relevant financial or non-financial interests to disclose and they have no conflicts of interest to declare that are relevant to the content of this manuscript.

AUTHORS' CONTRIBUTIONS

WH, NMS-M and MM designed the study. WH conducted sample collection and analysis. WH, MH & VD analysed the data. WH, MM and NMS-M interpreted the data. WH and MH drafted the manuscript, MM, VD and NMS-M edited and reviewed the manuscript. MM and NMS-M supervised implementation of the project. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

We acknowledge the Ministry of Health Provincial Health office and the Health Centres for approval of the study and provisions towards it. We further acknowledge all participants.

AUTHORS' INFORMATION AND AFFILIATION

WPH (Westone Hamwata[1,2]; phamwata@gmail.com) is a Medical Parasitologist and Entomologist based at the Tropical Diseases Research Centre and holds a MSc in Medical Parasitology and Master of Public Health.

MM (Mbanga Muleba[1]; mbangamuleba@gmail.com) is a Medical Entomologist at Tropical Diseases Research Centre and holds a PhD in Entomology

MH (Mwendalubi Hazyondo[2,3]; mwendalubihazyondo@gmail.com) is a Senior Wildlife Veterinary Officer at the Department of National Parks and Wildlife and hold a Doctor of Veterinary Medicine and Zootechnics

VD[4] (Victor Daka; dakavictorm@gmail.com) is Lecturer at The Copperbelt University, Michael Chilufya Sata School of Medicine, Department of Public Health and holds a PhD in Epidemiology.

NMS-M[2] (Nzooma M. Shimaponda-Mataa1; nzomataa@gmail.com) is a Medical Parasitologist and Lecturer at the University of Zambia and holds a PhD in Parasitology;

1. Tropical Diseases Research Centre, Department of Biomedical Sciences, Vector Biology and Parasitology Unit.

2. University of Zambia School of Health Sciences, Department of Biomedical Sciences, Ridgeway Campus, Lusaka, Zambia.

3. Department of National Parks & Wildlife, Chilanga, Zambia.

4. The Copperbelt University, Michael Chilufya Sata School of Medicine, Department of Public Health, Ndola Campus, Zambia.

LIST OF TABLES

Table 1: Entomological indices

Entomological Indices	Chipulukusu catchment		Kaniki catchment		Total
Species	Mapalo	Musalu	Kamalasha	Pima	
Species composition and mosquito abundance by site					
An. funestus s.l	45	23	15	40	123
An. gambiae s.l.	24	71	5	6	106
An. gibbinsi	0	0	1	1	2
Culex	88	181	45	78	392
Mansonia	51	63	1	6	121
Total	208	338	67	131	744
Mean densities of malaria vectors seeking a blood meal					
An. funestus s.l	35 (0.97)	2 (0.10)	10 (0.40)	10 (0.40)	57
An. gambiae s.l.	19 (1.83)	28 (1.26)	2 (0.10)	3 (0.14)	52
Mean densities of malaria vectors resting indoors					
An. funestus s.l	-	21 (0.72)	-	23 (1.2)	44
An. gambiae s.l.	-	34 (1.31)	-	1 (0.05)	35

Table 2: Mosquito larval habitats

Site	Type of larval habitat	No. of dips	# larvae collected (%)	Larval habitats identified	larval habitats with larvae	Mosquito genera	
						Anopheles larvae	*Culex larvae*
Mapalo	Foundation trenches	71	89 (3.37)	5	4	3	4
	Tyre marks	86	31 (1.17)	2	2	2	2
	Blocked drainages	18	13 (0.49)	1	1	1	1
	Subtotal	175	133 (5.03)	8	7	6	7
Musalu	irrigation canals	362	1690 (63.94)	19	11	9	11
	Tyre marks	147	258 (9.76)	3	2	2	2
	Garden ponds	203	553 (20.92)	4	3	3	3
	Blocked drainages	27	9 (0.34)	1	1	1	1
	Subtotal	739	2510 (94.97)	27	17	15	17
Kamalasha	Tyre marks	-*	0 (0.0)	5	0	0	0

	Subtotal	-*	0 (0.0)	5	0	0	0
Pima	Shallow wells	-*	0 (0.0)	2	0	0	0
	Stream	-*	0 (0.0)	1	0	0	0
	Subtotal	-*	0 (0.0)	3	0	0	0
Total from all sites		914	2643 (100)	43	24	21	24

**No larvae found after visual inspection followed by 10 dips*

Table 3: Predictors affecting Mosquito Counts of Anopheles funestus and Anopheles gambiae

Parameter	Regression coefficient (B)	Hypothesis Test			IRR	95% Wald Confidence Interval for Exp(B)	
		Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	1.919	3.876	1	0.049	6.816	1.009	46.067
No. of People	-0.023	0.134	1	0.714	0.978	0.866	1.104
No. of Animals	0.004	0.006	1	0.937	1.004	0.912	1.105
Type of Roof	-0.060	0.027	1	0.870	0.942	0.462	1.921
Type of Wall	0.234	0.342	1	0.559	1.264	0.577	2.769
Type of Eaves	0.203	0.345	1	0.557	1.225	0.622	2.412
No. of LLINs	-0.085	0.478	1	0.489	0.918	0.721	1.169
Spray Status	-0.956	10.513	1	0.001	0.384	0.216	0.685
(Scale)	1[a]						
(Negative binomial)	0.798						

Dependent Variable: No. of Malaria Vectors
Model: (Intercept), No. of People, No. of Animals, Type of Roof, Type of Wall, Type of Eaves, No. of LLINs, Spray Status
Fixed at the displayed value.

Table 4: Susceptibility Status of An. gambiae s.s from Musalu

Insecticide tested	# mosquitoes exposed	Knockdown @ 60min		Knockdown @ 24hours		Final Mortality (24 hours)
		Dead	Alive	Dead	alive	
Bendiocarb 0.1%	108	14	94	25	83	23%
DDT 4%	113	0	113	5	108	4%
Deltamethrin 0.05%	100	13	87	18	82	18%
Permethrin 0.75%	113	6	107	16	97	14%
Pirimiphos-methyl 0.25%	110	101	9	110	0	100%**
Malathion 5%	104	94	10	104	0	100%**
Clothianidin	107	104	3	107	0	100%**

**Fully susceptible

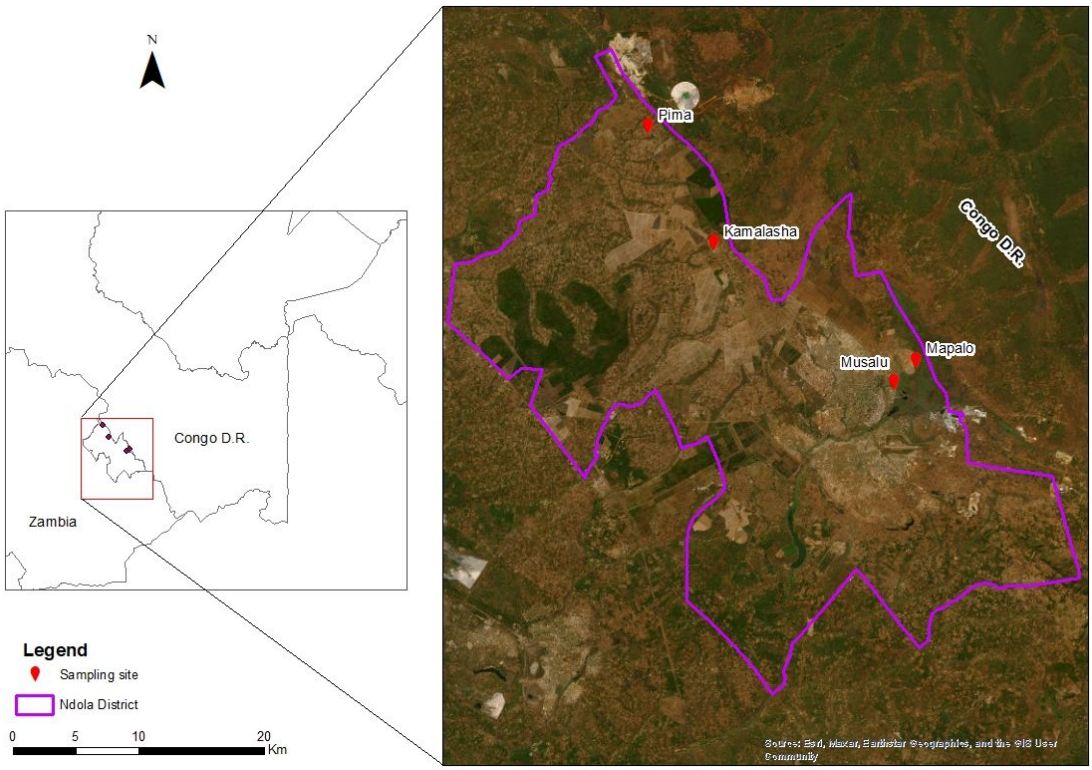

Figure 1: Map showing the location of the study sites

BMJ Open

Implications for Malaria Transmission: A Cross-sectional Study on the Bionomics and Susceptibility of Local Malaria Vectors in Urban and Peri-urban Settings of Ndola District.

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2024-091319.R1
Article Type:	Original research
Date Submitted by the Author:	03-Jan-2025
Complete List of Authors:	Hamwata, Westone; Tropical Diseases Research Centre, Department of Biomedical Sciences; University of Zambia - Ridgeway Campus, Biomedical Sciences Hazyondo, Mwendalubi; Department of National Parks and Wildlife Service; University of Zambia - Ridgeway Campus, Biomedical Sciences Daka, Victor; The Copperbelt University School of Medicine, Public Health Muleba, Mbanga; Tropical Diseases Research Centre, Department of Biomedical Sciences Shimaponda-Mataa, Nzooma ; University of Zambia - Ridgeway Campus, Biomedical Sciences
Primary Subject Heading:	Public health
Secondary Subject Heading:	Global health, Epidemiology, Infectious diseases
Keywords:	Malaria, Mosquito Vectors, Behavior, Entomology < TROPICAL MEDICINE

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Implications for Malaria Transmission: A Cross-sectional Study on the Bionomics and Susceptibility of Local Malaria Vectors in Urban and Peri-urban Settings of Ndola District.

Westone Hamwata[1,2]; Mwendalubi Hazyondo[2,3]; Victor Daka[4]; Mbanga Muleba[1]; Nzooma M. Shimaponda-Mataa[2];

1. Tropical Diseases Research Centre, Department of Biomedical Sciences, Vector Biology and Parasitology Unit
2. The University of Zambia, School of Health Sciences, Department of Biomedical Sciences
3. Department of National Parks and Wildlife, Chilanga, Lusaka, Zambia
4. The Copperbelt University, Michael Chilufya Sata School of Medicine, Department of Public Health

Corresponding Author:

Westone Hamwata

Tropical Diseases Research Centre, Department of Biomedical Sciences

Ndola, Zambia.

Telephone: +260972790935

Email: phamwata@gmail.com

ABSTRACT

Background: Malaria remains a public health issue in Zambia and insecticide-based vector control is the main malaria elimination strategy. Success of these strategies is dependent on a clear understanding of bionomics and susceptibility of the local vectors to insecticides used. Therefore, the objective of this study is to generate baseline data on vector behaviour and phenotypic resistance for effective vector control programming.

Methods: This was a cross-sectional study conducted in Ndola district between July and October 2021 across the four sites; two urban and two per-urban sites. Mosquitoes were collected using Center for Disease Control and prevention light trap (CDC-LT), Pyrethrum Spray Catch (PSC), Aspirations and larval collection. Mosquito identification was done using standard identification keys and Polymerase Chain Reaction (PCR). Williams's mean was used to determine mosquito densities and Kruskall Wallis H test was used to compare the distribution of mosquitoes. A negative binomial with a log link function was used to determine factors affecting mosquito counts. Susceptibility of the local vectors was determined using WHO tube and CDC bottle bioassays.

Results: The main breeding sites identified were irrigation trenches (4.67 larvae/dip) and garden ponds (2.72 larvae/dip) created from extensive urban agriculture practices. *Anopheles funestus* and *Anopheles gambiae* were found to coexist in all the four sites with *An. funestus* identified as the most dominant malaria vector. Densities of *An. gambiae s.s* seeking a blood meal ($\chi 2 = 12.566$, df = 3, p = 0.001) and resting indoors (Z = 56.5, p = 0.019) were found to be higher in urban than peri-urban sites compared to *An. funestus s.s* which had similar distribution across the study sites. Sprayed houses were significantly associated with reduced mosquito numbers (B = -0.956, IRR = 0.384, p = 0.001. *An. gambiae* s.s was fully susceptible to organophosphates and neonicotinoids but highly resistant to pyrethroids, carbamates and organochlorines.

Conclusions: The emergence of *An. funestus s.s* in an area previously dominated by *An. gambiae s.s* and its coexistence with *An. gambiae s.s* in the dry season pose a risk of sustaining malaria transmission all year round. Agriculture practices in urban areas resulted in highly productive mosquito breeding sites, thus the need for targeted vector control.

Key Words: *malaria vector coexistence, vector behaviour, insecticide resistance, Ndola, urban setting*

STRENGTHS AND LIMITATIONS OF THIS STUDY

- The study design and sampling strategies used allow for the determination of species composition, abundance, host-seeking and resting behaviour of malaria vectors.
- Presence of *An gambiae s.s* larval in the dry season facilitates mosquito breeding which may drive malaria transmission thus the need to plan for additional measures.
- The susceptibility of *An. gambiae s.s* the most efficient malaria vector was determined against seven different insecticides from five different classes but not for *An. funestus* s.s. due to limited numbers of adult *An. funestus* s.s due to the difficulty in finding larval habitats nor sufficient adults to conduct forced oviposition.
- This study was conducted in the dry season and the entomological indices determined may only be applicable to the dry season.

BACKGROUND

Malaria remains a public health challenge in Zambia accounting for approximately 1.4% of the global malaria disease burden. It is estimated that about four people die from malaria every day in Zambia [1,2]. High rainfall regions in northern Zambia experience the highest disease burden, while densely populated and arid regions in the south experience lower burden [3–6].

The primary malaria vectors in Zambia include *Anopheles funestus s.s, Anopheles gambiae s.s* and *Anopheles arabiensis* [7–9]. *Anopheles funestus s.s,* the most abundant and widely distributed malaria vector in the country thrives during the dry season whereas *An. gambiae s.s*, the most efficient malaria vector thrives predominantly in the wet season [8]. Historically, *An. gambiae s.s* has been the dominant malaria vector on the Copperbelt Province in the past decades [9]. In contrast, *An. arabiensis,* a more zoophilic mosquito, is the primary malaria vector in the southern regions and a secondary malaria vector in the eastern parts of the country – a region of moderate transmission [5,9–11].

Insecticide-treated bednets (ITNs), indoor residual spraying (IRS) and artemisinin combined therapy (ACTs) have played a vital role in reducing malaria disease burden in Zambia [12–14]. These interventions have been informed by entomological and parasite surveillance data generated from several parts of the country in the past two decades [13–15]. The Copperbelt province on the other hand has implemented IRS since the 1950s and over the past two decades scaled up IRS, ITNs and ACTs which were associated with a significant decrease in the malaria disease burden [16,17]. Furthermore, this success also led to a decline in entomological surveillance in the province. Since 2017, the Copperbelt province has experienced a rise in the number of malaria cases indicating a change in the epidemiological landscape, necessitating renewed entomological activities for informed vector control programming.

Vector surveillance across Zambia has revealed some level of heterogeneity in the behaviour and susceptibility of malaria vectors within and between selected districts [9,18]. Most active entomological sites are located in areas of high or low malaria transmission, with limited representation in settings of moderate transmission [19]. Additionally, over 95% of all entomological surveillance activities are conducted in rural settings yet a substantial number of reported malaria cases originate from peri-urban and urban areas [20–22]. To address these gaps, we conducted entomological studies in Ndola between July 2021 and October 2021, in two ecologically distinct settings representing the peri-urban and urban areas of Ndola district a moderate malaria transmission setting to assess vector behaviour and phenotypic resistance for effective vector control programming.

METHODS

Study design and study area

This was a cross sectional study was conducted in the dry season in Ndola district, the provincial capital of the Zambia's Copperbelt Province. The mean annual temperatures range from 12^{o}C to 25^{o}C, with mean annual rainfall ranging from 200 to 900 mm. The rainy season spans November to March, followed by a longer dry season from April to October.

Two catchment areas Chipulukusu and Kaniki were selected for their high malaria incidences rates in 2020. The malaria incidences for Chipulukusu and Kaniki health centres were 435 per 1000 population at risk and 971 per 1000 population at risk [2]. Chipulukusu is an urban catchment area with houses constructed with cement blocks, burnt bricks, or mud bricks and have iron or grass roofing. Mosquito collection in Chipulukusu was conducted in two zones: Musalu (-12.9524 S, 28.66012 E), a densely populated area with limited road access and extensive vegetable gardening

activities and Mapalo (-12.9374 S, 28.67564 E) an equally densely populated area but with road access and very minimal vegetable gardening activities. Kaniki is a peri-urban catchment consisting mainly of mud houses with thatched grass or iron sheet roofs. Mosquito collection in Kaniki was conducted in Kamalasha (-12.8556955 S, 28.5311082 E), a densely populated area near the Sakania border with a swamp on the western side of the Ndola-Mufulira Road, and Pima (-12.77416 S, 28.483865 E), a farming setting with houses organized in clusters. Both catchment areas serve as low-cost residential settings.

Sample size

A total of 166 houses were selected for adult mosquito collection; 56 houses for CDC light traps, 30 houses for pyrethrum spray catches and 80 houses for aspirations. An additional 60 collection efforts were made for larval collection from potential larval habitats.

Sample size justification

This study utilized WHO guidelines on mosquito sampling and the sample size used for this study follows previous modeling studies conducted on the minimum number of houses required to estimate mosquito abundance using a precision of 20% allowable for ecological studies [23,24].

Inclusion and exclusion criteria

The inclusion criteria for this study were twofold; firstly, only houses with an adult (16 years and above) were considered and houses where written consent was gotten. Houses were people cook using firewood from inside were excluded from the study.

House selection and adult mosquito collection

House selection was randomly done in each participating zone in the catchment area maintaining a minimum of 200m between two participating houses. Mosquito collection in each participating house was only done once and only one mosquito collection method was employed per house sampled. The collection of mosquitoes was conducted between July and September 2021 from 166 randomly selected houses; 83 houses from Chipulukusu catchment area, and the other 83 houses from Kaniki catchment area.

CDC light traps

The CDC-LT was used as a proxy for determining the biting density of mosquitoes to human hosts. The traps were set in randomly selected houses, and mosquito collection occurred from 18:00 hours to 06:00 hours the following morning. Each trap was set at a height of 1.5 metres from the ground, adjacent to a sleeping person and near their legs.

Aspirations

The live adult mosquitoes were collected using prokopack aspirator from 05:00 hours to 07:00 hours in the morning in houses where people slept. The live adult mosquitoes were then put in bugdom cages where they were supplied with 10% sugar solution and transported to the laboratory for identification.

Pyrethrum Spray Catches (PSC)

Adult mosquitoes resting indoors were collected indoors using PSC from 05:00 hours to 07:00 hours in the morning. Multiple pieces of white linen were spread over the floor, bed and furniture inside the house. Household members were asked to briefly exit the house and then the house was sprayed to saturation using pressurised two-in-one pyrethroid insecticide (imiprothrin 1.00 g/kg

and deltamethrin 0.51 g/kg) can. After 10 minutes, all the mosquitoes that were knocked down were picked using a pair of forceps and placed into properly labelled petri dishes.

Collection of immature mosquitoes (larvae)

Larval collection was carried out in October 2021. Potential larval habitats were initially visually inspected for the presence of larvae using a 350 ml capacity standard dippers (BioQuip Products, Inc., California, USA) followed by sampling. The number of dips and number of larvae scouped were recorded. Afterwards, the collected larvae was transported to the TDRC laboratory for rearing in a controlled microenvironment (temperature of 27°C ± 2°C and a relative humidity of 75% ± 10%).

Susceptibility testing

Adults, F0 *An. gambiae s.l* reared from field collected larvae from Musalu, were exposed to five different classes of insecticides. The mosquitoes, aged two to five days, obtained from wild collected larvae, were exposed to pirimiphos-methyl (0.25%), malathion (5%), deltamethrin (0.05%), permethrin (0.75%), bendiocarb (0.01%) and dichlorodiphenyltrichloroethane (4%) standard World Health Organisation (WHO) impregnated test paper. The bioassays were conducted in accordance with the WHO guidelines [25]. For Clothianidin, CDC bottle bioassay as described by Brogdon and Chan [26]. A minimum of 100 female *An. gambiae s.l* aged two to five days old were exposed each insecticide and 25 *An. gambiae s.l* were used as controls for each insecticide tested.

Experimental Procedures

1. **To determine species composition and abundance of malaria vectors in Ndola:** The species composition and abundance was determined by collections from CDC - LT, PSC, and aspirations.

2. **To assess the biting and resting behaviour of mosquitoes in Ndola:** The CDC – LT was used as a proxy for assessing the biting behaviour of malaria vectors. The densities of mosquitoes collected per trap per night (mosquitoes/trap/night) was used to assess the host seeking behaviour of the malaria vectors. The **Indoor Resting behaviour** is indicative of the mosquitoes that rest indoors. This indicator is an important one when considering whether to implement IRS or not. The mean indoor resting densities calculated in this study were determined by mosquito collections from PSC only.

3. **To determine the insecticide resistance status of primary malaria vectors:** The mosquitoes used for susceptibility testing were the first filial generation (F1) from the larvae collected.

Mosquito processing

Morphological identification

The female *Anopheles* mosquitoes collected were initially morphologically identified to the genus level using an identification key for Afrotropical *Anopheles* mosquitoes [27]. Thereafter, the mosquito samples were individually preserved in 1.5 mL Eppendorf tubes containing silica gel. These preserved samples were stored for molecular identification using PCR.

Molecular identification

A subset of adult mosquitoes reared from field collected larvae and those collected as adults, morphologically identified as *An. funestus s.l.* and *An. gambiae s.l.*, were further subjected to PCR for molecular confirmation of the IDs. Deoxyribose nucleic acid (DNA) extraction for this process was performed using the QIAGEN DNeasy Blood and Tissue kit for insects (QIAGEN Inc., USA). DNA amplification was performed using the Applied Biosystems GeneAmp PCR System 9700 thermocycler. For molecular identification, the methods described by *Koekemoer. L et al; 2002* [28] and *Scott W.G et al; 1993* [29] were used for *An. funestus s.l* and *An. gambiae s.l.* respectively.

STATISTICAL ANALYSIS

The data collected was entered in Microsoft Excel and mean densities excluding larval density were derived from log transformed data using Williams mean (M_w= [$(X_1 + 1)(X_2 + 1)(X_3 + 1)\ldots (X_n + 1)]^{1/n}$ to account for skewed (non-normal distribution) and count data [21,30]

The data were then exported to IBM SPSS statistics version 25. The Kruskal–Wallis H test was used to compare the means (Mw) of malaria vectors seeking a host. The Mann–Whitney U test was used to compare the densities of the malaria vectors resting indoors from the two sites where PSC was conducted. Additionally, a negative binomial model with a log function was used to identify factors associated with counts of malaria vectors in the sampled housing structures. Susceptibility status of *An. gambiae s.s.* was determined using WHO mortality scoring guidelines [23], [24].

Patient and Public Involvement

There was no direct patient and public involvement. The findings from this study will be shared with Ndola District Health Office and the Ministry of Health.

RESULTS

Species composition and abundance from adult mosquito surveys

A total of 166 houses were sampled, and a total of 744 female mosquitoes were collected. *Culex* accounted for 53% (392/744), *An. funestus s.l.* 17% (123/744), *Mansonia* 16% (106/744), *An. gambiae s.l.* 14% (106/744), and *An. gibbinsi s.l.* less than 1% (2/744) of the total mosquitoes collected (Table 1). Mosquito abundance by site showed the highest mosquito collections were from Musalu (338/744) and Mapalo (208/744), the two urban sites, followed by Pima (131/744) and Kamalasha (67/744), the two peri-urban sites. Notably, *Culex* mosquitoes comprised the largest proportion at each of the four sites (Table 1). Further, species composition by site shows that rural sites from Kaniki; Kamalasha and Pima had one more species A*n. gibbinsi* not found in the two sites from Chipulukusu (Mapalo and Musalu). *Culex* were the most abundant mosquito collected from each site with (Kamalasha 67%, Pima 59%, Musalu 53% and Mapalo 42%). The second most abundant mosquito in Kaniki (Kamalasha 22% and Pima 30%) was *An. funestus s.l.* This was followed by *An. gambiae s.l.* (Kamalasha 7% and Pima 5%). Whilst from Chipulukusu, *Mansonia (24%)* and *An. gambiae s.s* (21%) were the second most abundant mosquito species collected from Mapalo and Musalu respectively (table 1).

Molecular identification of sibling species

Eighty-nine percent (89%; 67/75) of the female *An. funestus s.l.* analysed for molecular identification amplified as *An. funestus s.s* at 505 base pairs and 85.5% (171/200) of *An. gambiae s.l* successfully amplified as *An. gambiae* s.s at 390 base pairs. Figure 1a and Figure 1b shows the

results of an agarose gel run for molecular identification of *An. funestus s.s* and *An. gambiae s.s* respectively.

Biting and resting behaviour of malaria vectors

The mean number of *An. funestus s.s* seeking a blood meal from Mapalo was 2.42 times higher (Mw = 0.97/trap/night) than in Kamalasha (0.4/trap/night) and Pima (0.4/trap/night) and 9.7 times higher than in Musalu (0.1/trap/night) (table 1). For *An. gambiae s.s* the mean number of mosquitoes seeking a blood meal from Musalu (1.26/trap/night) were 1.5 more than in Mapalo (0.83/trap/night) and higher than in Kamalasha (0.1/trap/night) and Pima (0.14/trap/night), with differences of 12.6 and 9 times, respectively. Despite these variations in mean densities, the Kruskal–Wallis H test revealed no statistical difference in the host seeking behaviour of *An. funestus s.l.* ($\chi2 = 4.598$, df = 3, $p = 0.204$) across the four sites. However, a statistical difference was observed in the host seeking behaviour of *An. gambiae s.l.* ($\chi2 = 12.566$, df=3, $p < 0.001$).

The indoor resting density of *An. funestus s.l.* in Pima (1.2 mosquitoes per house) was 1.67 times higher than in Musalu (0.72 mosquitoes per house) whereas for *An. gambiae s.l.* in Musalu (1.31 mosquitoes per house), the indoor resting density was 262 times higher than in Pima (0.05 vectors per house) (Table 1). The Mann–Whitney U test indicated no statistical difference in the resting densities of *An. funestus s.l* between Pima and Musalu ($Z = 143.5$, $p = 0.202$), but statistical difference in the resting densities of *An. gambiae s.l* ($Z = 56.5$, $p < 0.019$) was observed between the two sites.

***Anopheles* mosquito larval habitats**

A total of 43 potential *anopheline* larval habitats were identified, and 55.81% (*n=24*; 95 CI: 40% – 71%) of these found to contain larvae. All the larval habitats found to contain larvae were from

either Musalu (70.83%; 95% CI: 49% – 87%) or Mapalo (29.12%; 95% CI: 13% – 51%) sites. Seven different categories of potential larval habitats identified included blocked trenches, foundation trenches, garden ponds, irrigation canals (channels), shallow wells, streams and tyre marks. From the different larval habitats, 2,643 larvae were collected from a total of 914 dips. The proportion of larvae collected from Musalu was 94.97% (2,510/2,643; 95% CI: 94% – 96%), whereas the remaining 5.03% (133/2,643; 95% CI: 4.2% – 5.9%) were collected from Mapalo (Table 2). Additionally, 63.94% (1,690/2,643; 95% CI: 62% – 66%) of the collected larvae were from irrigation canals, 20.92% (553/2,643; 95% CI: 19% – 22%) were from garden ponds, 10.93% (289/2,643; 95% CI: 9.7% – 12%) were from tire marks, 3.37% (89/2,643; 95% CI: 2.7 – 4.1%) were from foundation trenches and 0.83% (22/2,643%; 95% CI: 0.52% – 1.3%) were from blocked trenches.

The larval density was highest in irrigation canals, with 4.67 larvae per dip, this was followed by garden ponds with 2.72 larvae per dip, tyre marks with 1.30 larvae per dip, foundation trenches with 1.25 larvae per dip and blocked drainages with 0.49 larvae per dip.

Factors affecting mosquito counts in housing structures

Seven predictors were utilised to identify associations with mosquito counts of *An. funestus s.s* and *An. gambiae s.s* in households, and only the spray status with Fludora® Fusion (B = -0.956, IRR = 0.384, p = 0.001) was found to be statistically significant, (Table 3). While three other predictors were associated with reduced mosquito counts, including the number of people who slept in a house the previous night (B = -0.023, IRR = 0.978, p = 0.714), housing structures with a thatched roof (B = -0.060, IRR = 0.942, p = 0.870, and the number of LLINs in a housing structure (B = -0.085, IRR = 0.918, p = 0.489, these predictors were not statistically significant. On the other

hand, the other three predictors, number of animals that slept in a house the previous night (B = 0.004, IRR = 1.004, p = 0.937, housing structures plastered with mud walls or unburnt bricks (B = 0.234, IRR = 1.264, p = 0.559) and housing structures with open eaves (B = 0.203, IRR = 1.225, p = 0.557), were associated with increased mosquito counts, but they were not statistically significant.

Susceptibility status of *An. gambiae s.s*

The study showed full susceptibility 24 hours post exposure (100% mortality) to organophosphate (malathion 5% and pirimiphos-methyl 0.25%) and neonicotinoids (clothianidin). Conversely, resistance was confirmed to bendiocarb 0.1%, permethrin 0.75%, deltamethrin 0.75% and Dichlorodiphenyltrichloroethane 4% (DDT) with corresponding mortalities of 23%, 14%, 18% and 4% respectively (Table 4). The area where the larvae used for susceptibility testing were collected from Musalu, an area predominantly known for urban agriculture practices.

DISCUSSION

This study reveals that the two main malaria vectors in Zambia *An. funestus s.l.* and *An. gambiae s.l.* were found in all four sites and these were molecularly identified as *An. funestus* s.s. and *An. gambiae* s.s. respectively. In Zambia, these mosquitoes have been implicated as the main vectors responsible for malaria transmission and have been found to exist in sympatry [20,21,31,32]. Surprisingly, *Anopheles funestus* was found to be the most abundant malaria vector in Ndola. Historically, the province has been dominated by *An. gambiae s.s* but this study found *An. funestus s.s* as the dominant malaria vector in Ndola. This finding is similar to other entomological findings in other districts within the Copperbelt province were *An. funestus s.s* is the more dominant vector [9,15]. However, *An. gambiae s.s.* remained the more dominant malaria vector in urban areas,

whereas *An. funestus s.s.* was more abundant in peri-urban areas, consistent with earlier studies conducted in sub-Saharan Africa [18,21]. This disparity in vector abundance could be attributed to variations in ecological habitats. *Anopheles gambiae* prefers to breed in man-made water habitats such as drainages, tire tracks, small pools and agriculture sites, whilst *An. funestus s.s.* prefers to breed in permanent and semi-permanent water habitats with some vegetative cover [33,34]. An earlier study in the northern parts of the country identified *An. funestus s.s* as the primary driver of malaria transmission in the dry season whereas *An. gambiae* s.s as the primary driver in the wet season [31]. Nonetheless, the existence of breeding grounds for *Anopheles gambiae s.s* in urban areas implies that even during the dry season, *An. gambiae s.s* will continue to be the primary driver of malaria transmission. The coexistence of these two malaria vectors pose an increased year round risk of malaria transmission in the area. The recent increase in the incidences of malaria reported in Ndola could be attributed to the changing vector bionomics that now includes *An. funestus s.s.* not reported previously in the area.

Mosquito diversity was observed to be higher in peri-urban than urban sites with the inclusion of *An. gibbinsi,* a potential secondary malaria vector. This vector has been reported in other parts of the country as a potential secondary malaria vector [35–37]. Secondary malaria vectors have not been adequately considered in most vector control programming yet they contribute to 5% of malaria transmission in the southern African region [38]. Their contribution to transmission is significant making the need to incorporate interventions targeting secondary malaria vectors into vector control toolkits inevitable.

The host-seeking behaviours of *An. funestus s.s.* and *An. gambiae s.s.* were different. The host-seeking behaviour of *An. funestus* s.s. was found to be homogeneous across the four sites, whereas the host-seeking behaviour of *An. gambiae s.s.* was found to be much higher in urban sites with

vast larval habitats. This heightened host-seeking behaviour of *An. gambiae s.s.* indicates an increased risk of disease transmission in urban sites compared to per-urban sites thus the [39]. As such the need for enhanced vector control methods in urban settings with extensive larval habitats due to the elevated risk cannot be overemphasized.

The mean densities of *An. funestus* s.s found resting indoors were generally low across the peri-urban and urban sites. However, the indoor resting density of *An. gambiae* s.s in the urban site was much higher than that in the peri-urban site. Variations in the indoor resting behaviour of *An. funestus s.s.* and *An. gambiae s.s.* could be influenced by the presence of vast *An. gambiae s.s* breeding sites in urban sites. Therefore, vector control interventions such as IRS and LLINs in such settings may need to be supplemented with larval source management [40].

The larval habitats that were active breeding sites were all from the two urban sites adjacent to a dambo. The larval habitats identified included irrigation canals (or irrigation channels), garden ponds, tire marks, foundation trenches and blocked drainages. However, irrigation channels and garden ponds were found to be the main mosquito breeding sites, similar to studies conducted in Ghana, Tanzania, Cote d'Ivoire and China [41]. However, the larval densities found in this study were higher than that found in China, possibly due to differences in the climatic conditions, variations in the bacterial diversity and physicochemical composition of the larval habitats [42]. These factors have been found to influence mosquito oviposition, survival, and development into competent malaria vectors, thereby potentially impacting malaria incidence [3,43]. Unfortunately, this study only identified the different types of larval habitats; future research is needed to fully characterize larval habitats in order to generate additional information valuable for an effective and targeted larval source management programme.

The four predictors associated with reduced counts of malaria vectors in housing structures were; the number of people who slept in a housing structure, housing structures with a thatched roof, the number of LLINs used the previous night and housing structures sprayed with *Fludora*® *Fusion*. However, only housing structures sprayed were found to be statistically associated with reduced counts of malaria vectors, similar to what was found in Sao Tome and Principe [40]. Individuals who sleep in sprayed houses experience a lower vector-to-host contact, which entails reduced exposure to infectious mosquito bites unlike those sleeping in unsprayed houses. Additionally, maximum benefit is derived when at least 85% of houses are sprayed with an efficacious insecticide to kill host seeking mosquitoes that rest indoors [44]. On the other hand, number of animals in a housing structure, housing structures with mud wall surfaces and open eaves were associated with increased counts of malaria vectors but were not statistically significant. Elsewhere, a study conducted in Cameroon associated open eaves and holes in the walls to increased mosquito counts [45]. Another study in Gambia also found that closing the eaves reduces mosquitoes entering thatched houses but increases mosquito entry into metal-roofed houses [46].

Susceptibility tests in this study reveal that *An. gambiae* s.s. was fully susceptible to organophosphates (malathion and pirimiphos-methyl) and neonicotinoids (clothianidin). This was also observed in several other districts in Zambia, where *An. funestus s.s.* and *An. gambiae s.s.* was found to be susceptible to these two classes of insecticides [20,22]. In that regard, organophosphates and neonicotinoids could be effective at controlling mosquito populations of *An. gambiae s.s* in Ndola and several other districts in Zambia, with evidence of susceptibility. However, resistance of *An. gambiae* s.s. to pyrethroids (permethrin and deltamethrin) and carbamates (bendiocarb) was confirmed and this could be attributed to the extensive use of

pesticides and insecticides for agriculture and public health purposes. These results align with previous studies that found extensive insecticide resistance to pyrethroids and carbamates in the Copperbelt province [9,18]. In the wake of widespread resistance to pyrethroids and carbamates, there is reduced efficacy of the malaria vector control tools used and lowered community protection where carbamate and pyrethroid-only active ingredients are used. As such, this has the potential to drive transmission in Ndola District despite implementing these interventions.

CONCLUSION

The two primary malaria vectors *An. funestus* s.s. and *An. gambiae* s.s. were found to coexist in the two ecologically distinct settings, with *An. funestus* s.s. being the dominant malaria vector. This coexistence has the potential of sustaining high malaria transmission throughout the year especially in urban areas. Urban agriculture practices created *An. gambiae s.s* breeding sites during the dry season, contributing to the high host seeking and indoor resting behaviour in the urban sites. Sprayed housing structures were associated with reduced counts of malaria vectors. *Anopheles gambiae* was found to be susceptible to organophosphates and neonicotinoids, but resistance to pyrethroids, carbamates and organochlorides was confirmed. Additional studies are needed to investigate the different mechanisms of pyrethroid resistance in the area.

REFERENCES

1. WHO. World malaria report 2022. Geneva; 2022.
2. MOH. Health Management Information System of zambia [Internet]. Lusaka; 2020. Available from: https://www.nmec.org.zm/
3. Shimaponda-Mataa NM, Tembo-Mwase E, Gebreslasie M, Achia TNO, Mukaratirwa S. Modelling the influence of temperature and rainfall on malaria incidence in four endemic provinces of Zambia using semiparametric Poisson regression. Acta Trop. 2017 Feb 1;166:81–91.
4. Sitali L, Miller JM, Mwenda MC, Bridges DJ, Hawela MB, Hamainza B, et al. Distribution of Plasmodium species and assessment of performance of diagnostic tools used during a malaria

survey in Southern and Western Provinces of Zambia. Malar J [Internet]. 2019;18(1):1–9. Available from: https://doi.org/10.1186/s12936-019-2766-2

5. MOH. Zambia National Malaria Indicator Survey 2021: MINISTRY OF HEALTH. Natl Malar Elimin Centre, Minist Heal Zambia [Internet]. 2021; Available from: papers2://publication/uuid/2E7FD35E-174F-4868-8A7F-7910082D3A11

6. Chipoya MN, Shimaponda-Mataa NM. Prevalence, characteristics and risk factors of imported and local malaria cases in North-Western Province, Zambia: a cross-sectional study. Malar J 2020 191 [Internet]. 2020 Nov 23 [cited 2021 Jul 24];19(1):1–12. Available from: https://malariajournal.biomedcentral.com/articles/10.1186/s12936-020-03504-1

7. Cross DE, Thomas C, McKeown N, Siaziyu V, Healey A, Willis T, et al. Geographically extensive larval surveys reveal an unexpected scarcity of primary vector mosquitoes in a region of persistent malaria transmission in western Zambia. Parasites and Vectors [Internet]. 2021 Dec 1 [cited 2021 Apr 17];14(1):91. Available from: https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-020-04540-1

8. Das S, Muleba M, Stevenson JC, Norris DE. Habitat partitioning of malaria vectors in nchelenge district, Zambia. Am J Trop Med Hyg. 2016;94(6):1234–44.

9. NMEC. Insecticide Resistance Management and Monitoring Plan, Ministry of Health, Government Republic of Zambia. In 2019.

10. Fornadel CM, Norris LC, Franco V, Norris DE. Unexpected Anthropophily in the Potential Secondary Malaria Vectors An coustani s.l and An squamosus in Macha, Zambia. 2011;11(8).

11. Saili K, de Jager C, Sangoro OP, Nkya TE, Masaninga F, Mwenya M, et al. Anopheles rufipes implicated in malaria transmission both indoors and outdoors alongside Anopheles funestus and Anopheles arabiensis in rural south-east Zambia. Malar J [Internet]. 2023;22(1):1–12. Available from: https://doi.org/10.1186/s12936-023-04489-3

12. Chanda E, Masaninga F, Coleman M, Sikaala C, Katebe C, MacDonald M, et al. Integrated vector management: The Zambian experience. Malar J. 2008;7:1–8.

13. Chizema-Kawesha E, Miller JM, Steketee RW, Mukonka VM, Mukuka C, Mohamed AD, et al. Scaling Up Malaria Control in Zambia: Progress and Impact 2005–2008. Am J Trop Med Hyg [Internet]. 2010 Sep 7 [cited 2023 Apr 23];83(3):480–8. Available from: https://www.ajtmh.org/view/journals/tpmd/83/3/article-p480.xml

14. Masaninga F, Chanda E, Chanda-kapata P, Hamainza B, Masendu HT, Kamuliwo M, et al. Review of the malaria epidemiology and trends in Zambia. 2013;3(2):89–94.

15. PMI USAID. The President's Malaria Initiative (PMI) / Vectorlink; Zambia 2019-2020 Entomology Annual Report. Rockville, MD PMI VectorLink Proj Abt Assoc. 2020;

16. Barnes KI, Chanda P, Barnabas GA. Impact of the large-scale deployment of artemether / lumefantrine on the malaria disease burden in Africa : case studies of South. Malar J. 2009;7(October 2005):1–7.

17. Masaninga F, Chanda E, Chanda-kapata P, Hamainza B, Masendu HT, Kamuliwo M, et al. Review of the malaria epidemiology and trends in Zambia. 2013;3(2):89–94.

18. Thomsen EK, Strode C, Hemmings K, Hughes AJ, Chanda E, Musapa M, et al. Underpinning sustainable vector control through informed insecticide resistance management. PLoS One. 2014;9(6).

19. NMEC. National Malaria Elimination Strategic Plan 2022-2026. 2022;1(1):62.

20. PMI USAID. The President's Malaria Initiative (PMI) / Vectorlink; Zambia 2018-2019 Entomology Annual Report. Rockville, MD PMI VectorLink Proj Abt Assoc. 2019;(November 2018):37.

21. Stevenson JC, Pinchoff J, Muleba M, Lupiya J, Chilusu H, Mwelwa I, et al. Spatio-temporal heterogeneity of malaria vectors in northern Zambia : implications for vector control. Parasit Vectors [Internet]. 2016;1–15. Available from: http://dx.doi.org/10.1186/s13071-016-1786-9

22. Chanda J, Saili K, Phiri F, Stevenson JC, Mwenda M, Chishimba S, et al. Pyrethroid and Carbamate Resistance in Anopheles funestus Giles along Lake Kariba in Southern Zambia. 2020;103(Suppl 2):90–7.

23. World Health Organization(WHO). Malaria surveillance, monitoring & evaluation: a reference manual [Internet]. Licence: C. 2018. Available from: http://apps.who.int/iris.%0ASales,

24. Zhou G, Minakawa N, Githeko A, Yan G. Spatial distribution patterns of malaria vectors and sample size determination in spatially heterogeneous environments: A case study in the west Kenyan highland. J Med Entomol. 2004;41(6):1001–9.

25. World Health Organization(WHO). Test procedures for insecticide resistance monitoring in malaria vector mosquitoes Second edition. 2018;

26. Brogdon W, Chan A. Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay. 2010;1–28.

27. Coetzee M. Key to the females of Afrotropical Anopheles mosquitoes (Diptera: Culicidae). Malar J [Internet]. 2020;19(1):1–20. Available from: https://doi.org/10.1186/s12936-020-3144-9

28. Koekemoer LL, Kamau L, Hunt RH, Coetzee M. A cocktail polymerase chain reaction assay to identify members of the Anopheles funestus (Diptera: Culicidae) group. Am J Trop Med Hyg. 2002;66(6):804–11.

29. Scott JA, Brogdon WG, Collins FH. Identification of single specimens of the Anopheles gambiae complex by the polymerase chain reaction. Am J Trop Med Hyg [Internet]. 1993 [cited 2021 Apr 30];49(4):520–9. Available from: https://pubmed.ncbi.nlm.nih.gov/8214283/

30. William C. THE USE OF LOGARITHMS IN THE INTERPRETATION OF CERTAIN ENTOMOLOGICAL PROBLEMS. Ann Appl Biol [Internet]. 1937;24(March 1936):404–14. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1744-7348.1937.tb05042.x

31. Muleba M, Mbata KJ, Stevenson JC, Norris DE. Spatial–temporal vector abundance and malaria transmission dynamics in Nchelenge and Lake Mweru islands, a region with a high burden of malaria in northern Zambia. Malar J [Internet]. 2023;22(1):1–11. Available from: https://doi.org/10.1186/s12936-023-04746-5

32. Fornadel CM, Norris LC, Glass GE, Norris DE. Analysis of Anopheles arabiensis blood feeding behavior in southern zambia during the two years after introduction of insecticide-treated bed nets. Am J Trop Med Hyg. 2010;83(4):848–53.

33. Doumbe-Belisse P, Kopya E, Ngadjeu CS, Sonhafouo-Chiana N, Talipouo A, Djamouko-Djonkam L, et al. Urban malaria in sub-Saharan Africa: dynamic of the vectorial system and the entomological inoculation rate. Malar J [Internet]. 2021;20(1):1–18. Available from: https://doi.org/10.1186/s12936-021-03891-z

34. Nambunga IH, Ngowo HS, Mapua SA, Hape EE, Msugupakulya BJ, Msaky DS, et al. Aquatic habitats of the malaria vector Anopheles funestus in rural south-eastern Tanzania. Malar J [Internet]. 2020;19(1):1–11. Available from: https://doi.org/10.1186/s12936-020-03295-5

35. Lobo NF, St. Laurent B, Sikaala CH, Hamainza B, Chanda J, Chinula D, et al. Unexpected diversity of Anopheles species in Eastern Zambia: implications for evaluating vector behavior and interventions using molecular tools. Sci Rep. 2015 Dec 9;5:17952.

36. Gebhardt ME, Krizek RS, Coetzee M, Koekemoer LL, Dahan-Moss Y, Mbewe D, et al. Expanded geographic distribution and host preference of Anopheles gibbinsi (Anopheles species 6) in northern Zambia. Malar J [Internet]. 2022;21(1):1–10. Available from: https://doi.org/10.1186/s12936-022-04231-5

37. Cooke MK, Kahindi SC, Oriango RM, Owaga C, Ayoma E, Mabuka D, et al. 'A bite before bed': exposure to malaria vectors outside the times of net use in the highlands of western Kenya. Malar J. 2015 Jun 25;14(1):259.

38. Afrane YA, Bonizzoni M, Yan G. Secondary Malaria Vectors of Sub-Saharan Africa: Threat to Malaria Elimination on the Continent? Curr Top Malar. 2016;(November).

39. Cummins B, Cortez R, Foppa IM, Walbeck J, Hyman JM. A spatial model of mosquito host-seeking behavior. PLoS Comput Biol. 2012;8(5).

40. Chen YA, Lien JC, Tseng LF, Cheng CF, Lin WY, Wang HY, et al. Effects of indoor residual spraying and outdoor larval control on Anopheles coluzzii from São Tomé and Príncipe, two islands with pre-eliminated malaria. Malar J [Internet]. 2019;18(1):1–16. Available from: https://doi.org/10.1186/s12936-019-3037-y

41. Wang Y, Cheng P, Jiao B, Song X, Wang H, Wang H, et al. Investigation of mosquito larval habitats and insecticide resistance in an area with a high incidence of mosquito-borne diseases in Jining, Shandong Province. PLoS One [Internet]. 2020;15(3):1–14. Available from: http://dx.doi.org/10.1371/journal.pone.0229764

42. Wang H, Wang Y, Cheng P, Wang H, Wang H, Liu H, et al. The Larval Density of Mosquitos (Diptera: Culicidae) in Jiaxiang County, Shandong Province, China: Influence of Bacterial Diversity, Richness, and Physicochemical Factors. Front Ecol Evol. 2021;9(February):1–12.

43. Hinne IA, Attah SK, Mensah BA, Forson AO, Afrane YA. Larval habitat diversity and Anopheles mosquito species distribution in different ecological zones in Ghana. Parasit Vectors [Internet]. 2021;1–14. Available from: https://doi.org/10.1186/s13071-021-04701-w

44. World Health Organization(WHO). an Operational Manual for Indoor Residual Spraying (Irs) for Malaria Transmission Control and Elimination Second Edition Indoor Residual Spraying. 2015;(2).

45. Ngadjeu CS, Ngadjeu CS, Doumbe-Belisse P, Doumbe-Belisse P, Talipouo A, Talipouo A, et al. Influence of house characteristics on mosquito distribution and malaria transmission in the city of Yaoundé, Cameroon. Malar J [Internet]. 2020;19(1):1–14. Available from: https://doi.org/10.1186/s12936-020-3133-z

46. Jatta E, Jawara M, Bradley J, Jeffries D, Kandeh B, Knudsen JB, et al. Articles How house design affects malaria mosquito density , temperature , and relative humidity : an experimental study in rural Gambia. Lancet Planet Heal [Internet]. 2015;2(11):e498–508. Available from: http://dx.doi.org/10.1016/S2542-5196(18)30234-1

47. Syme T, Gbegbo M, Obuobi D, Fongnikin A, Agbevo A, Todjinou D, et al. Pyrethroid-piperonyl

butoxide (PBO) nets reduce the efficacy of indoor residual spraying with pirimiphos-methyl against pyrethroid-resistant malaria vectors. Sci Rep [Internet]. 2022;12(1):1–13. Available from: https://doi.org/10.1038/s41598-022-10953-y

ABBREVIATIONS

ACTs : artemisinin combined therapy; B : Regression Coefficient; CDC – LT : Centres for Disease Control and Prevention light traps; CI : Confidence Interval; DDT : Dichlorodiphenyltrichloroethane; df : degrees of freedom; DNA : Deoxyribonucleic acid; IRR : Incidence rate ratio; IRS : Indoor residual spraying; ITNs : Insecticide treated bednet; LLINs : Long lasting insecticide treated nets; Mw : Williams mean; NHRA : National Health Research Authority; NMEC : National Malaria Elimination Centre; P : Probability value; PBO : piperonyl butoxide; PCR : Polymerase Chain Reaction; PSC : Pyrethrum Spray Catches; *s.l : sensu lato; s.s : sentu stricto;* SPSS : Statistical Package for Social Sciences; TDRC : Tropical Diseases Research Centre; WHO : World Health Organisation.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study involved collecting mosquitoes from households with no direct contact with humans. As such, ethics clearance for the study protocol (Supplementary file 1) to undertake this study was obtained from the Tropical Diseases Research Centre Ethics Committee (Supplementary file 2) and the National Health Research Authority (Supplementary file 3). Written consent (supplementary file 4) was obtained from the head of the house prior to mosquito and larvae collection from their houses and their gardens, respectively. All data that was collected was restricted to the investigators and confidentiality was strictly maintained.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

FUNDING

This work did not receive any funding

CONFLICTS OF INTEREST / COMPETING INTERESTS

Financial interests: The authors as well as the funders declare that they have no financial or non-financial interests to disclose and they have no conflicts of interest to declare that are relevant to the content of this manuscript.

AUTHORS' CONTRIBUTIONS

WH: Participated in study design, conducted sample collection, analysis and interpretation. Participated in drafting the manuscript and also final approval of the revised manuscript. Westone Hamwata / WH acted as guarantor.

NMS-M: Participated in study design, supervised implementation of the project, interpretation of the results, review of the manuscript and final approval of the revised manuscript.

MM: Participated in study design, supervised implementation of the project, interpretation of the results, review of the manuscript and final approval of the revised manuscript.

VD: Participated in data analysis, review of the manuscript and final approval of the revised manuscript

MH: Participated in data analysis, drafting of the manuscript and final approval of the revised manuscript

ACKNOWLEDGEMENTS

We acknowledge the Ministry of Health Provincial Health office and the Health Centres for approval of the study and provisions towards it. Additionally, we acknowledge the support rendered by Tropical Diseases Research Centre (TDRC) and a Pan African Mosquito Control Association (PAMCA) for the laboratory supplies used in processing the samples.

AUTHORS' INFORMATION AND AFFILIATION

WH (Westone Hamwata[1,2]; phamwata@gmail.com) is a Medical Parasitologist and Entomologist based at the Tropical Diseases Research Centre and holds a MSc in Medical Parasitology and Master of Public Health.

MM (Mbanga Muleba[1]; mbangamuleba@gmail.com) is a Medical Entomologist at Tropical Diseases Research Centre and holds a PhD in Entomology

MH (Mwendalubi Hazyondo[2,3]; mwendalubihazyondo@gmail.com) is a Principal Wildlife Veterinary Officer at the Department of National Parks and Wildlife and hold a Doctor of Veterinary Medicine and Zootechnics

VD[4] (Victor Daka; dakavictorm@gmail.com) is Lecturer at The Copperbelt University, Michael Chilufya Sata School of Medicine, Department of Public Health and holds a PhD in Epidemiology.

NMS-M[2] (Nzooma M. Shimaponda-Mataa1; nzomataa@gmail.com) is a Medical Parasitologist and Lecturer at the University of Zambia and holds a PhD in Parasitology;

1. Tropical Diseases Research Centre, Department of Biomedical Sciences, Vector Biology and Parasitology Unit.

2. University of Zambia School of Health Sciences, Department of Biomedical Sciences, Ridgeway Campus, Lusaka, Zambia.

3. Department of National Parks & Wildlife, Chilanga, Zambia.

4. The Copperbelt University, Michael Chilufya Sata School of Medicine, Department of Public Health, Ndola Campus, Zambia.

LIST OF TABLES

Table 1: Entomological indices

Entomological Indices	Chipulukusu catchment		Kaniki catchment		Total
	Mapalo	Musalu	Kamalasha	Pima	
Species composition					
An. funestus s.l	45	23	15	40	123
An. gambiae s.l.	24	71	5	6	106
An. gibbinsi	0	0	1	1	2
Culex	88	181	45	78	392
Mansonia	51	63	1	6	121
Total	208	338	67	131	744
Mean densities of malaria vectors seeking a blood meal *(Mw)*					
An. funestus s.l	0.97*	0.10*	0.40*	0.40*	-
An. gambiae s.l.	0.83*	1.26*	0.10*	0.14*	-
Mean densities of malaria vectors resting indoors *(Mw)*					
An. funestus s.l	-	0.72*	-	1.2*	-
An. gambiae s.l.	-	1.31*	-	0.05*	-

*Mw = densities using Williams mean

Table 2: Mosquito larval habitats

Site	Type of larval habitat	No. of dips	Number of larvae collected (%)	Larval habitats identified	larval habitats with larvae	Mosquito genera	
						Anopheles larvae	*Culex larvae*
Mapalo	Foundation trenches	71	89 (3.37)	5	4	3	4
	Tyre marks	86	31 (1.17)	2	2	2	2
	Blocked drainages	18	13 (0.49)	1	1	1	1
	Subtotal	175	133 (5.03)	8	7	6	7
Musalu	irrigation canals	362	1690 (63.94)	19	11	9	11
	Tyre marks	147	258 (9.76)	3	2	2	2
	Garden ponds	203	553 (20.92)	4	3	3	3
	Blocked drainages	27	9 (0.34)	1	1	1	1
	Subtotal	739	2510 (94.97)	27	17	15	17
Kamalasha	Tyre marks	-*	0 (0.0)	5	0	0	0
	Subtotal	-*	0 (0.0)	5	0	0	0
Pima	Shallow wells	-*	0 (0.0)	2	0	0	0
	Stream	-*	0 (0.0)	1	0	0	0
	Subtotal	-*	0 (0.0)	3	0	0	0
Total from all sites		914	2643 (100)	43	24	21	24

**No larvae found after visual inspection followed by 10 dips*

Table 3: Predictors affecting Mosquito Counts of *An. funestus s.s* and *An. gambiae s.s*

Parameter	Regression coefficient (B)	Hypothesis Test			IRR**	95% Wald Confidence Interval for Exp(B)	
		Wald Chi-Square	df*	Sig.		Lower	Upper
(Intercept)	1.919	3.876	1	0.049	6.816	1.009	46.067
Number of People	-0.023	0.134	1	0.714	0.978	0.866	1.104
Number of Animals	0.004	0.006	1	0.937	1.004	0.912	1.105

Type of Roof	-0.060	0.027	1	0.870	0.942	0.462	1.921
Type of Wall	0.234	0.342	1	0.559	1.264	0.577	2.769
Type of Eaves	0.203	0.345	1	0.557	1.225	0.622	2.412
Number of LLINs***	-0.085	0.478	1	0.489	0.918	0.721	1.169
Spray Status	-0.956	10.513	1	0.001	0.384	0.216	0.685
(Scale)	1[a]						
(Negative binomial)	0.798						

Dependent Variable: No. of Malaria Vectors
Model: (Intercept), No. of People, No. of Animals, Type of Roof, Type of Wall, Type of Eaves, No. of LLINs, Spray Status
Fixed at the displayed value.
*df = degrees of freedom
**IRR = Incidence rate ratio
***LLINs = long lasting insecticide nets

Table 4: Susceptibility Status of *An. gambiae s.s* from Musalu

Insecticide tested	Number of mosquitoes exposed	Knockdown at 60min		Knockdown at 24hours		Final Mortality (24 hours)
		Dead	Alive	Dead	alive	
Bendiocarb 0.1%	108	14	94	25	83	23%
DDT 4%	113	0	113	5	108	4%
Deltamethrin 0.05%	100	13	87	18	82	18%
Permethrin 0.75%	113	6	107	16	97	14%
Pirimiphos-methyl 0.25%	110	101	9	110	0	100%**
Malathion 5%	104	94	10	104	0	100%**
Clothianidin	107	104	3	107	0	100%**

**Fully susceptible

Figure Legends

Figure 1: Molecular detection of An. funestus s.l and An. gambiae s.l

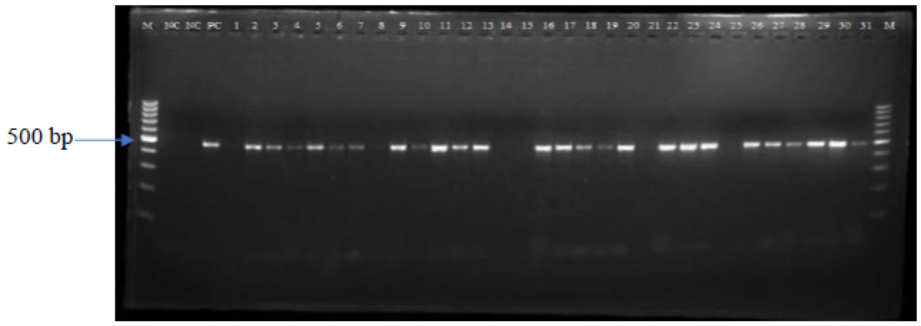

Figure 1a: 2% agarose electrophoresis of An. funestus PCR amplified products: M – 100 bp ladder; NC – Negative Control PCR; NC - Negative Control Extraction; PC – Positive Control; lanes 1 – 31 tested samples. The expected band was 505 bp.

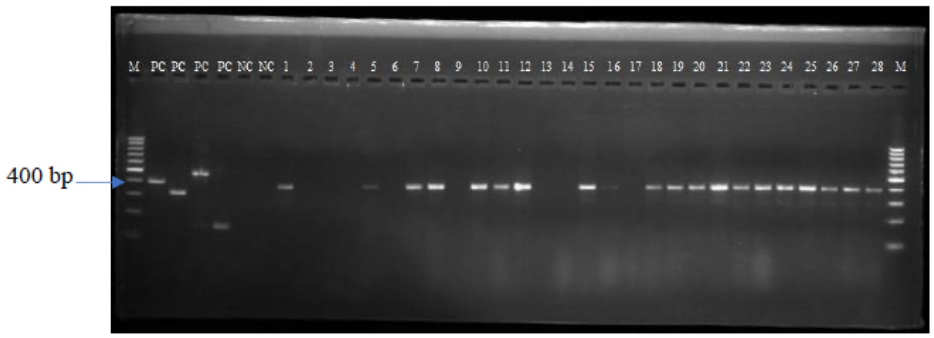

Figure 1b: 2% agarose gel electrophoresis of An. gambiae s.l. PCR amplified products M – 100 bp ladder; PC – Positive Control An. gambiae s.s.; PC – Positive Control An. arabiensis; PC – Positive Control An. merus; PC – An quadramulatus; NC – Negative Control Extraction; NC - Negative Control PCR and lanes 1 – 28 tested samples. The expected band was 390 bp.

Molecular detection of An. funestus s.l and An. gambiae s.l

149x139mm (120 x 120 DPI)

University of Zambia
School of Health Sciences
Department of Biomedical Sciences

Malaria Vector Bionomics and Phenotypic Resistance Status to Insecticides Used in Vector Control in Ndola District.

A Research Project Proposal Submitted in Partial Fulfilment for the Master of Science Degree in Medical Parasitology

Westone Phanuel Hamwata
19000762

Name of Supervisor: Dr. N. M. Shimaponda-Mataa

Name of Co-Supervisor: Mr Mbanga Muleba (TDRC)

Lusaka
(April, 2021)

EXECUTIVE SUMMARY

Malaria is a vector-borne disease transmitted to human through the bite of an infected female *Anopheles* mosquito. It is endemic in the tropical and subtropical regions of the world with 94% of the disease burden found in Africa. Zambia contributes to 2% of the global burden of Malaria and is among the top 20 countries with the highest malaria disease burden in the world. The use of insecticide-based vector control has shown great success at controlling malaria from the 1950s to date and this has relied on a clear understanding of mosquito ecology and behaviour of the local malaria vectors.

Insecticide-based vector control is the main malaria elimination strategy identified to achieve a malaria free Zambia. However, the extensive use of insecticide-based vector control results into an increase in the population of resistant malaria vectors and behavior modification for mosquito survival. Therefore, this study will assess the malaria vector bionomics and phenotypic resistance to insecticides used in vector control in Ndola.

This will be a cross sectional study conducted in Ndola district and mosquitoes will be collected using CDC light traps, Pyrethrum Spray Catches, Aspirations and larval collection. Multiplex PCR will be used for determination of sibling species of the *An gambiae* and *An funestus* complex and for determination of blood meal sources. Data analysis will be done in Microsoft excel and stata version 14. A zero-inflated negative binomial regression model will used to analyze the mosquito count against the independent variables.

Contents

1.0 Introduction

1.1 Background

Malaria is one of the diseases of public health importance in the tropical regions. In 2019, the burden was estimated at 229 million malaria cases and 409, 000 deaths. The highest malaria disease burden (94%)of these cases and deaths) was from Africa and Zambia contributed 2% of this global malaria disease burden (WHO, 2020). This disease is caused by a protozoan parasite of the genus *Plasmodium* and is transmitted to humans through the bite of an infected *Anopheles* mosquitoes. There are about 40 species from the *Anopheles* genera known to transmit malaria (WHO, 2019). In Zambia the two main malaria vectors are *An funestus s.l* and *An gambiae s.l* (Das *et al.*, 2016). However, other *Anopheles* mosquitoes *An coustani* and *An squamosus* have shown to be more anthropophilic but tested negative for circumsporozoites (Fornadel *et al.*, 2011).

Vector control has played a significant role in reducing the global malaria disease burden (Wilson *et al.*, 2020). An estimated 1.5 billion malaria cases and 7.6 million malaria related deaths have been averted since 2000. Africa has recorded a reduction in the number of malaria cases and malaria deaths from 362.8 to 225.2 per 1000 and 121.1 to 40.3 per 100, 000 respectively (WHO, 2020). This has to a greater extent been attributed to concerted efforts in vector control (Wilson *et al.*, 2020).

To achieve a malaria free Zambia, the National Malaria Elimination Centre (NMEC) has adopted the use of Indoor Residual Spraying (IRS) and use of long-lasting insecticide treated nets (LLINs) as the main vector control strategies (Chipoya and Shimaponda-Mataa, 2020; NMEC, 2017). These interventions are aimed at reducing the human to vector interactions by creating a barrier and killing host seeking mosquitoes that rest on a sprayed wall surface or on LLINs. By doing this malaria infected host seeking mosquitoes die without making contact with the human host, thus

reducing the population of malaria vectors infected with the parasite as well as the humans getting infected.

The successful implementation of vector control strategies is hinged on a clear understanding of the ecology and behavior of malaria vectors in different geographical locations (Wilson *et al.*, 2020). Once their ecology and behaviour are known, the vector control strategies employed target one or more vulnerabilities in the behavior of the vector. Further, mosquito behaviours have been shown to change when exposed to elements that threaten their survival (Sougoufara *et al*., 2020). Therefore, routine vector surveillance studies should be conducted to ensure that malaria vector behaviours and their ecology are well understood prior to deployment of any vector control strategy. It is against this background that this study is proposed, to generate knowledge on the behavior and phenotypic resistance of malaria vectors in Zambia.

1.2 Statement of the Problem

Zambia is among the top 20 countries with a high malaria disease burden contributing 2% of the global malaria burden (WHO, 2020). These malaria cases are mainly in the provinces closer to the Democratic Republic of Congo (DRC) and in the northern parts of the country (MOH, 2019).

The government has identified vector control as the main malaria elimination strategy. This strategy heavily relies on spraying at least 80% of eligible structures through IRS and universal coverage of LLINs (NMEC, 2017). However, the extensive use of insecticide-based vector control leads to mosquito behavior modification and increase in the selection of resistant malaria vectors (WHO, 2019). Therefore, vector surveillance should be conducted to monitor the malaria vector behaviour and selected resistant malaria vectors. However, the last entomological activity conducted in Ndola was 8 years ago to determine the susceptibility status of malaria vectors and it was found that *An gambiae s.l* was resistant to carbamates (Thomsen *et al.*, 2014). As it stands,

there is no data on mosquito species composition, their behavior and insecticide resistance status of the malaria vectors. Therefore, it is uncertain whether the vector control interventions implemented target the malaria vectors and their current behavior.

1.3 Rationale of the Study

The IRS and LLINs strategy adopted for malaria control should be guided by data generated on the current malaria vector behaviour and their susceptibility status to insecticides used in vector control. Therefore, the proposed survey will identify the mosquitoes responsible for malaria transmission in Ndola to subspecies level, assess their current behavior and determine their phenotypic resistance status to the different insecticides used in malaria control.

The results generated will be useful in ensuring that vector control planning is based on updated information on the species, behavior and susceptibility status of local malaria vectors to insecticides used in malaria control. Further, the study findings will serve as baseline entomological survey to guide the possible resumption of regular surveys.

2.0 Research Questions

1. What is the species composition and density of malaria vectors in Ndola?
2. What are the biting and resting behaviours of mosquitoes in Ndola District?
3. What are the blood meal sources for mosquitoes in Ndola?
4. What is the insecticide resistance status of primary malaria vectors?

3.0 Objectives

3.1 General Objective

The main objective is to evaluate malaria vector behavior and their phenotypic resistance status to insecticides used in vector control in Ndola.

3.2 Specific Objectives

1. To determine species composition and vector density of malaria vectors in Ndola
2. To assess the biting and resting behaviour of malaria vectors in Ndola District
3. To assess host preference of blood feeding mosquitoes in Ndola
4. To evaluate the insecticide resistance status of primary malaria vectors in Ndola.

4.0 Literature Review

4.1 Global Malaria Situation

Diseases transmitted by vectors account for 17% of all infectious diseases in the world (Eder *et al.*, 2018). About 700,000 deaths occur annually from pathogens transmitted by different vectors including mosquitoes, ticks, triatome bugs, snails, fleas, sandflies, tsetse flies, lice and black flies (Benelli *et al.*, 2020). Mosquitoes alone are responsible for 8 infections affecting man and these are chikungunya, dengue, lymphatic filariasis, rift valley fever, yellow fever, malaria, Japanese encephalitis and west nile fever. In 2019, 229 million cases of malaria were recorded and there were 385,000 deaths as a result of malaria infections (WHO, 2020). The WHO African region recorded the highest malaria cases of 215 million cases accounting for 94% of the global malaria diseases burden. The majority of these cases where only in 29 countries with Nigeria (27%) and the Democratic Republic of congo (12%) having the most malaria cases in the world. The remaining WHO regions South East Asia, Eastern Mediterranean, Western Pacific and Region of the Americans contributed to 6% of the global malaria disease burden.

The implementation of malaria control interventions has seen a significant reduction in the malaria incidence from 80 per 1000 in 2000 to 58 per 1000 in 2019 (WHO, 2020). This has been to a greater extent been attributed to vector control and the World Health Organization continue to put great emphasis on the need for improving these tool in order to effectively achieve malaria control (Wilson *et al.*, 2020).

Zambia has also recorded a reduction in the malaria prevalence in under five children from 15% in 2015 to 9% in 2018. This reduction could be attributed to the increase in the number of household that have received a treated bed net and/or IRS. However, Luapula province recorded the highest malaria prevalence of 30%. Copperbelt on the other hand recorded a reduction in the

prevalence from 15.2% in 2015 to 7.7% in 2018 despite having the lowest bed net ownership rate of 55.3% (MOH, 2015)(MOH, 2019).

Further, a study showed that in the first 10 months of 2020, Zambia recorded an estimated 3.7 million cases of confirmed malaria cases. This rise in the number of confirmed malaria cases could be associated to the effects that COVID-19 had on efforts aimed at curbing malaria (Chisaya *et al.*, 2020).

4.2 Introduction to Mosquitoes

Mosquitoes taxonomically belong to the class insecta, order diptera, family Culicidae. These belong to five mosquito genera namely *Anopheles*, *Culex*, *Aedes*, *Mansonia* and *Conquillettidia* (Service, 1996; WHO, 2014). There about 3500 different species of mosquitoes worldwide, 400 of these belong to the *Anopheles* genera and only 40 of them have been implicated as malaria vectors (WHO, 2019). Mosquitoes require blood for the maturation of their eggs and its this host seeking behaviour that makes them ideal vectors of disease (O'Donnell *et al.*, 2019; Barry, 1996).

4.2.1 Life cycle of *Anopheles* Mosquito

The mosquito undergoes four distinct stages in their development; the egg, larvae, pupa and the adult stage. The first three stages of the life cycle of the mosquito are aquatic and the adult stage is the only terrestrial (Durden and Mullen, 2018). The time taken for the mosquito to metamorphose from one development stage to the next is dependent on a number of factors but most importantly is temperature, humidity and diet (Service, 2012).

a) **Egg Stage**: *Anopheles* mosquitoes lay an estimated 200 eggs in one oviposition in water. These eggs are laid singly and have floaters (Service, 2012). These eggs hatch in 2 – 3 at

optimum environmental conditions (27 °C ± 2, 80% ± 10 relative humidity (RH)) but can take as long as 14 days in cold temperatures (Mazigo *et al.*, 2019).

b) Larval Stage: The eggs hatch into larvae and the larvae will pass through 4 larval instars in this stage. This is the feeding stage of the immature stage of the mosquito and lasts for a period of 8 to 10 days at optimum environmental conditions. The larvae lay horizontal to the water surface and they breath through the spiracles along the body of the larvae. From this stage the larval will undergo metamorphosis and develop into pupa (Durden and Mullen, 2018).

c) Pupa Stage: This is the non-feeding stage in the life cycle of the mosquito and exist as a *cephalothorax.* The pupa breath through the respiratory trumpets on the apical end of the cephalothorax. The pupa speeds most of its time on the water surface taking in air and if disturbed they swim in a jerk fashion (up and down). This stage also takes 2 to 3 days at optimum environmental conditions (Service, 2012).

d) Adult Stage: The pupae then emerge into adult mosquitoes normally at dusk and then the females fly into a swam of males for mating. Once the female mosquito mate, they lay eggs throughout their lifespan. They have several blood meals depending on the length of their gonotrophic cycle (Service, 2012).

4.3 Malaria Vectors

4.3.1 Global Malaria Vectors

The distribution of malaria vectors in the world differ from continent to continent. In the Americans region *An darlingi* is the most efficient malaria vector of *P. falciparum* and *P. vivax.*. It is a highly anthropophilic malaria vector and its natural habitats are the shady areas of streams and ponds. This mosquito can breed in both clear and muddy water with floating vegetation. Other

malaria vectors in this region are *An nuneztovaris s.l, An albitarsis* found in South America. (Alimi *et al.*, 2015). In Asia, the most efficient malaria vector is *An stephensi* found to thrive most in urban areas (Alimi *et al.*, 2015). The breeding sites for this mosquito include man made water habitats such as wells, water receptacles, overhead tanks, fountains, barrels and tins and polluted water in drainages (Thomas *et al.*, 2016). Other malaria vectors include *An culicifacies s.l, An darius s.l* and *An maculatus s.l* (Sinka *et al.*, 2012).

4.3.2 Malaria Vectors in Africa

In Africa *An gambiae* and *An funestus* complex are the main malaria vectors of malaria (Mzilahowa *et al.*, 2012). *An gambiae s.l* prefers to breed in small temporal water collections such as gardens, hoof prints, tire marks and a dirty road (Ndiaye *et al.*, 2020). *Anopheles funestus s.l* on the other hand breeds in semi-permanent water bodies such as dams created from road construction, swamps, marshes and in the edges of a stream where the water is not moving (Fillinger *et al.*, 2009) (Mattah *et al.*, 2017).

There is now a threat of *An stephensi* a primary malaria vector in Asia (Balkew *et al.*, 2020). This new malaria vector poses a threat of increased urban malaria in Africa as it thrives in urban areas (Sinka *et al.*, 2020). In African countries *An. funestus* complex has shown to be abundant throughout the year and *An. gambiae* complex has shown to be most abundant in the wet season (Mzilahowa *et al.*, 2012). These two *Anopheles* complexes have been well studied and understood and this led to discovery of sibling species within these complexes. The *An. funestus* complex has 9 sibling species in Africa and these include *An funestus s.s, An rivolurum, An leesoni, An vaneedeni, An aruni, An fuscivenous, An parensis* and *An brucei*. Studies further show that *An funestus s.s* is the predominant and widely distributed malaria vector in Africa. *An leesoni, An*

revolurum, An parensis and *An vaneedeni* are the other sibling species of the *An funestus* complex in Africa (Kweka, et al, 2013).

On the other hand, there has been a reduction in the malaria vector densities and species composition of the *An gambiae* complex due to vector control interventions (Derua *et al.*, 2015). *An gambiae s.s, An arabiensis* and *An coluzzi* are the other malaria vectors in the *An gambiae* complex transmitting *Plasmodium falciparum* and *Plasmodium vivax* (Wiebe *et al.*, 2017). Other sibling species in the *An gambiae* complex are *An quadrianulatus A, An. amharicus, An merus, An melas* and *An bwambae* (Ebenezer *et al.*, 2014). *An gambiae s.s, An arabiensis, An coluzzi, An quadrianulatus A, An quadrianulatus B* are fresh water malaria vectors. *An melas* and *An merus* are salt water malaria vectors and *An bwambae* is only found in Uganda (Bartilol *et al.*, 2021).

The number of secondary malaria vectors in Africa vary with geographical locations and very little is known about these vectors (Mwangangi *et al.*, 2013b). Studies have shown that due to the lack of a proper understanding of their ecology and behavior they have contributed greatly to residual transmission in areas with low malaria transmission (Ayuya *et al* 2021). The secondary malaria vectors of public health importance in Africa include *An coustani, An Ziemani, An pharoensis, An maculipalpis* (Afrane *et al.*, 2016).

4.3.3 Malaria vectors in Zambia

An. funestus and *An. gambiae* complexes are the primary malaria vectors in Zambia (Cross *et al.*, 2021). *An. funestus s.l* is the predominant malaria vector in 8 of the 10 provinces in Zambia. The main malaria vector on the Copperbelt province is *An gambiae s.s* and *An arabiensis* in the Southern Province (NMEC, 2019). The provinces were *An funestus s.l* is the predominant malaria vector are the same provinces with the highest malaria prevalence. Other malaria vectors

potentially identified as secondary vectors have been identified through their highly anthropophilic and endophagic behaviours. *Anopheles leesoni* a zoophilic mosquito mainly found in Luapula province assumes the role of a malaria vector when its preferred blood meal source is not available (Das *et al.*, 2016). Other anopheles mosquitoes that are mainly zoophilic and exhibits a high exophagic behaviour are *An squamosus, An rufipes, An coustani, An pretoriensis* and *An quadrianuratus* (Stevenson *et al.*, 2016) (Lobo *et al.*, 2015). Further, studies have shown that other than being more zoophilic and exophagic *An arabiensis, An coustani* and *An pretoriensis* are early outdoor bitters (NMEC, 2019) (Fornadel *et al.*, 2011).

4.4 Malaria Vector Bionomics

Vector bionomics refers to the ecology of malaria vectors and their resting, biting and host preference (Massey *et al.*, 2016). Mosquitoes have been known to modify their behavior when exposed to elements that do not favour their proliferation (Sougoufara *et al*., 2020). This to a larger extent has been attributed to vector control interventions that are implemented to control mosquito borne diseases (Thomsen *et al.*, 2017). The most efficient malaria vectors in the sub-Saharan Africa have been found to be highly anthropophilic. They feed indoors and rest indoors after having their blood meals (Sougoufara, *et al*., 2020). However, in Tanzania, *An arabiensis* a zoophilic mosquito was found to prefer biting outdoors and resting outdoors (Limwagu *et al.*, 2019). The deployment of LLITNs in Senegal was associated with a change in biting time from biting in the night to biting in broad day time. Further, there was no indication of the change in their behaviour from being highly anthropophilic and endophilic (Sougoufara *et al.*, 2014). Another study in Tanzania revealed a change in biting behaviour within *An gambiae* complex from 1997 to 2009. The night biting activity of the *An. gambiae* complex was high in 1997 but in 2009 there was a shift to only *An gambiae s.s* and *An arabiensis* being the only malaria vectors

predominantly biting late in the night (Russell *et al.*, 2011). Another study in Benin revealed that there was an increase in mosquito biting in broad day light after the introduction of LLITNs (Moiroux *et al.*, 2012).

A study in the Pan American Health Organization region found that land use change, climate change, deforestation, loss of forest cover has affected distribution of malaria vectors causing a shift in behaviour thereby increasing human to vector contact (Alimi *et al.*, 2015).

4.4.1 Species Composition

A study in Kenya showed that there was a change in the predominant malaria vector from *An funestus s.s* and *An gambiae s.s* to *An arabiensis* and *An merus* over a period of 20 years. Further, a notable shift in the feeding behaviour from humans to animals of *An gambiae s.l* (99% to 16%) and *An funestus s.l* (100% to 3%). This could be attributed to the shift in the species composition recorded from *An gambiae s.s* as the predominant malaria vector to *An arabiensis* (Mwangangi *et al.*, 2013a). Further, another study in Uganda showed a change in the species composition of malaria vector after the implementation of IRS and LLITNs. Before the interventions the predominant malaria vector was *An. gambiae s.s* (76.7%) but after the intervention, 99.5% of the mosquitoes collected were *An arabiensis* and *An gambiae s.s* was 0.5% (Musiime *et al.*, 2019).

4.4.2 Malaria Vector Density

A study in Uganda revealed that the distribution of LLITNs was associated with a reduction in the *An funestus s.l* vector density from 0.07 per house per night to 0.02 per house per night but not for *An gambiae s.l*. This revealed that *An funestus s.l* was more affected as compared to *An gambiae s.l* (Mawejje *et al.*, 2021). The introduction of vector control interventions in Uganda led to the reduction in the vector density of *An. gambiae s.l* from 76.7% before the intervention to 0.5% post intervention (Musiime *et al.*, 2019). A study in Zambia revealed that there was a reduction in

vector density of *An quariannulatus* from 95.1% to 69.7% following implementation of IRS. However, there was a proportionate increase in number of *An. arabiensis* collected from 3.9% to 95.1% from the total mosquitoes collected in the *An gambiae* complex (Chinula *et al.*, 2018).

4.4.3 Resting Behaviour

Following the deployment of LLITNS in Western Kenya, a study revealed that there was a higher indoor resting density for *An. gambiae s.l* and *An. funestus s.l*. The introduction of LLITNs did not have an effect on the indoor resting behaviour of malaria vectors (Machani *et al.*, 2020). The results from this study indicate that there is need to implement an intervention that will also target indoor resting malaria vectors. A study in Western Kenya were a large proportion of the malaria vectors rest indoors revealed a reduction in the indoor resting density post IRS implementation (Abong'o *et al.*, 2020).

4.4.4 Biting Behaviour

Use of LLINs is the most appropriate vector control tool to deploy where mosquitoes predominantly endophilic. However, a study conducted in Senegal revealed that the deployment of vector control had no effect on the endophilic behaviour of the malaria vectors (Sougoufara *et al.*, 2014). Further, a study revealed that 60% of *An. Arabiensis* were able to successfully have their blood meals and an estimated 50% of these blood meals were taken from outside the house (Killeen *et al.*, 2016).

4.4.5 Host preference

Malaria vectors have their preferred hosts for their blood meals and they will only feed on another host in the event that their preferred host is unavailable. *An. arabiensis* a highly zoophilic mosquito prefers to feed on cattle but in the event that there is no cattle nearby they will feed on humans (Killeen *et al.*, 2016). Another study in conducted in Kenya were human and domestic animals

were sharing the house revealed that mosquitoes fed on any available blood meal source host human, goat or bovine. 53.1% of the *An gambiae s.s* fed on humans, 26.5% fed on goats and 18.4% fed on bovine (Ndenga *et al.*, 2016). In Honduras it was found that most of *Anopheles* mosquitoes collected had multiple blood meals and that only 24.9% had fed on a single host. The Anopheles mosquitoes preferred to feed on either chickens or bovine. The Human blood index in this study was found to be 22.1% (Escobar *et al.*, 2020).

4.5 Insecticide-Based Vector Control

Vector Control is the main method of controlling malaria and it has played a significant role in the reduction of vector-borne diseases from the 1800s. This was achieved through a clear understanding of the behaviour and ecology of the different vectors of disease. However, after the 1940s to date, malaria vector control has significantly depended on insecticide-based interventions and this has brought in the challenge of insecticide resistance (Wilson *et al.*, 2020). Historically, organophosphates, organochlorines, carbamates and pyrethroids were the four classes of insecticides approved by WHO for use in public health vector control (Corrêa *et al.*, 2019). Pyrrole (Chlorfenapyr 240 SC) and Neonicotinoids (Chlothianidin WG) have been prequalified by WHO for use in public health vector control (WHO, 2013). In Africa, the main vector control strategies employed target the adults mosquitoes and the immature stages to a lesser extent.

4.5.1 Control of Adult Mosquitoes

a) **Use of treated bed nets:** Pyrethroids and pyrroles are the only two classes of insecticides approved by WHO for use in LLINs. These classes of insecticides have shown to pose very low risk to humans yet providing the desired lethal effect to arthropods of public health importance (CDC, 2019). The use of bed nets has shown to significantly reduce malaria prevalence in areas where the malaria vectors predominantly bite indoors and late at night

(Ntonifor and Veyufambom, 2016; Steinhardt *et al.*, 2017). The treated bed net creates a barrier between the vector and the human host and produces a toxic effect to the malaria vectors that rests on the bed net (Paaijmans and Huijben, 2020).

b) Indoor Residual Spraying: Indoor Residual Spraying involves the application of a predetermined amount of insecticide with a residual effect on a wall surface (Tangena *et al.*, 2020). In this intervention organophosphates, organochlorines, carbamates, pyrethroids, pyrroles and neonicotinoid have been prequalified for use in IRS (WHO, 2013). This intervention targets host seeking mosquitoes that rest indoors after a bloodmeal. Once the mosquito rests on a sprayed wall surface, the mosquito absorbs the insecticide through its legs and the mosquito dies (Phiri *et al.*, 2015). This intervention protects the next person from getting an infectious bite by killing the infected mosquito (Pinchoff *et al.*, 2016).

c) **Use of symbionts:** The use of symbionts for the control of mosquito borne diseases still remain largely unexplored (Ricci *et al.*, 2012). Endosymbionts have been shown to interrupt transmission in their natural or engineered form. In Anopheles a Wolbachia strain (wAnga-Mali) significantly reduces the prevalence and intensity of sporozoites on field collected mosquitoes (Gomes *et al.*, 2017). Another bacteria in its engineered form *Pantoea agglomerans* successfully inhibits the development of plasmodium falciparum by 98% in *Anopheles gambiae s.l.*(Wang *et al.*, 2012) . In a more recent study, *Microsporidia MB* naturally occurring in *An arabiensis* was found to completely interrupt transmission of the malaria parasite (Herren *et al.*, 2020).

4.5.2 Control of immature stages of the malaria vectors

a) **Use of predators:** The use of predator fish in malaria control has not shown any statistical significant result on the malaria infection rates, entomological inoculation rate or the adult vector density in the areas where they have been studied (Walshe *et al.*, 2017). However, a study in western Kenya revealed that biocontrol was able to reduce the density of larvae and pupae (Howard *et al.*, 2007).

b) **Pathogens and parasites:** This is a widely practiced larval source management intervention targeting the larval stages of the mosquito (Service, 2012). *Bacillus thuringiensis var. israelensis (Bti)* and *Bacillus spharicus* have been found to be effective at controlling larvae and was well received and accepted in communities. These pathogen has been found to be ecofriendly and ease to produce in large numbers (Derua *et al.*, 2019). This intervention has been shown to be effective especially with the rise of insecticide resistance to pyrethroids (Zhou *et al.*, 2020).

c) **Use of Oils and Surface films:** This is an ancient mosquito larvae control that was mainly practiced in the Americas and India. It involves the application of a petroleum oil or isotearyl alcohol on the surface of the water so to kill the larvae through suffocation or toxic effect of the oil (Service, 2012). The use of monomolecular films is a more recent larvicidal strategy which works by forming an ultrathin film on the surface of mosquito breeding areas thus suffocating the larvae to death (Antonio-Nkondjio *et al.*, 2018).

4.6 Vector Control in Zambia

In Zambia, Indoor Residual Spraying and use of LLINs are the main malaria elimination strategies identified to achieve a malaria free Zambia (NMEC, 2017). These two vector control interventions have received massive funding from both the Government and Cooperating partners (PMI, 2019).

However, larval source management is mainly implemented by Local authorities and mining companies.

In the quest to have a malaria free Zambia, there has been wide spread scaling up of these insecticide-based vector control (LLINs and IRS) to all the Districts in the country. Some districts have received both treated bed nets and IRS whereas others have only received one of the two interventions (PMI, 2019). However, these interventions have the ability to increase selection pressure on the population of mosquitoes that have resistant alleles (Nkya *et al.*, 2013).

4.7 Insecticide Resistance

The ability mosquitoes have to withstand toxic effects of an insecticide through natural selection or mutation is referred to as insecticide resistance. Usually this arises from repeated exposure of mosquitoes to insecticides or selection of individual mosquitoes that are able to detoxify the insecticide takes place. The mosquitoes selected survive and pass their ability to detoxify insecticides to their progeny (Riveron *et al.*, 2018). The widespread in insecticide resistance world over has been attributed to the extensive use of insecticides in agriculture and public health (Mouhamadou *et al.*, 2019).

Insecticide resistance has been reported against the four different classes of insecticides used for public health vector control and these are pyrethroids, organophosphates, organochlorines and carbamates (Mint Mohamed Lemine *et al.*, 2018)(Fang *et al.*, 2019)(Ondeto *et al.*, 2017).

Insecticide resistance is widespread in Zambia and has been recorded against pyrethroids, organochlorines and carbamates. Organophosphates and neonicotinoids are the only two classes of insecticides used in public health where local malaria vectors are still susceptible to (NMEC, 2019).

4.7.1 Mechanism of resistance

Routine monitoring of the susceptibility status of malaria vectors is cardinal for the successful implementation vector control (WHO, 2018). Area earmarked for insecticide-based vector control should be preceded by resistance surveillance to help determine most appropriate insecticide for use in vector control based on the susceptibility status of local malaria vectors. This monitoring of malaria vector susceptibility should be conducted once every year and in the event that there are several malaria vectors in the area with seasonal variations, monitoring at the beginning and end of the control effort should be done (McAllister and Scott, 2020).

There are basically four mechanisms of resistance and reports in some African countries indicate that the frequency of the presence of multiple mechanism of resistance is on the rise (Kwiatkowska *et al.*, 2013).

The figure below illustrates the different mechanisms of resistance (1) Reduced Penetration- Changes to the cuticle of mosquito exoskeleton prevents absorption of the insecticide. (2) Target-site- When the target-site for the insecticide is modified, the insecticide will no longer bind. (3) Metabolic- enzymes breakdown the insecticide before they can have a toxic effect on the mosquito

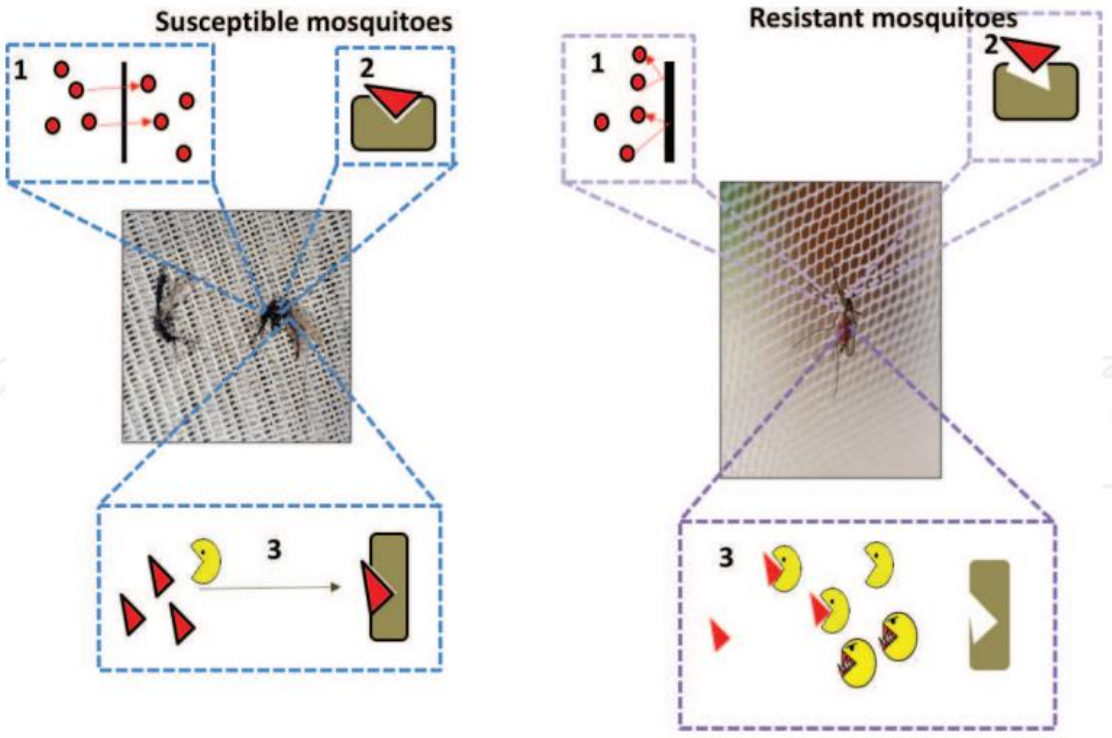

Figure 1: Summary of the 3 Main Mechanism of resistance

Source: (Riveron *et al.*, 2018)

Reduced Penetration: This type of resistance occurs when the cuticular structure composition is altered to reduce the amount of insecticide that is absorbed into the mosquito. Studies have shown that resistant mosquitoes have a thicker cuticle than susceptible mosquitoes (Riveron *et al.*, 2018). A study conducted in west Africa associated cuticle thickening to pyrethroid resistance (Yahouédo *et al.*, 2017). Additionally, the legs of resistant mosquitoes are sealed with large amounts of cuticular hydrocarbons compared to the susceptible mosquitoes (Balabanidou *et al.*, 2019). This mechanism of resistance is also one of the least studied yet it is possess a serious threat to vector control (Huang *et al.*, 2018).

Target-Site: In this mechanism of resistance, the target-site in the mosquito were the insecticide binds is modified and the insecticide can no longer bind. Insecticide molecules that enter the body

of the mosquito fail to bind to the target-site because it has been altered. This is the most common mechanism of resistance in Africa and it has contributed greatly to the malaria disease burden (Yewhalaw and Kweka, 2015).

Metabolic Resistance: This type of resistance arises when the body enzymes digest the insecticide molecules before attaching to the target-site where it will produce the desired toxic effect. This is one of the most common type of insecticide resistance report in the sub-Saharan Africa (Riveron *et al.*, 2018). This mechanism of resistance is widespread across most African countries and has been reported in at least one of the four classes of insecticides used in public health vector control (Diouf *et al.*, 2020)

Behavioural Resistance: This type of resistance if not monitored and has the potential to negatively impact the vector control strategies employed (Sokhna *et al*., 2013). Mosquitoes in this type of resistance tend to avoid surfaces that contain insecticides including sprayed wall surfaces and treated bed net surfaces (Gatton *et al.*, 2013). Other studies conducted have shown that the use of insecticidal nets has led to the change in feeding behaviours of mosquitoes with a rise in the biting times at dusk and dawn (Killeen and Chitnis, 2014).

4.7.2 Insecticide Resistance Management

To slow down development of resistance, the NMEP has provided guidelines to rotate IRS products and implement a mosaic approach. This approach is based on the data generated from mosquito susceptibility assays conducted on mosquitoes from different locations benefitting from IRS (PMI, 2019). However, the NMEP has a limited number of sites were susceptibility data is collected from and these are mostly sites that are supported by partners including PMI sites, MACEPA sites and Global Fund coordinated by NMEP. On the Copperbelt there are only two active sites operated by PMI which are used as entomological surveillance sites (PMI, 2020).

5.0 Materials and Methods

5.1 Study Design

This will be a cross sectional survey involving the collection of mosquitoes from two catchment areas (one urban and one rural) of Ndola District both with a malaria high burden.

5.2 Study Site

The study will be conducted in Ndola District (12.9726° S, 28.6265° E), the provincial capital of the Copperbelt Province. The district shares a boundary with the Democratic Republic of Congo on the eastern side.

Chipulukusu Urban Health Centre and Kaniki Rural Health Centre are the two catchment areas that will participate in this study. According to the Health Management Information System (HMIS) data for 2020, there were 19618 malaria confirmed cases in Chipulukusu and 10,679 malaria confirmed cases in Kaniki catchment area (Ndola, 2020). Chipulukusu Urban Health Centre serves a low cost area with an estimated population of 49,730 people (CSO, 2020).

Chipulukusu is one of the catchment areas found at the centre of the District. The housing structure comprise of cement block, burnt brick and mud brick walled houses with both iron and grass roofing. Most families in Chipulukusu access kitchen and toilet facilities in standalone structures apart from the main house used mostly for sleeping (CSO, 2020).

On the other hand, Kaniki Rural Health Centre shares a border post with the Democratic Republic of Congo at Sakanya border post. The majority of the housing structure in the area are mud house roofed with grass or iron sheets. The catchment has an estimated population of 11, 716 people. The sleeping house, Kitchen and toilet facilities are stand-alone structures (CSO, 2020).

5.3 Study Population

The study population will include all the housing structures occupied by people in the selected catchment areas of Chipulukusu and Kaniki. The estimated number of household in Chipulukusu is 16000 and the total number of household in Kaniki is 4412.

5.3.1 Inclusion Criteria

The houses where mosquitoes will be sampled include those houses where people sleep in and there is an adult (16 years and above)

5.3.2 Exclusion Criteria

The houses in which occupants will not consent to participate and / or cook from inside the house using firewood will be excluded from this study.

5.4 Sample Size Determination

Total Collection efforts 225 (CDC LT 56 houses, PSC 30 houses, Aspirations 80 houses and 60 Larvae collection efforts)

Sampling for this study is based on vector sampling guidelines by WHO (WHO, 2019). However, the sample size has been determined based on the minimum number of structures per square meters and the predominant malaria vector. The minimum houses to be sampled for areas where *An gambiae s.l* is the predominant vector is 17 houses per square km and 42 houses per square km where *An funestus s.l* is the predominant vector. (Zhou *et al.*, 2004). A study further revealed that sampling 120 houses (120 collection efforts) gives precision equivalent to 200 houses for mosquito surveillance (Sedda et al, 2019)

For vector susceptibility assays, a minimum of 100 adult female mosquitoes of a given species per insecticide is required. These mosquitoes will be tested in 4 replicates of 25 – 30 mosquitoes per WHO tube or CDC bottle (WHO, 2018).

5.6 Sampling Method

Purposive sampling will be used to select the two catchment areas that will participate in the study. Houses where adult mosquitoes will be collected will be randomly selected from each section maintaining at least 200 m between two participating houses. The collection tools that will be used for mosquito collection are;

5.6.1 CDC light traps

The traps will be set in randomly selected houses and mosquito collection will start at 18:00 hours and end at 06:00 hours the following morning. The traps will be set at a height of 1.5m from the ground next to a sleeping space. After every collection the mosquito collection cups will be properly labelled and transported to the laboratory for processing. At the laboratory, the mosquito collection cups will be put in a freezer at -4°C for 1 hour to kill mosquitoes that would still be alive.

5.5.2 Pyrethrum Spray Collections (PSC)

Adult mosquitoes resting indoors will be collected indoors using PSC from 05: 00 hours to 07: 00 hours in 20 houses per catchment area. The Collector will enter the house spread a 3m X 2m white linen over the floor, bed and furniture. The collector outside the house starts by spraying the eaves and other openings and once he is done the collector inside (wearing a nose mask) will saturate the house with insecticide to knockdown the mosquitoes. After the 10 minutes the collector will carefully fold the sheets towards the centre and take the sheets outside. The collector will pick the mosquitoes and place them in properly labelled petri dishes.

5.5.3 Aspirations

The live adult mosquitoes will be collected from 05:00 hours to 08:00 hours in houses where people sleep using mouth aspirator and prokopack. The live adult mosquitoes will then be put in bugdom cages and fed with a 10% sugar solution and transported to the laboratory for processing.

5.5.4 Larval collection

The larvae will be collected from breeding areas around houses where people live using dippers for larger breeding sites and Pasteur pipette for small breeding sites. The larvae will then be carefully transported to the laboratory where they will be reared into adult mosquitoes in a controlled microenvironment.

5.6 Variables

Variable type	Variable	Indicator
Outcome Variable	Mosquito Bionomics	Species Composition
		Malaria Vector Density
		Indoor Resting Density
		Man Biting Rate
Independent	Type of Wall	Mud Cement
	Roof type	Thatch Iron sheets
	Type of eaves	Open Closed
	Human Host	No. of People
	Animal Host	No. of animals
	Spray status	Sprayed Not Sprayed

	LLINs	No. of LLINs

5.7 Experimental Procedures

1. **To determine species composition and vector density of malaria vectors in Ndola:** Mosquitoes collected from houses using CDC Light traps and aspiration will be quantified by calculating total catches per household. All mosquitoes collected will be morphologically identified using a standard mosquito identification key for Afrotropical *Anopheles* mosquitoes (Coetzee, 2020). Confirmation of mosquito identification and determination of the subling species from the *An gambiae s.l* and *An funestus s.l* complexes will be done using multiplex PCR (Scott *et al.*, 1993) (Koekemoer *et al.*, 2002). DNA extraction will be done using the xygem DNA extraction kit. The PCR master mix prepared will include specific primers for sibling species. The PCR mix will then be run in the thermocycler and after amplification, the amplicons will be run on a gel.

   Malaria vector density: This is a very important indicator used in vector surveillance to assess the behaviour of malaria vectors or to assess the effectiveness of a vector control intervention. It is a mean of the malaria vectors collected from a define number of houses surveyed. It is calculated by dividing the total number of mosquitoes collected by the total number of houses surveyed.

   $$\textbf{Malaria vector density} = \frac{Number\ of\ malaria\ vectors\ collected}{Total\ number\ of\ houses\ surveyed}$$

2. **To assess the biting and resting behaviour of mosquitoes in Ndola**

The Man Biting Rate helps to estimate the potential risk of exposure to infectious bites. The formular for calculating the human biting rate or man biting rate is

$$\text{Man biting rate} = \frac{Number\ of\ mosquitoes\ collected\ using\ light\ trap}{\text{Total Number of sleepers}}\ X\ HBI$$

The Indoor Resting behaviour is indicative of the mosquitoes that rest indoors. This indicator is an important one when considering whether to implement IRS or not. This is calculated by dividing the total number of mosquitoes collected using PSC over the total number of houses surveyed.

3. **To assess host preference of blood feeding mosquitoes:** Blood fed female anopheles mosquitoes collected will be analysed using multiplex PCR for the determination of blood meal sources (Kent and Norris, 2010). Extraction of DNA will be done using a xygen DNA extraction kit and the samples will be stored at -20°C prior to running the PCR. After PCR the amplicons will be run on a gel for determination of blood meal source. The Human Blood index will be calculated using excel using the formula;

$$\text{Human Blood Index} = \frac{No.of\ female\ mosquitoes\ with\ human\ blood}{Total\ number\ of\ mosquitoes\ analysed}$$ (Escobar *et al.*, 2020). See appendi ????? for primers that will be used in multiplex PCR for determination of bloodmeal sources.

4. **To determine the insecticide resistance status of primary malaria vectors:** Live adult female anopheles mosquitoes will be exposed to various insecticides used in vector control using the using WHO Tube Bioassays and CDC bottle assays (WHO, 2018; Brogdon and Chan, 2010). The selected mosquitoes will be placed in WHO tubes lined with insecticide impregnated paper and left to sit for 60 minutes. Thereafter, knockdown will be observed

at 60 minutes, 12 hours and final mortality will be read at 24 hours post exposure. For the CDC bottle assay, mosquitoes will be put in whatton bottles coated with insecticides and knockdown will be read every 15 minutes for 2 hours and final mortality will be determined 24 hours post exposure.

5.8 Data Analysis

The data collected will entered in excel for determination of species composition, vector density, Indoor Resting Density, Human Blood Index and Man Biting Rate.

Analysis of variance will be used to determine any statistical difference in the malaria vector density, indoor resting behaviour and man biting rate between the two areas. Susceptibility status of the local malaria vectors will be determined using WHO guidelines provided (98% - 100% means susceptible; 90% - 97% means suspected resistance and less than 90% means confirmed resistance) that is if mortality in the controls is less than 5%. A corrected mortality using Abbotts formula will be computed where mortality in the controls will be between 5% and 20%. However, if the mortality in the controls will be greater than 20%, the findings will be discarded and the assay will be repeated.

6.0 Ethics Considerations

Ethics clearance to undertake this study will be obtained from the Tropical Diseases Research Centre Ethics Committee and National Health Research Authority. This study will assure protection of collectors and staff working during mosquito collection by ensuring that proper training is given to them and that they wear proper protective wear. It will also assure for

preservation of ecology by sampling without total depletion. Permission to collect mosquitoes from the District will be sought from Ndola District Health Office. Additionally, informed consent will be obtained from the head of the house before collecting mosquitoes and larvae from their houses and their gardens respectively. This study will involve invading participants personal space during mosquito collection and assurance will be given that their health, safety and privacy will be preserved.

7.0 Work Plan

Description of Activity	Apr-21				May-21				Jun-21				Jul-21				Aug-21			
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12	Week 13	Week 14	Week 15	Week 16	Week 17	Week 18	Week 19	Week 20
Proposal Writing	■	■																		
Proposal Presentation			■																	
Ethics Clearance				■	■	■														
Data Collection							■	■	■	■	■	■								
Data Analysis								■	■	■	■	■	■	■	■	■				
Report Writing										■	■	■	■	■	■	■	■	■	■	
Dissemination of Findings																				■

8.0 Activity Based Budget

Description of Activity	Amount
Communication and Internet connectivity	ZMW 2,000.00
Mosquito collection	ZMW 5,000.00
Molecular Analysis of Mosquitoes	ZMW 45,000.00
Data Analysis	ZMW 3,500.00
Ethics Clearance	ZMW 1,000.00
Publication	ZMW 2,500.00
Total Amount	ZMW 59,000.00

9.0 References

Abong'o, B., Gimnig, J. E., Torr, S. J., Longman, B., Omoke, D., Muchoki, M., *et al.* (2020) Impact of indoor residual spraying with pirimiphos-methyl (Actellic 300CS) on entomological indicators of transmission and malaria case burden in Migori County, western Kenya, *Scientific Reports*, 10 (1), pp. 1–14. DOI:10.1038/s41598-020-61350-2.

Afrane, Y. A., Bonizzoni, M. and Yan, G. (2016) Secondary Malaria Vectors of Sub-Saharan Africa: Threat to Malaria Elimination on the Continent?, *Current Topics in Malaria*, (November). DOI:10.5772/65359.

Alimi, T. O., Fuller, D. O., Qualls, W. A., Herrera, S. V., Arevalo-Herrera, M., Quinones, M. L., Lacerda, M. V. G. and Beier, J. C. (2015) Predicting potential ranges of primary malaria vectors and malaria in northern South America based on projected changes in climate, land cover and human population, *Parasites and Vectors*, 8 (1), pp. 1–16. DOI:10.1186/s13071-015-1033-9.

Antonio-Nkondjio, C., Sandjo, N. N., Awono-Ambene, P. and Wondji, C. S. (2018) Implementing a larviciding efficacy or effectiveness control intervention against malaria vectors: Key parameters for success, *Parasites and Vectors*, 11 (1), pp. 1–12. DOI:10.1186/s13071-018-2627-9.

Ayuya Stephen, Kitungulu Nicholas, Annette O. Busula, Mark Kilongosi Webale, E. O. (2021) Detection of Plasmodium sporozoites in Anopheles coustani s.l; a hindrance to malaria control strategies in highlands of western Kenya, *Biorix*, 33 (1976).

Balabanidou, V., Kefi, M., Aivaliotis, M., Koidou, V., Girotti, J. R., Mijailovsky, S. J., *et al.*

(2019) Mosquitoes cloak their legs to resist insecticides, *Proceedings of the Royal Society B: Biological Sciences*, 286 (1907). DOI:10.1098/rspb.2019.1091.

Balkew, M., Mumba, P., Dengela, D., Yohannes, G., Getachew, D., Yared, S., *et al.* (2020) Geographical distribution of Anopheles stephensi in eastern Ethiopia, *Parasites and Vectors*, 13 (1), pp. 1–8. DOI:10.1186/s13071-020-3904-y.

Bartilol, B., Omedo, I., Mbogo, C., Mwangangi, J. and Rono, M. K. (2021) Bionomics and ecology of Anopheles merus along the East and Southern Africa coast, *Parasites & Vectors*, pp. 1–11. DOI:10.1186/s13071-021-04582-z.

Benelli, G., Petrelli, R. and Canale, A. (2020) Arthropod-Borne Disease Control at a Glance: What's New on Drug Development?, *Molecules (Basel, Switzerland)*, 25 (21). DOI:10.3390/molecules25215175.

Brogdon, W. and Chan, A. (2010) Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay, pp. 1–28.

Chinula, D., Hamainza, B., Chizema, E., Kavishe, D. R., Sikaala, C. H. and Killeen, G. F. (2018) Proportional decline of Anopheles quadriannulatus and increased contribution of An. arabiensis to the An. gambiae complex following introduction of indoor residual spraying with pirimiphos-methyl: An observational, retrospective secondary analysis of pre-, *Parasites and Vectors*, 11 (1), pp. 1–7. DOI:10.1186/s13071-018-3121-0.

Chipoya, M. N. and Shimaponda-Mataa, N. M. (2020) Prevalence, characteristics and risk factors of imported and local malaria cases in North-Western Province, Zambia: a cross-sectional study, *Malaria Journal*, 19 (1), pp. 1–12. DOI:10.1186/s12936-020-03504-1.

Chisaya, M., Phiri, M. and Ngomah, M. (2020) AN UPDATE ON MALARIA TRENDS IN ZAMBIA (2019 TO 2020); A DESCRIPTIVE STUDY, *Health Press Zambia*, pp. 13–18.

Coetzee, M. (2020) Key to the females of Afrotropical Anopheles mosquitoes (Diptera: Culicidae), *Malaria Journal*, 19 (1), pp. 1–20. DOI:10.1186/s12936-020-3144-9.

Corrêa, A. P. S. A., Galardo, A. K. R., Lima, L. A., Câmara, D. C. P., Müller, J. N., Barroso, J. F. S., *et al.* (2019) Efficacy of insecticides used in indoor residual spraying for malaria control: An experimental trial on various surfaces in a 'test house', *Malaria Journal*, 18 (1), pp. 1–14.

DOI:10.1186/s12936-019-2969-6.

Cross, D. E., Thomas, C., Mckeown, N., Siaziyu, V., Healey, A., Willis, T., *et al.* (2021) Geographically extensive larval surveys reveal an unexpected scarcity of primary vector mosquitoes in a region of persistent malaria transmission in western Zambia, *Parasites & Vectors*, pp. 1–14. DOI:10.1186/s13071-020-04540-1.

Das, S., Muleba, M., Stevenson, J. C. and Norris, D. E. (2016) Habitat partitioning of malaria vectors in nchelenge district, Zambia, *American Journal of Tropical Medicine and Hygiene*, 94 (6), pp. 1234–1244. DOI:10.4269/ajtmh.15-0735.

Derua, Y. A., Alifrangis, M., Magesa, S. M., Kisinza, W. N. and Simonsen, P. E. (2015) Sibling species of the Anopheles funestus group, and their infection with malaria and lymphatic filarial parasites, in archived and newly collected specimens from northeastern Tanzania, *Malaria Journal*, 14 (1), pp. 1–8. DOI:10.1186/s12936-015-0616-4.

Derua, Y. A., Kweka, E. J., Kisinza, W. N., Githeko, A. K. and Mosha, F. W. (2019) Bacterial larvicides used for malaria vector control in sub-Saharan Africa: Review of their effectiveness and operational feasibility, *Parasites and Vectors*, 12 (1), pp. 1–18. DOI:10.1186/s13071-019-3683-5.

Diouf, E. hadji, Niang, E. hadji A., Samb, B., Diagne, C. T., Diouf, M., Konaté, A., Dia, I., Faye, O. and Konaté, L. (2020) Multiple insecticide resistance target sites in adult field strains of An. gambiae (s.l.) from southeastern Senegal, *Parasites and Vectors*, 13 (1), pp. 1–10. DOI:10.1186/s13071-020-04437-z.

Durden, L. A. and Mullen, G. R. (2018) *Medical and Veterinary Entomology*, *Medical and Veterinary Entomology*. DOI:10.1016/B978-0-12-814043-7.00001-7.

Ebenezer, A., Noutcha, A. E. M., Agi, P. I., Okiwelu, S. N. and Commander, T. (2014) Spatial distribution of the sibling species of Anopheles gambiae sensu lato (Diptera: Culicidae) and malaria prevalence in Bayelsa State, Nigeria, *Parasites and Vectors*, 7 (1), pp. 1–6. DOI:10.1186/1756-3305-7-32.

Eder, M., Cortes, F., Teixeira de Siqueira Filha, N., Araújo de França, G. V., Degroote, S., Braga, C., Ridde, V. and Turchi Martelli, C. M. (2018) Scoping review on vector-borne diseases

in urban areas: Transmission dynamics, vectorial capacity and co-infection, *Infectious Diseases of Poverty*, 7 (1), pp. 1–24. DOI:10.1186/s40249-018-0475-7.

Escobar, D., Ascencio, K., Ortiz, A., Palma, A., Sánchez, A. and Fontecha, G. (2020) Blood meal sources of anopheles spp. In malaria endemic areas of honduras, *Insects*, 11 (7), pp. 1–12. DOI:10.3390/insects11070450.

Fang, Y., Shi, W. Q., Wu, J. T., Li, Y. Y., Xue, J. B. and Zhang, Y. (2019) Resistance to pyrethroid and organophosphate insecticides, and the geographical distribution and polymorphisms of target-site mutations in voltage-gated sodium channel and acetylcholinesterase 1 genes in Anopheles sinensis populations in Shanghai, China, *Parasites and Vectors*, 12 (1), pp. 1–13. DOI:10.1186/s13071-019-3657-7.

Fillinger, U., Sombroek, H., Majambere, S., Van Loon, E., Takken, W. and Lindsay, S. W. (2009) Identifying the most productive breeding sites for malaria mosquitoes in the Gambia, *Malaria Journal*, 8 (1), pp. 1–14. DOI:10.1186/1475-2875-8-62.

Fornadel, C. M., Norris, L. C., Franco, V. and Norris, D. E. (2011) Unexpected Anthropophily in the Potential Secondary Malaria Vectors An coustani s.l and An squamosus in Macha, Zambia., 11 (8). DOI:10.1089/vbz.2010.0082.

Gatton, M. L., Chitnis, N., Churcher, T., Donnelly, M. J., Ghani, A. C., Godfray, H. C. J., *et al.* (2013) The importance of mosquito behavioural adaptations to malaria control in Africa, *Evolution*, 67 (4), pp. 1218–1230. DOI:10.1111/evo.12063.

Gomes, F. M., Hixson, B. L., Tyner, M. D. W., Ramirez, J. L., Canepa, G. E., Alves e Silva, T. L., *et al.* (2017) Effect of naturally occurring Wolbachia in Anopheles gambiae s.l. mosquitoes from Mali on Plasmodium falciparum malaria transmission, *Proceedings of the National Academy of Sciences of the United States of America*, 114 (47), pp. 12566–12571. DOI:10.1073/pnas.1716181114.

Herren, J. K., Mbaisi, L., Mararo, E., Makhulu, E. E., Mobegi, V. A., Butungi, H., *et al.* (2020) A microsporidian impairs Plasmodium falciparum transmission in Anopheles arabiensis mosquitoes, *Nature Communications*, 11 (1). DOI:10.1038/s41467-020-16121-y.

Howard, A. F. V., Zhou, G. and Omlin, F. X. (2007) Malaria mosquito control using edible fish

in western Kenya: Preliminary findings of a controlled study, *BMC Public Health*, 7, pp. 1–6. DOI:10.1186/1471-2458-7-199.

Huang, Y., Guo, Q., Sun, X., Zhang, C., Xu, N., Xu, Y., *et al.* (2018) Culex pipiens pallens cuticular protein CPLCG5 participates in pyrethroid resistance by forming a rigid matrix, *Parasites and Vectors*, 11 (1), pp. 1–10. DOI:10.1186/s13071-017-2567-9.

Kent, R. J. and Norris, D. E. (2010) Identification of Mammalian Blood Meals in Mosquitoes By a Multiplexed Polymerase Chain Reaction Targeting Cytochrome B, *Human Development*, 45 (6), pp. 1654–1668. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16103600%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4147110 [Accessed

Killeen, G. F. and Chitnis, N. (2014) Potential causes and consequences of behavioural resilience and resistance in malaria vector populations: A mathematical modelling analysis, *Malaria Journal*, 13 (1), pp. 1–16. DOI:10.1186/1475-2875-13-97.

Killeen, G. F., Govella, N. J., Lwetoijera, D. W. and Okumu, F. O. (2016) Most outdoor malaria transmission by behaviourally - resistant Anopheles arabiensis is mediated by mosquitoes that have previously been inside houses, *Malaria Journal*, pp. 1–10. DOI:10.1186/s12936-016-1280-z.

Koekemoer, L. L., Kamau, L., Hunt, R. H. and Coetzee, M. (2002) A cocktail polymerase chain reaction assay to identify members of the Anopheles funestus (Diptera: Culicidae) group, *American Journal of Tropical Medicine and Hygiene*, 66 (6), pp. 804–811. DOI:10.4269/ajtmh.2002.66.804.

Kwiatkowska, R. M., Platt, N., Poupardin, R., Irving, H., Dabire, R. K., Mitchell, S., *et al.* (2013) Dissecting the mechanisms responsible for the multiple insecticide resistance phenotype in Anopheles gambiae s.s., M form, from Vallée du Kou, Burkina Faso, *Gene*, 519 (1), pp. 98–106. DOI:10.1016/j.gene.2013.01.036.

Limwagu, A. J., Kaindoa, E. W., Ngowo, H. S., Hape, E., Finda, M., Mkandawile, G., *et al.* (2019) Using a miniaturized double-net trap (DN-Mini) to assess relationships between indoor-outdoor biting preferences and physiological ages of two malaria vectors, Anopheles arabiensis

and Anopheles funestus, *Malaria Journal*, 18 (1), pp. 1–15. DOI:10.1186/s12936-019-2913-9.

Lobo, N. F., St. Laurent, B., Sikaala, C. H., Hamainza, B., Chanda, J., Chinula, D., *et al.* (2015) Unexpected diversity of Anopheles species in Eastern Zambia: Implications for evaluating vector behavior and interventions using molecular tools, *Scientific Reports*, 5 (December), pp. 1–10. DOI:10.1038/srep17952.

Machani, M. G., Ochomo, E., Amimo, F., Kosgei, J., Munga, S., Zhou, G., Githeko, A. K., Yan, G. and Afrane, Y. A. (2020) Resting behaviour of malaria vectors in highland and lowland sites of western Kenya : Implication on malaria vector control measures, pp. 55–66. DOI:10.1371/journal.pone.0224718.

Massey, N. C., Garrod, G., Wiebe, A., Henry, A. J., Huang, Z., Moyes, C. L. and Sinka, M. E. (2016) A global bionomic database for the dominant vectors of human malaria, *Scientific Data*, 3, pp. 1–13. DOI:10.1038/sdata.2016.14.

Mattah, P. A. D., Futagbi, G., Amekudzi, L. K., Mattah, M. M., De Souza, D. K., Kartey-Attipoe, W. D., Bimi, L. and Wilson, M. D. (2017) Diversity in breeding sites and distribution of Anopheles mosquitoes in selected urban areas of southern Ghana, *Parasites and Vectors*, 10 (1), pp. 1–15. DOI:10.1186/s13071-016-1941-3.

Mawejje, H. D., Kilama, M., Kigozi, S. P., Musiime, A. K., Kamya, M., Lines, J., *et al.* (2021) Impact of seasonality and malaria control interventions on Anopheles density and species composition from three areas of Uganda with differing malaria endemicity, *Malaria Journal*, pp. 1–13. DOI:10.1186/s12936-021-03675-5.

Mazigo, E., Kidima, W., Myamba, J. and Kweka, E. J. (2019) The impact of Anopheles gambiae egg storage for mass rearing and production success, *Malaria Journal*, 18 (1), pp. 1–11. DOI:10.1186/s12936-019-2691-4.

McAllister, J. C. and Scott, M. (2020) CONUS manual for evaluating insecticide resistance in mosquitoes using the CDC bottle bioassay kit, pp. 1–19. Available from: https://www.cdc.gov/zika/pdfs/CONUS-508.pdf [Accessed

Mint Mohamed Lemine, A., Ould Lemrabott, M. A., Niang, E. H. A., Basco, L. K., Bogreau, H., Faye, O. and Ould Mohamed Salem Boukhary, A. (2018) Pyrethroid resistance in the major

malaria vector Anopheles arabiensis in Nouakchott, Mauritania, *Parasites and Vectors*, 11 (1), pp. 1–8. DOI:10.1186/s13071-018-2923-4.

MOH (2015) Zambia malaria indicator survey, *National Malaria Elimination Centre, Ministry Of Health, Zambia*. Available from: http://www.makingmalariahistory.org/wp-content/uploads/2017/06/Zambia-MIS2015_Jan20-nosigs.pdf [Accessed

MOH (2019) Zambia National Malaria Indicator Survey 2018: MINISTRY OF HEALTH, *National Malaria Elimination Centre, Ministry Of Health, Zambia*, (May), pp. 2009–2015.

Moiroux, N., Gomez, M. B., Pennetier, C., Elanga, E., Djènontin, A., Chandre, F., Djègbé, I., Guis, H. and Corbel, V. (2012) Changes in anopheles funestus biting behavior following universal coverage of long-lasting insecticidal nets in benin, *Journal of Infectious Diseases*, 206 (10), pp. 1622–1629. DOI:10.1093/infdis/jis565.

Mouhamadou, C. S., De Souza, S. S., Fodjo, B. K., Zoh, M. G., Bli, N. K. and Koudou, B. G. (2019) Evidence of insecticide resistance selection in wild Anopheles coluzzii mosquitoes due to agricultural pesticide use, *Infectious Diseases of Poverty*, 8 (1), pp. 64. DOI:10.1186/s40249-019-0572-2.

Musiime, A. K., Smith, D. L., Kilama, M., Rek, J., Arinaitwe, E., Nankabirwa, J. I., *et al.* (2019) Impact of vector control interventions on malaria transmission intensity , outdoor vector biting rates and Anopheles mosquito species composition in Tororo , Uganda, *Malaria Journal*, pp. 1–9. DOI:10.1186/s12936-019-3076-4.

Mwangangi, J. M., Mbogo, C. M., Orindi, B. O., Muturi, E. J., Midega, J. T., Nzovu, J., *et al.* (2013a) Shifts in malaria vector species composition and transmission dynamics along the Kenyan coast over the past 20 years, pp. 1–9.

Mwangangi, J. M., Muturi, E. J., Muriu, S. M., Nzovu, J., Midega, J. T. and Mbogo, C. (2013b) The role of Anopheles arabiensis and Anopheles coustani in indoor and outdoor malaria transmission in Taveta District , Kenya, pp. 1–9.

Mzilahowa, T., Hastings, I. M., Molyneux, M. E. and McCall, P. J. (2012) Entomological indices of malaria transmission in Chikhwawa district, Southern Malawi, *Malaria Journal*, 11, pp. 1–9. DOI:10.1186/1475-2875-11-380.

Ndenga, B. A., Mulaya, N. L., Musaki, S. K., Shiroko, J. N., Dongus, S. and Fillinger, U. (2016) Malaria vectors and their blood - meal sources in an area of high bed net ownership in the western Kenya highlands, *Malaria Journal*, pp. 6–8. DOI:10.1186/s12936-016-1115-y.

Ndiaye, A., Amadou Niang, E. H., Diène, A. N., Nourdine, M. A., Sarr, P. C., Konaté, L., Faye, O., Gaye, O. and Sy, O. (2020) Mapping the breeding sites of Anopheles gambiae s. l. In areas of residual malaria transmission in central western Senegal, *PLoS ONE*, 15 (12 December), pp. 1–16. DOI:10.1371/journal.pone.0236607.

Nkya, T. E., Akhouayri, I., Kisinza, W. and David, J. P. (2013) Impact of environment on mosquito response to pyrethroid insecticides: Facts, evidences and prospects, *Insect Biochemistry and Molecular Biology*, 43 (4), pp. 407–416. DOI:10.1016/j.ibmb.2012.10.006.

NMEC (2017) National Malaria Elimination Strategic Plan 2017 - 2021, Ministry of Health, Government Republic of Zambia, 70 (10), pp. 1113. Available from: https://static1.squarespace.com/static/58d002f017bffcf99fe21889/t/5b28d7f1575d1ff0942dbce1/1529403401067/National+Malaria+Elimination+Strategic+Plan+2017-Final_PRINT.pdf [Accessed

NMEC (2019) Insecticide Resistance Management and Monitoring Plan, Ministry of Health, Government Republic of Zambia.,

Ntonifor, N. H. and Veyufambom, S. (2016) Assessing the effective use of mosquito nets in the prevention of malaria in some parts of Mezam division, Northwest Region Cameroon, *Malaria Journal*, 15 (1), pp. 1–8. DOI:10.1186/s12936-016-1419-y.

O'Donnell, A. J., Rund, S. S. C. and Reece, S. E. (2019) Time-of-day of blood-feeding: Effects on mosquito life history and malaria transmission, *Parasites and Vectors*, 12 (1), pp. 1–16. DOI:10.1186/s13071-019-3513-9.

Ondeto, B. M., Nyundo, C., Kamau, L., Muriu, S. M., Mwangangi, J. M., Njagi, K., Mathenge, E. M., Ochanda, H. and Mbogo, C. M. (2017) Current status of insecticide resistance among malaria vectors in Kenya, *Parasites and Vectors*, 10 (1), pp. 1–13. DOI:10.1186/s13071-017-2361-8.

Paaijmans, K. P. and Huijben, S. (2020) Taking the 'I' out of LLINs: Using insecticides in vector

control tools other than long-lasting nets to fight malaria, *Malaria Journal*, 19 (1), pp. 1–6. DOI:10.1186/s12936-020-3151-x.

Phiri, E., Baboo, K. S. and Miller, J. (2015) Effect of Indoor Residual Spraying on the Incidence of Malaria in Kaoma District of Western Zambia, *Medical Journal of Zambia*, 42 (4), pp. 150–158.

Pinchoff, J., Larsen, D. A., Renn, S., Pollard, D., Fornadel, C., Maire, M., *et al.* (2016) Targeting indoor residual spraying for malaria using epidemiological data : a case study of the Zambia experience, *Malaria Journal*, pp. 1–6. DOI:10.1186/s12936-015-1073-9.

PMI (2019) PRESIDENT ' S MALARIA INITIATIVE; Zambia Malaria Operational Plan FY 2019, *Operational Plan*. Available from: http://www.pmi.gov/countries/mops/fy14/nigeria_mop_fy14.pdf [Accessed

Prevention, C.-C. for D. C. and (2019) CDC - Malaria - Malaria Worldwide - How Can Malaria Cases and Deaths Be Reduced? - Insecticide-Treated Bed Nets.

Ricci, I., Valzano, M., Ulissi, U., Epis, S., Cappelli, A. and Favia, G. (2012) Symbiotic control of mosquito borne disease, *Pathogens and Global Health*, 106 (7), pp. 380–385. DOI:10.1179/2047773212Y.0000000051.

Riveron, J., Tchouakui, M., Mugenzi, L., Menze, B., Chiang, M.-C. and Wondji, C. (2018) Insecticide Resistance in Malaria Vectors: An Update at Global Scale, in: *IntechOpen*.32, pp. 137–144.

Russell, T. L., Govella, N. J., Azizi, S., Drakeley, C. J., Kachur, S. P. and Killeen, G. F. (2011) Increased proportions of outdoor feeding among residual malaria vector populations following increased use of insecticide-treated nets in rural Tanzania, *Malaria Journal*, 10, pp. 1–10. DOI:10.1186/1475-2875-10-80.

Scott, J. A., Brogdon, W. G. and Collins, F. H. (1993) Identification of single specimens of the Anopheles gambiae complex by the polymerase chain reaction, *American Journal of Tropical Medicine and Hygiene*, 49 (4), pp. 520–529. DOI:10.4269/ajtmh.1993.49.520.

Service, M. (2012) *Medical entomology for students, fourth edition*, *Medical Entomology for Students, Fourth Edition*. DOI:10.1017/CBO9780511811012.

Sinka, M. E., Bangs, M. J., Manguin, S., Rubio-Palis, Y., Chareonviriyaphap, T., Coetzee, M., *et al.* (2012) A global map of dominant malaria vectors, *Parasites and Vectors*, 5 (1), pp. 69. DOI:10.1186/1756-3305-5-69.

Sinka, M. E., Pironon, S., Massey, N. C., Longbottom, J., Hemingway, J., Moyes, C. L. and Willis, K. J. (2020) A new malaria vector in Africa: Predicting the expansion range of Anopheles stephensi and identifying the urban populations at risk, *Proceedings of the National Academy of Sciences of the United States of America*, 117 (40), pp. 24900–24908. DOI:10.1073/pnas.2003976117.

Sokhna, C., Ndiath, M. O. and Rogier, C. (2013) The changes in mosquito vector behaviour and the emerging resistance to insecticides will challenge the decline of malaria, *Clinical Microbiology and Infection*, 19 (10), pp. 902–907. DOI:10.1111/1469-0691.12314.

Sougoufara, S., Diédhiou, S. M., Doucouré, S., Diagne, N., Sembène, P. M., Harry, M., Trape, J. F., Sokhna, C. and Ndiath, M. O. (2014) Biting by Anopheles funestus in broad daylight after use of long-lasting insecticidal nets: A new challenge to malaria elimination, *Malaria Journal*, 13 (1), pp. 1–7. DOI:10.1186/1475-2875-13-125.

Sougoufara, S., Ottih, E. C. and Tripet, F. (2020) The need for new vector control approaches targeting outdoor biting anopheline malaria vector communities, *Parasites & Vectors*, pp. 1–15. DOI:10.1186/s13071-020-04170-7.

Steinhardt, L. C., Jean, Y. S., Impoinvil, D., Mace, K. E., Wiegand, R., Huber, C. S., *et al.* (2017) Effectiveness of insecticide-treated bednets in malaria prevention in Haiti: a case-control study, *The Lancet Global Health*, 5 (1), pp. e96–e103. DOI:10.1016/S2214-109X(16)30238-8.

Stevenson, J. C., Pinchoff, J., Muleba, M., Lupiya, J., Chilusu, H., Mwelwa, I., *et al.* (2016) Spatio-temporal heterogeneity of malaria vectors in northern Zambia : implications for vector control, *Parasites & Vectors*, pp. 1–15. DOI:10.1186/s13071-016-1786-9.

Tangena, J. A. A., Hendriks, C. M. J., Devine, M., Tammaro, M., Trett, A. E., Williams, I., *et al.* (2020) Indoor residual spraying for malaria control in sub-Saharan Africa 1997 to 2017: An adjusted retrospective analysis, *Malaria Journal*, 19 (1), pp. 1–15. DOI:10.1186/s12936-020-03216-6.

The President's Malaria Initiative (PMI) / Vectorlink (2020) Zambia Annual Entomology Report (June 2019-August 2020), *Rockville, MD. The PMI VectorLink Project, Abt Associates*.

Thomas, S., Ravishankaran, S., Justin, J. A., Asokan, A., Mathai, M. T., Valecha, N., Thomas, M. B. and Eapen, A. (2016) Overhead tank is the potential breeding habitat of Anopheles stephensi in an urban transmission setting of Chennai, India, *Malaria Journal*, 15 (1), pp. 1–10. DOI:10.1186/s12936-016-1321-7.

Thomsen, E. K., Koimbu, G., Pulford, J., Jamea-Maiasa, S., Ura, Y., Keven, J. B., *et al.* (2017) Mosquito behavior change after distribution of bednets results in decreased protection against malaria exposure, *Journal of Infectious Diseases*, 215 (5), pp. 790–797. DOI:10.1093/infdis/jiw615.

Thomsen, E. K., Strode, C., Hemmings, K., Hughes, A. J., Chanda, E., Musapa, M., *et al.* (2014) Underpinning sustainable vector control through informed insecticide resistance management, *PLoS ONE*, 9 (6). DOI:10.1371/journal.pone.0099822.

Walshe, D. P., Garner, P., Adeel, A. A., Pyke, G. H. and Burkot, T. R. (2017) Larvivorous fish for preventing malaria transmission, *Cochrane Database of Systematic Reviews*, 2017 (12). DOI:10.1002/14651858.CD008090.pub3.

Wang, S., Ghosh, A. K., Bongio, N., Stebbings, K. A., Lampe, D. J. and Jacobs-Lorena, M. (2012) Fighting malaria with engineered symbiotic bacteria from vector mosquitoes, *Proceedings of the National Academy of Sciences of the United States of America*, 109 (31), pp. 12734–12739. DOI:10.1073/pnas.1204158109.

WHO (2019) *Guidelines for Malaria Vector Control*, *Guidelines for Malaria Vector Control*. Available from: https://apps.who.int/iris/bitstream/handle/10665/310862/9789241550499-eng.pdf?ua=1%0Ahttp://www.ncbi.nlm.nih.gov/pubmed/30844152

WHO (2020) *World malaria report 2020*.

Wiebe, A., Longbottom, J., Gleave, K., Shearer, F. M., Sinka, M. E., Massey, N. C., *et al.* (2017) Geographical distributions of African malaria vector sibling species and evidence for insecticide resistance, *Malaria Journal*, 16 (1), pp. 1–10. DOI:10.1186/s12936-017-1734-y.

Wilson, A. L., Courtenay, O., Kelly-hope, L. A., Id, T. W. S., Takken, W., Torr, S. J. and

Lindsay, S. W. (2020) *The importance of vector control for the control and elimination of vector-borne diseases*.

World Health Organization(WHO) (2014) *Malaria entomology and vector control - GUIDE FOR PARTICIPANTS, 2014 11th International Conference on Fuzzy Systems and Knowledge Discovery, FSKD 2014*. 1 st Editi. Malta: World Health Organization. DOI:10.1109/FSKD.2014.6980805.

World Health Organization(WHO) (2018) Test procedures for insecticide resistance monitoring in malaria vector mosquitoes Second edition.

World Health Organization (2013) WHO recommended insecticides for indoor residual spraying against malaria vectors, (October), pp. 2013. Available from: http://www.who.int/whopes/Insecticides_IRS_Malaria_25_Oct_2013.pdf [Accessed

World Health Organization (2019) Malaria and related entomological and vector control concepts, *Guidelines for Malaria Vector Control*. Available from: https://www.ncbi.nlm.nih.gov/books/NBK538113/ [Accessed 17 April 2021].

Yahouédo, G. A., Chandre, F., Rossignol, M., Ginibre, C., Balabanidou, V., Mendez, N. G. A., Pigeon, O., Vontas, J. and Cornelie, S. (2017) Contributions of cuticle permeability and enzyme detoxification to pyrethroid resistance in the major malaria vector Anopheles gambiae, *Scientific Reports*, 7 (1), pp. 1–10. DOI:10.1038/s41598-017-11357-z.

Yewhalaw, D. and Kweka, E. J. (2015) Insecticide Resistance in East Africa — History, Distribution and Drawbacks on Malaria Vectors and Disease Control, *Intech*, 32 (July), pp. 137–144. Available from: http://www.intechopen.com/books/trends-in-telecommunications-technologies/gps-total-electron-content-tec- prediction-at-ionosphere-layer-over-the-equatorial-region%0AInTec%0Ahttp://www.asociatiamhc.ro/wp-content/uploads/2013/11/Guide-to-Hydropower.pdf [Accessed

Zhou, G., Lo, E., Githeko, A. K., Afrane, Y. A. and Yan, G. (2020) Long-lasting microbial larvicides for controlling insecticide resistant and outdoor transmitting vectors: a cost-effective supplement for malaria interventions, *Infectious Diseases of Poverty*, 9 (1), pp. 1–8. DOI:10.1186/s40249-020-00767-3.

Zhou, G., Minakawa, N., Githeko, A. and Yan, G. (2004) Spatial distribution patterns of malaria vectors and sample size determination in spatially heterogeneous environments: A case study in the west Kenyan highland, *Journal of Medical Entomology*, 41 (6), pp. 1001–1009. DOI:10.1603/0022-2585-41.6.1001.

10.0 Appendices

Mosquito Collection Form

University of Zambia
School of Health Sciences
Department of Biomedical Sciences

Province: _____ District: _____ Study site: _____ Date of Collection: _____ Collection Type: _____ GPS Coordinates: Latitude _____ Longitude _____ Altitude (m): _____

SN	Head of Household Name	No. of People	No. of Animals	Roof Type	Wall Type	Type of eaves	No. of LLINs	Brand of LLINs	Spray status	If Sprayed Insecticide used	Mosquito Species	Abdominal Status		
												U	F	G

136/bmjopen-2024-091319 on 5 March 2025. Downloaded from

PARTICIPANT INFORMATION SHEET

University of Zambia

School of Health Sciences

Department of Biomedical Sciences

Principal Investigator: **Mr Westone Hamwata**

Co-Investigators: **Dr N.M Shimaponda-Mataa**

Mr Mbanga Muleba

Introduction

You are invited to take part in a survey titled ***Malaria Vector Bionomics and Phenotypic Resistance Status to Insecticides Used in Vector Control in Ndola District.*** You will be provided with enough information about the study which will help you decide if you will participate or not.

Purpose of the Study

This study will evaluate the behaviour of malaria vectors and their susceptibility status to insecticides used in vector control. Specifically, species composition, malaria vector density, resting behaviour, biting behaviour, host preference and mosquito susceptibility status will be determined.

What will happen if you participate in this study

You must be provided with enough information about the study to help you understand why the research is being done and what it involves. This will help you decide whether to participate or not. Please take time to read or listen as I read the document to you.

Mosquito collection will be conducted at your premises and you will be asked questions which will just take 5 minutes of your time to complete the mosquito collection form.

How long will this study last?

This study will last 5 months. However, mosquito collection at your premise will only be conducted once.

What are the risks of participating in this study?

There are no major risks to being in this study. You will only be asked to move out of the house when conducting pyrethrum spray catches in a period of about 20 minutes then you can go back in between 05:00 hrs to 07:00 hrs. If you feel uneasy leaving investigators alone, you will be provided with a face mask so that you accompany the investigator inside house during saturation of the house with the pyrethroid insecticide.

The study involves invading your personal space and as such utmost confidentiality will be adhered to by the investigators. Further, necessary protective measures against COVID-19 are put in place during our interaction with you. We will ensure physical distancing, availability of hand sanitizer, strict use of masks by both you and the investigator.

What is the study Procedure?

By consenting to this study you will allow the investigators to access your premises to collect mosquitoes. Mosquitoes will be collected using four different collection methods. CDC light traps will be set in your house around 16:00 and retrieved the following morning around 06:00hrs. Mosquito collection using pyrethrum spray catches and aspirations will be done from 05:00 hours to 08:00hours. Larval collection will be conducted in breeding sites near your house. The mosquitoes collected will then be taken to the laboratory where they will be morphologically identified and molecular identification will be conducted for determination of sibling species in the *An gambiae* and *An funestus* complex.

What are the benefits of participating in this study?

There are no direct program benefits to you for participating in the study. You may find an indirect benefit in knowing you have participated in an important study that could help generate baseline data for informed vector control programming.

Compensation

Participants in the study will not be compensated for the time taken to participate in this study. Despite this, we would be grateful if you could allow the team to collect mosquitoes from your premises.

What freedom and rights do you have in participating in the study?

The decision to participate in this study is entirely yours, and no one else should make it for you. You are free not to join this study or to stop participating in the study at any time. You will not receive any punishment now or in the future because of this. We will respect your freedom of choice. We will not share any of your information with your family, friends, or parent.

How is your confidentiality protected in this study?

The information that is collected during the study will be kept private. No one will be told that you have participated in the study or what your answers are to the questions. The study team will make every effort to protect your privacy and maintain the confidentiality of all the information that you provide. Your name or other identifiers will not be included in reports from this study. The information will be stored on a secure computer system.

At the end of the study, the report from the study will be made available to researchers or others

who are interested in using it to know more about the behaviour of malaria vectors and their phenotypic resistance status to insecticides used in vector control in Ndola. This means that others people besides the study staff will be able to see the information you provided.

What will happen to the results of the research study?

We will write a report combining all of the responses from all participants who have participated in this study. This report will be shared with The University of Zambia School of Health Sciences, Tropical Diseases Research Centre, Ndola District Health Office, National Malaria Elimination Centre and other key stakeholders.

Who has approved the research study?

This study has been reviewed and approved by the Tropical Diseases Research Centre Ethics Committee and National Health Research Authority.

Who else can you contact about the study and how do you contact them?

If you have any questions on the study or you being in the study, you or your selected relative/friend can contact Mr. Westone Hamwata, the Principal Investigator of the study on 0972790935. If you have a question about your rights as a research subject you or your selected relative/friend can contact the Secretary of the Tropical Diseases Research Centre Ethics Committee.

Declaration of Consent

I understand the contents of this Consent Form, and I agree to participate in this research study. I have had the opportunity to ask questions in an information session and all my questions have been answered to my satisfaction. I have been given sufficient time to consider the above information and to seek advice if I choose to do so. I understand that I am taking part in the study freely and

that I can stop being part of this study at any time and for any reason. By signing this consent form, I agree to participate in the research study.

I agree to participate in this study Yes ☐ No ☐

_____ _____ ____/___/_____

Head of Household Signature Date

Witness (*if the participant was not able to read and understand the Consent Information Sheet and Informed Consent Document*).

I affirm that the Informed Consent Document has been read to the participant, and she understands the study and I have witnessed her consent to study participation.

_____ _____ ____/___/_____

Name of Witness Signature Date

INFORMED CONSENT FORM

University of Zambia

School of Health Sciences

Department of Biomedical Sciences

Name of Head of Household: _____

Surveillance Activity Type: _____

House Number: _____

I can confirm that I have read (read to me) the information sheet and understand the objectives of this study. I had the opportunity to consider the information, ask questions and have had these answered fully.	Tick:
.....(name of investigator) has explained to me the nature and purpose of the activities to be undertaken. I understand fully what is to be done.	Tick:
I understand that my participation is voluntary and I am free to withdraw at any time, without giving any reason.	Tick:
I agree to take part in the above activity.	Tick:

Signature of Head of Household: _____ Date: _____

I confirm that I have explained to the person named above the nature and purpose of the activities to be undertaken.

Signature of Investigator: _____ Date: _____

1 signed copy of this form is given to the head of household;

1 signed copy of this form is kept for the Principal Investigator

TROPICAL DISEASES
Tel/Fax +260212 615444
tdrc-ethics@tdrc.org.zm

RESEARCH CENTRE
P O Box 71769
Ndola,
ZAMBIA

TDRC ETHICS REVIEW COMMITTEE
IRB REGISTRATION NUMBER : 00002911
FWA NUMBER : 00003729

TRC/C4/06/2021

22nd June 2021

Westone Phanuel Hamwata
Student No: 19000762
Tropical Diseases Research Centre
P.O. Box 71769
Ndola

RE: ETHICAL APPROVAL OF STUDY PROTOCOL

Reference is made to the protocol entitled **"Malaria Vector Bionomics and Phenotypic Resistance Status to Insecticides used in Vector Control in Ndola District",** which was submitted to the TDRC Ethics Review Committee for review.

On behalf of the Chairperson of the Committee, I wish to inform you that the Committee reviewed and approved your study protocol.

You are further required to submit progress reports to the TDRC ERC twice a year.

Should there be any protocol modifications or amendments, you are required to notify the ERC and submit protocol amendments for approval.

You are now required to submit your protocol to the National Health Research Authority for final approval following the link: https://www.nhra.org.zm. A final report of the study should be submitted to the Ethics Review Committee Secretariat at the end of the study.

This approval is valid for the period 22nd June, 2021 to 22nd June, 2022.

The Committee wishes you success in the execution of the study.

Yours faithfully,
TROPICAL DISEASES RESEARCH CENTRE

Sydney Mwanza
DEPUTY SECRETARY – TDRC ETHICS REVIEW COMMITTEE

TROPICAL DISEASES RESEARCH CENTRE
ETHICS REVIEW COMMITTEE
22 JUN 2021
P.O. BOX 71769
NDOLA, ZAMBIA

NATIONAL HEALTH RESEARCH AUTHORITY

Paediatric Centre of Excellence, University Teaching Hospital, P.O. Box 30075, LUSAKA

Tell: +260211 250309 | Email: znhrasec@gmail.com | www.nhra.org.zm

Ref No: NHRA000016/29/06/2021 **Date: 29th June, 2021**

The Principal Investigator,
Mr. Westone Hamwata,
University of Zambia
School of Health Sciences
PO Box 50110
Ridgeway Campus
Lusaka, ZAMBIA.

Dear Mr. Hamwata,

Re: Request for Authority to Conduct Research

The National Health Research Authority is in receipt of your request for authority to conduct research titled **"Malaria Vector Bionomics and Phenotypic Resistance Status to Insecticides Used in Vector Control in Ndola District."** I wish to inform you that following submission of your request to the Authority, our review of the same and in view of the ethical clearance, this study has been **approved** on condition that:

1. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
2. Progress updates are provided to NHRA quarterly from the date of commencement of the study;
3. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
4. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, University leadership, and all key respondents.

Yours sincerely,

Prof. Godfrey Biemba
Director/CEO
National Health Research Authority

8.2 PARTICIPANT INFORMATION SHEET

University of Zambia

School of Health Sciences

Department of Biomedical Sciences

Principal Investigator: **Mr Westone Hamwata**

Co-Investigators: **Dr N.M Shimaponda-Mataa**

Mr Mbanga Muleba

Introduction

You are invited to take part in a survey titled ***Malaria Vector Bionomics and Phenotypic Resistance Status to Insecticides Used in Vector Control in Ndola District.*** You will be provided with enough information about the study which will help you decide if you will participate or not.

Purpose of the Study

This study will evaluate the behaviour of malaria vectors and their susceptibility status to insecticides used in vector control. Specifically, species composition, malaria vector density, resting behaviour, biting behaviour, host preference and mosquito susceptibility status will be determined.

What will happen if you participate in this study

You must be provided with enough information about the study to help you understand why the research is being done and what it involves. This will help you decide whether to participate or not. Please take time to read or listen as I read the document to you.

Mosquito collection will be conducted at your premises and you will be asked questions which will just take 5 minutes of your time to complete the mosquito collection form.

How long will this study last?

This study will last 5 months. However, mosquito collection at your premise will only be conducted once.

What are the risks of participating in this study?

There are no major risks to being in this study. You will only be asked to move out of the house when conducting pyrethrum spray catches in a period of about 20 minutes then you can go back in between 05:00 hrs to 07:00 hrs. If you feel uneasy leaving investigators alone, you will be provided with a face mask so that you accompany the investigator inside house during saturation of the house with the pyrethroid insecticide.

The study involves invading your personal space and as such utmost confidentiality will be adhered to by the investigators. Further, necessary protective measures against COVID-19 are put in place during our interaction with you. We will ensure physical distancing, availability of hand sanitizer, strict use of masks by both you and the investigator.

What is the study Procedure?

By consenting to this study you will allow the investigators to access your premises to collect mosquitoes. Mosquitoes will be collected using four different collection methods. CDC light traps will be set in your house around 16:00 and retrieved the following morning around 06:00hrs. Mosquito collection using pyrethrum spray catches and aspirations will be done from 05:00 hours to 08:00hours. Larval collection will be conducted in breeding sites near your house. The mosquitoes collected will then be taken to the laboratory where they will be morphologically identified and molecular identification will be conducted for determination of sibling species in the *An gambiae* and *An funestus* complex.

What are the benefits of participating in this study?

There are no direct program benefits to you for participating in the study. You may find an indirect benefit in knowing you have participated in an important study that could help generate baseline data for informed vector control programming.

Compensation

Participants in the study will not be compensated for the time taken to participate in this study. Despite this, we would be grateful if you could allow the team to collect mosquitoes from your premises.

What freedom and rights do you have in participating in the study?

The decision to participate in this study is entirely yours, and no one else should make it for you. You are free not to join this study or to stop participating in the study at any time. You will not receive any punishment now or in the future because of this. We will respect your freedom of choice. We will not share any of your information with your family, friends, or parent.

How is your confidentiality protected in this study?

The information that is collected during the study will be kept private. No one will be told that you have participated in the study or what your answers are to the questions. The study team will make every effort to protect your privacy and maintain the confidentiality of all the information that you provide. Your name or other identifiers will not be included in reports from this study. The information will be stored on a secure computer system.

At the end of the study, the report from the study will be made available to researchers or others

who are interested in using it to know more about the behaviour of malaria vectors and their phenotypic resistance status to insecticides used in vector control in Ndola. This means that others people besides the study staff will be able to see the information you provided.

What will happen to the results of the research study?

We will write a report combining all of the responses from all participants who have participated in this study. This report will be shared with The University of Zambia School of Health Sciences, Tropical Diseases Research Centre, Ndola District Health Office, National Malaria Elimination Centre and other key stakeholders.

Who has approved the research study?

This study has been reviewed and approved by the Tropical Diseases Research Centre Ethics Committee and National Health Research Authority.

Who else can you contact about the study and how do you contact them?

If you have any questions on the study or you being in the study, you or your selected relative/friend can contact Mr. Westone Hamwata, the Principal Investigator of the study on 0972790935. If you have a question about your rights as a research subject you or your selected relative/friend can contact the Secretary of the Tropical Diseases Research Centre Ethics Committee.

Declaration of Consent

I understand the contents of this Consent Form, and I agree to participate in this research study. I have had the opportunity to ask questions in an information session and all my questions have been answered to my satisfaction. I have been given sufficient time to consider the above information and to seek advice if I choose to do so. I understand that I am taking part in the study freely and

that I can stop being part of this study at any time and for any reason. By signing this consent form, I agree to participate in the research study.

I agree to participate in this study Yes ☐ No ☐

_____ _____ ____/___/_____

Head of Household Signature Date

Witness (*if the participant was not able to read and understand the Consent Information Sheet and Informed Consent Document*).

I affirm that the Informed Consent Document has been read to the participant, and she understands the study and I have witnessed her consent to study participation.

_____ _____ ____/___/_____

Name of Witness Signature Date

8.3 INFORMED CONSENT FORM

University of Zambia

School of Health Sciences

Department of Biomedical Sciences

Name of Head of Household: _____

Surveillance Activity Type: _____

House Number: _____

I can confirm that I have read (read to me) the information sheet and understand the objectives of this study. I had the opportunity to consider the information, ask questions and have had these answered fully.	Tick:
.....(name of investigator) has explained to me the nature and purpose of the activities to be undertaken. I understand fully what is to be done.	Tick:
I understand that my participation is voluntary and I am free to withdraw at any time, without giving any reason.	Tick:
I agree to take part in the above activity.	Tick:

Signature of Head of Household: _____ Date: _____

I confirm that I have explained to the person named above the nature and purpose of the activities to be undertaken.

Signature of Investigator: _____ Date: _____

1 signed copy of this form is given to the head of household;

1 signed copy of this form is kept for the Principal Investigator

BMJ Open

Implications for Malaria Transmission: A Cross-sectional Study on the Bionomics and Susceptibility of Local Malaria Vectors in Urban and Peri-urban Settings of Ndola District.

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2024-091319.R2
Article Type:	Original research
Date Submitted by the Author:	14-Feb-2025
Complete List of Authors:	Hamwata, Westone; Tropical Diseases Research Centre, Department of Biomedical Sciences; University of Zambia - Ridgeway Campus, Biomedical Sciences Hazyondo, Mwendalubi; Department of National Parks and Wildlife Service; University of Zambia - Ridgeway Campus, Biomedical Sciences Daka, Victor; The Copperbelt University School of Medicine, Public Health Muleba, Mbanga; Tropical Diseases Research Centre, Department of Biomedical Sciences Shimaponda-Mataa, Nzooma ; University of Zambia - Ridgeway Campus, Biomedical Sciences
Primary Subject Heading:	Public health
Secondary Subject Heading:	Global health, Epidemiology, Infectious diseases
Keywords:	Malaria, Mosquito Vectors, Behavior, Entomology < TROPICAL MEDICINE

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Implications for Malaria Transmission: A cross-sectional study on the bionomics and susceptibility of local malaria vectors in urban and peri-urban settings of Ndola district.

Westone Hamwata[1,2]; Mwendalubi Hazyondo[2,3]; Victor Daka[4]; Mbanga Muleba[1]; Nzooma M. Shimaponda-Mataa[2];

1. Tropical Diseases Research Centre, Department of Biomedical Sciences, Vector Biology and Parasitology Unit
2. The University of Zambia, School of Health Sciences, Department of Biomedical Sciences
3. Department of National Parks and Wildlife, Chilanga, Lusaka, Zambia
4. The Copperbelt University, Michael Chilufya Sata School of Medicine, Department of Public Health

Corresponding Author:

Westone Hamwata

Tropical Diseases Research Centre, Department of Biomedical Sciences

Ndola, Zambia.

Telephone: +260972790935

Email: phamwata@gmail.com

ABSTRACT

Objectives: To assess vector behaviour and phenotypic resistance for effective vector control programming.

Design: This was a cross-sectional study**.**

Setting: This study was conducted in the urban and peri-urban areas of Ndola district, Zambia.

Participants / Study units: A total of 166 houses were selected for adult mosquito collection and an additional 60 collection efforts were made for larval collection from potential larval habitats.

Primary and secondary outcome measures: The primary outcome was the behaviour of the malaria vectors and the secondary outcome was their phenotypic resistance status.

Results: The main breeding sites identified were irrigation trenches (4.67 larvae/dip) and garden ponds (2.72 larvae/dip) created from extensive urban agriculture practices. *Anopheles funestus* and *Anopheles gambiae* were found to coexist in all the four sites with *An. funestus* identified as the most dominant malaria vector. Densities of *An. gambiae s.s* seeking a blood meal ($\chi 2 = 12.566$, df = 3, p = 0.001) and resting indoors (Z = 56.5, p = 0.019) were found to be higher in urban than peri-urban sites compared to *An. funestus s.s* which had similar distribution across the study sites. Sprayed houses were significantly associated with reduced mosquito numbers (B = -0.956, IRR = 0.384, p = 0.001. *An. gambiae* s.s was fully susceptible to organophosphates and neonicotinoids but highly resistant to pyrethroids, carbamates and organochlorines.

Conclusions: The emergence of *An. funestus s.s* in an area previously dominated by *An. gambiae s.s* and its coexistence with *An. gambiae s.s* in the dry season pose a risk of sustaining malaria transmission all year round. Agriculture practices in urban areas resulted in highly productive mosquito breeding sites, thus the need for targeted vector control.

Key Words: *malaria vector coexistence, vector behaviour, insecticide resistance, Ndola, urban setting*

STRENGTHS AND LIMITATIONS OF THIS STUDY

- The study design and sampling strategies used allow for the determination of species composition, abundance, host-seeking and resting behaviour of malaria vectors.
- Presence of *An gambiae s.s* larval in the dry season facilitates mosquito breeding which may drive malaria transmission thus the need to plan for additional measures.
- The susceptibility of *An. gambiae s.s* the most efficient malaria vector was determined against seven different insecticides from five different classes but not for *An. funestus* s.s. due to limited numbers of adult *An. funestus* s.s and difficulty in finding larval habitats nor sufficient adults to conduct forced oviposition.
- This study was conducted in the dry season and the entomological indices determined may only be applicable to the dry season.

BACKGROUND

Malaria remains a public health challenge in Zambia accounting for approximately 1.4% of the global malaria disease burden. It is estimated that about four people die from malaria every day in Zambia [1,2]. High rainfall regions in northern Zambia experience the highest disease burden, while densely populated and arid regions in the south experience lower burden [3–6].

The primary malaria vectors in Zambia include *Anopheles funestus s.s, Anopheles gambiae s.s* and *Anopheles arabiensis* [7–9]. *Anopheles funestus s.s,* the most abundant and widely distributed malaria vector in the country thrives during the dry season whereas *An. gambiae s.s*, the most efficient malaria vector thrives predominantly in the wet season [8]. Historically, *An. gambiae s.s* has been the dominant malaria vector on the Copperbelt Province in the past decades [9]. In contrast, *An. arabiensis,* a more zoophilic mosquito, is the primary malaria vector in the southern regions and a secondary malaria vector in the eastern parts of the country – a region of moderate transmission [5,9–11].

Insecticide-treated bednets (ITNs), indoor residual spraying (IRS) and artemisinin combined therapy (ACTs) have played a vital role in reducing malaria disease burden in Zambia [12–14]. These interventions have been informed by entomological and parasite surveillance data generated from several parts of the country in the past two decades [13–15]. The Copperbelt province on the other hand has implemented IRS since the 1950s and over the past two decades scaled up IRS, ITNs and ACTs which were associated with a significant decrease in the malaria disease burden [16,17]. Furthermore, this success also led to a decline in entomological surveillance in the province. Since 2017, the Copperbelt province has experienced a rise in the number of malaria cases indicating a change in the epidemiological landscape, necessitating renewed entomological activities for informed vector control programming.

Vector surveillance across Zambia has revealed some level of heterogeneity in the behaviour and susceptibility of malaria vectors within and between selected districts [9,18]. Most active entomological sites are located in areas of high or low malaria transmission, with limited representation in settings of moderate transmission [19]. Additionally, over 95% of all entomological surveillance activities are conducted in rural settings yet a substantial number of reported malaria cases originate from peri-urban and urban areas [20–22]. To address these gaps, we conducted entomological studies in Ndola between July 2021 and October 2021, in two ecologically distinct settings representing the peri-urban and urban areas of Ndola district a moderate malaria transmission setting to assess vector behaviour and phenotypic resistance for effective vector control programming.

METHODS

Study design and study area

This was a cross sectional study was conducted in the dry season in Ndola district, the provincial capital of the Copperbelt Province. The mean annual temperatures range from 12^{o}C to 25^{o}C, with mean annual rainfall ranging from 200 to 900 mm. The rainy season spans November to March, followed by a longer dry season from April to October.

Two catchment areas Chipulukusu and Kaniki were selected for their high malaria incidences rates in 2020. The malaria incidences for Chipulukusu and Kaniki health centres were 435 per 1000 population at risk and 971 per 1000 population at risk [2]. Chipulukusu is an urban catchment area with houses constructed with cement blocks, burnt bricks, or mud bricks and have iron or grass roofing. Mosquito collection in Chipulukusu was conducted in two zones: Musalu (-12.9524 S, 28.66012 E), a densely populated area with limited road access and extensive vegetable gardening

activities and Mapalo (-12.9374 S, 28.67564 E) an equally densely populated area but with road access and very minimal vegetable gardening activities. Kaniki is a peri-urban catchment consisting mainly of mud houses with thatched grass or iron sheet roofs. Mosquito collection in Kaniki was conducted in Kamalasha (-12.8556955 S, 28.5311082 E), a densely populated area near the Sakania border with a swamp on the western side of the Ndola-Mufulira Road, and Pima (-12.77416 S, 28.483865 E), a farming setting with houses organized in clusters. Both catchment areas serve as low-cost residential settings.

Sample size

A total of 166 houses were selected for adult mosquito collection; 56 houses for CDC light traps, 30 houses for pyrethrum spray catches and 80 houses for aspirations. An additional 60 collection efforts were made for larval collection from potential larval habitats.

Sample size justification

This study utilized WHO guidelines on mosquito sampling and the sample size used for this study follows previous modeling studies conducted on the minimum number of houses required to estimate mosquito abundance using a precision of 20% allowable for ecological studies [23,24].

Inclusion and exclusion criteria

The inclusion criteria for this study were twofold; firstly, only houses with an adult (16 years and above) were considered and houses where written consent was gotten. Houses were people cook using firewood from inside were excluded from the study.

House selection and adult mosquito collection

House selection was randomly done in each participating zone in the catchment area maintaining a minimum of 200m between two participating houses. Mosquito collection in each participating

house was only done once and only one mosquito collection method was employed per house sampled. The collection of mosquitoes was conducted between July and September 2021 from 166 randomly selected houses; 83 houses from Chipulukusu catchment area, and the other 83 houses from Kaniki catchment area.

CDC light traps

The CDC-LT was used as a proxy for determining the biting density of mosquitoes to human hosts. The traps were set in randomly selected houses, and mosquito collection occurred from 18:00 hours to 06:00 hours the following morning. Each trap was set at a height of 1.5 metres from the ground, adjacent to a sleeping person and near their legs.

Aspirations

The live adult mosquitoes were collected using prokopack aspirator from 05:00 hours to 07:00 hours in the morning in houses where people slept. The live adult mosquitoes were then put in bugdom cages where they were supplied with 10% sugar solution and transported to the laboratory for identification.

Pyrethrum Spray Catches (PSC)

Adult mosquitoes resting indoors were collected using PSC from 05:00 hours to 07:00 hours in the morning. Multiple pieces of white linen were spread over the floor, bed and furniture inside the house. Household members were asked to briefly exit the house and then the house was sprayed to saturation using pressurised two-in-one pyrethroid insecticide (imiprothrin 1.00 g/kg and deltamethrin 0.51 g/kg) can. After 10 minutes, all the mosquitoes that were knocked down were picked using a pair of forceps and placed into properly labelled petri dishes.

Collection of immature mosquitoes (larvae)

Larval collection was carried out in October 2021. Potential larval habitats were initially visually inspected for the presence of larvae using a 350 ml capacity standard dippers (BioQuip Products, Inc., California, USA) followed by sampling. The number of dips and number of larvae scooped were recorded. Afterwards, the collected larvae was transported to the TDRC laboratory for rearing in a controlled microenvironment (temperature of 27°C ± 2°C and a relative humidity of 75% ± 10%).

Susceptibility testing

Adults, F0 *An. gambiae s.l* reared from field collected larvae from Musalu, were exposed to five different classes of insecticides. The mosquitoes aged two to five days obtained from wild collected larvae were exposed to pirimiphos-methyl (0.25%), malathion (5%), deltamethrin (0.05%), permethrin (0.75%), bendiocarb (0.01%) and dichlorodiphenyltrichloroethane (4%) standard World Health Organisation (WHO) impregnated test paper. The bioassays were conducted in accordance with the WHO guidelines [25]. For Clothianidin, CDC bottle bioassay as described by Brogdon and Chan [26]. A minimum of 100 female *An. gambiae s.l* aged two to five days old were exposed each insecticide and 25 *An. gambiae s.l* were used as controls for each insecticide tested.

Experimental Procedures

1. **To determine species composition and abundance of malaria vectors in Ndola:** The species composition and abundance was determined by collections from CDC - LT, PSC, and aspirations.

2. **To assess the biting and resting behaviour of mosquitoes in Ndola:** The densities of mosquitoes collected per trap per night (mosquitoes/trap/night) was used to assess the host seeking behaviour of the malaria vectors. The **Indoor Resting behaviour** is indicative of the mosquitoes that rest indoors. This indicator is an important one when considering whether to implement IRS or not. The mean indoor resting densities calculated in this study were determined by mosquito collections from PSC only.

3. **To determine the insecticide resistance status of primary malaria vectors:** The mosquitoes used for susceptibility testing were the first filial generation (F1) from the larvae collected.

Mosquito processing

Morphological identification

The female *Anopheles* mosquitoes collected were initially morphologically identified to the genus level using an identification key for Afrotropical *Anopheles* mosquitoes [27]. Thereafter, the mosquito samples were individually preserved in 1.5 mL Eppendorf tubes containing silica gel. These preserved samples were stored for molecular identification using PCR.

Molecular identification

A subset of adult mosquitoes reared from field collected larvae and those collected as adults, morphologically identified as *An. funestus s.l.* and *An. gambiae s.l.*, were further subjected to PCR for molecular confirmation of the IDs. Deoxyribose nucleic acid (DNA) extraction for this process was performed using the QIAGEN DNeasy Blood and Tissue kit for insects (QIAGEN Inc., USA). DNA amplification was performed using the Applied Biosystems GeneAmp PCR System 9700

thermocycler. For molecular identification, the methods described by *Koekemoer. L et al; 2002* [28] and *Scott W.G et al; 1993* [29] were used for *An. funestus s.l* and *An. gambiae s.l.* respectively.

STATISTICAL ANALYSIS

The data collected was entered in Microsoft Excel and mean densities excluding larval density were derived from log transformed data using Williams mean (M_w= [$(X_1+1)(X_2+1)(X_3+1)....(X_n+1)]^{1/n}$ to account for skewed (non-normal distribution) and count data [21,30]

The dataset was then exported to IBM SPSS statistics version 25. The Kruskal–Wallis H test was used to compare the means (*Mw*) of malaria vectors seeking a host. The Mann–Whitney U test was used to compare the densities of the malaria vectors resting indoors from the two sites where PSC was conducted. Additionally, a negative binomial model with a log function was used to identify factors associated with counts of malaria vectors in the sampled housing structures. Susceptibility status of *An. gambiae s.s.* was determined using WHO mortality scoring guidelines [23], [24].

Patient and Public Involvement

There was no direct patient and public involvement. The findings from this study will be shared with Ndola District Health Office and the Ministry of Health.

RESULTS

Species composition and abundance from adult mosquito surveys

A total of 166 houses were sampled and from these 744 female mosquitoes were collected. *Culex* accounted for 53% (392/744), *An. funestus s.l.* 17% (123/744), *Mansonia* 16% (106/744), *An. gambiae s.l.* 14% (106/744), and *An. gibbinsi s.l.* less than 1% (2/744) of the total mosquitoes

collected (Table 1). Mosquito abundance by site showed the highest mosquito collections were from Musalu (338/744) and Mapalo (208/744) the two urban sites, followed by Pima (131/744) and Kamalasha (67/744) the two peri-urban sites. Notably, *Culex* mosquitoes comprised the largest proportion at each of the four sites (Table 1). Further, species composition by site shows that peri-urban sites from Kaniki; Kamalasha and Pima had one more species A*n. gibbinsi* not found in the two sites from Chipulukusu (Mapalo and Musalu). *Culex* were the most abundant mosquito collected from each site with (Kamalasha 67%, Pima 59%, Musalu 53% and Mapalo 42%). The second most abundant mosquito in Kaniki (Kamalasha 22% and Pima 30%) was *An. funestus s.l.* This was followed by *An. gambiae s.l.* (Kamalasha 7% and Pima 5%). Whilst from Chipulukusu, *Mansonia (24%)* and *An. gambiae s.s* (21%) were the second most abundant mosquito species collected from Mapalo and Musalu respectively (table 1).

Molecular identification of the malaria vectors collected revealed that 89%; (67/75) of the female *An. funestus s.l.* analysed amplified as *An. funestus s.s* at 505 base pairs and 85.5% (171/200) of *An. gambiae s.l* successfully amplified as *An. gambiae* s.s at 390 base pairs.

Biting and resting behaviour of malaria vectors

The mean number of *An. funestus s.s* seeking a blood meal from Mapalo was 2.42 times higher (*Mw* = 0.97/trap/night) than in Kamalasha (0.4/trap/night) and Pima (0.4/trap/night) and 9.7 times higher than in Musalu (0.1/trap/night) (table 1). For *An. gambiae s.s* the mean number of mosquitoes seeking a blood meal from Musalu (1.26/trap/night) were 1.5 more than in Mapalo (0.83/trap/night) and higher than in Kamalasha (0.1/trap/night) and Pima (0.14/trap/night), with differences of 12.6 and 9 times, respectively. Despite these variations in mean densities, the Kruskal–Wallis H test revealed no statistical difference in the host seeking behaviour of *An.*

funestus s.l. ($\chi 2 = 4.598$, df = 3, p = 0.204) across the four sites. However, a statistical difference was observed in the host seeking behaviour of *An. gambiae s.l.* ($\chi 2 = 12.566$, df=3, $p < 0.001$).

Indoor Resting Density of Malaria Vectors – PSC

The indoor resting density of *An. funestus s.l.* in Pima (1.2 mosquitoes per house) was 1.67 times higher than in Musalu (0.72 mosquitoes per house) whereas for *An. gambiae s.l.* in Musalu (1.31 mosquitoes per house), the indoor resting density was 262 times higher than in Pima (0.05 vectors per house) (Table 1). The Mann–Whitney U test indicated no statistical difference in the resting densities of *An. funestus s.l* between Pima and Musalu (Z = 143.5, p = 0.202), but statistical difference in the resting densities of *An. gambiae s.l* (Z = 56.5, $p < 0.019$) was observed between the two sites.

***Anopheles* mosquito larval habitats**

A total of 43 potential *anopheline* larval habitats were identified and 55.81% (*n=24*; 95 CI: 40% – 71%) of these found to contain larvae. All the larval habitats found to contain larvae were from either Musalu (70.83%; 95% CI: 49% – 87%) or Mapalo (29.12%; 95% CI: 13% – 51%) sites. Seven different categories of potential larval habitats identified included blocked trenches, foundation trenches, garden ponds, irrigation canals (channels), shallow wells, streams and tyre marks. From the different larval habitats, 2,643 larvae were collected from a total of 914 dips. The proportion of larvae collected from Musalu was 94.97% (2,510/2,643; 95% CI: 94% – 96%), whereas the remaining 5.03% (133/2,643; 95% CI: 4.2% – 5.9%) were collected from Mapalo (Table 2). Additionally, 63.94% (1,690/2,643; 95% CI: 62% – 66%) of the collected larvae were from irrigation canals, 20.92% (553/2,643; 95% CI: 19% – 22%) were from garden ponds, 10.93% (289/2,643; 95% CI: 9.7% – 12%) were from tire marks, 3.37% (89/2,643; 95% CI: 2.7 – 4.1%)

were from foundation trenches and 0.83% (22/2,643%; 95% CI: 0.52% – 1.3%) were from blocked trenches.

The larval density was highest in irrigation canals, with 4.67 larvae per dip, this was followed by garden ponds with 2.72 larvae per dip, tyre marks with 1.30 larvae per dip, foundation trenches with 1.25 larvae per dip and blocked drainages with 0.49 larvae per dip.

Factors affecting mosquito counts in housing structures

Seven predictors were utilised to identify associations with mosquito counts of *An. funestus s.s* and *An. gambiae s.s* in households, and only the spray status with Fludora® Fusion (B = -0.956, IRR = 0.384, p = 0.001) was found to be statistically significant (Table 3). While three other predictors were associated with reduced mosquito counts, including the number of people who slept in a house the previous night (B = -0.023, IRR = 0.978, p = 0.714), housing structures with a thatched roof (B = -0.060, IRR = 0.942, p = 0.870) and the number of LLINs in a housing structure (B = -0.085, IRR = 0.918, p = 0.489) these predictors were not statistically significant. On the other hand, the other three predictors; number of animals that slept in a house the previous night (B = 0.004, IRR = 1.004, p = 0.937), housing structures plastered with mud walls or unburnt bricks (B = 0.234, IRR = 1.264, p = 0.559) and housing structures with open eaves (B = 0.203, IRR = 1.225, p = 0.557) were associated with increased mosquito counts but not statistically significant.

Susceptibility status of *An. gambiae s.s*

The study showed full susceptibility 24 hours post exposure (100% mortality) to organophosphate (malathion 5% and pirimiphos-methyl 0.25%) and neonicotinoids (clothianidin). Conversely, resistance was confirmed to bendiocarb 0.1%, permethrin 0.75%, deltamethrin 0.75% and

Dichlorodiphenyltrichloroethane 4% (DDT) with corresponding mortalities of 23%, 14%, 18% and 4% respectively (Table 4). The area where the larvae used for susceptibility testing were collected from Musalu, an area predominantly known for urban agriculture practices.

DISCUSSION

This study reveals that the two main malaria vectors in Zambia *An. funestus s.l.* and *An. gambiae s.l.* were found in all four sites and these were molecularly identified as *An. funestus* s.s. and *An. gambiae* s.s. respectively. In Zambia, these mosquitoes have been implicated as the main vectors responsible for malaria transmission and have been found to exist in sympatry [20,21,31,32]. Surprisingly, *Anopheles funestus* was found to be the most abundant malaria vector in Ndola. Historically, the province has been dominated by *An. gambiae s.s* but this study found *An. funestus s.s* as the dominant malaria vector in Ndola. This finding is similar to other entomological findings in other districts within the Copperbelt province were *An. funestus s.s* is the more dominant vector [9,15]. However, *An. gambiae s.s.* remained the more dominant malaria vector in urban areas whereas *An. funestus s.s.* was more abundant in peri-urban areas, consistent with earlier studies conducted in sub-Saharan Africa [18,21]. This disparity in vector abundance could be attributed to variations in ecological habitats. *Anopheles gambiae* prefers to breed in man-made water habitats such as drainages, tire tracks, small pools and agriculture sites, whilst *An. funestus s.s.* prefers to breed in permanent and semi-permanent water habitats with some vegetative cover [33,34]. An earlier study in the northern parts of the country identified *An. funestus s.s* as the primary driver of malaria transmission in the dry season whereas *An. gambiae* s.s as the primary driver in the wet season [31]. Nonetheless, the existence of breeding grounds for *Anopheles gambiae s.s* in urban areas implies that even during the dry season, *An. gambiae s.s* will continue to be the primary driver of malaria transmission. The coexistence of these two malaria vectors pose

an increased year round risk of malaria transmission in the area. The recent increase in the incidences of malaria reported in Ndola could be attributed to the changing vector bionomics that now includes *An. funestus s.s.* not reported previously in the area.

Mosquito diversity was observed to be higher in peri-urban than urban sites with the inclusion of *An. gibbinsi,* a potential secondary malaria vector. This vector has been reported in other parts of the country as a potential secondary malaria vector [35–37]. Secondary malaria vectors have not been adequately considered in most vector control programming yet they contribute to 5% of malaria transmission in the southern African region [38]. Their contribution to transmission is significant making the need to incorporate interventions targeting secondary malaria vectors into vector control toolkits inevitable.

The host-seeking behaviours of *An. funestus s.s.* and *An. gambiae s.s.* were different. The host-seeking behaviour of *An. funestus* s.s. was found to be homogeneous across the four sites, whereas the host-seeking behaviour of *An. gambiae s.s.* was found to be much higher in urban sites with vast larval habitats. This heightened host-seeking behaviour of *An. gambiae s.s.* indicates an increased risk of disease transmission in urban sites compared to per-urban sites [39]. As such the need for enhanced vector control methods in urban settings with extensive larval habitats due to the elevated risk cannot be overemphasized.

The mean densities of *An. funestus* s.s found resting indoors were generally low across the peri-urban and urban sites. However, the indoor resting density of *An. gambiae* s.s in the urban site was much higher than that in the peri-urban site. Variations in the indoor resting behaviour of *An. funestus s.s.* and *An. gambiae s.s.* could be influenced by the presence of vast *An. gambiae s.s*

breeding sites in urban sites. Therefore, vector control interventions such as IRS and LLINs in such settings may need to be supplemented with larval source management [40].

The larval habitats that were active breeding sites were all from the two urban sites adjacent to a dambo. The larval habitats identified included irrigation canals (or irrigation channels), garden ponds, tire marks, foundation trenches and blocked drainages. However, irrigation channels and garden ponds were found to be the main mosquito breeding sites, similar to studies conducted in Ghana, Tanzania, Cote d'Ivoire and China [41]. However, the larval densities found in this study were higher than that found in China, possibly due to differences in the climatic conditions, variations in the bacterial diversity and physicochemical composition of the larval habitats [42]. These factors have been found to influence mosquito oviposition, survival and development into competent malaria vectors, thereby potentially impacting malaria incidence [3,43]. Unfortunately, this study only identified the different types of larval habitats; future research is needed to fully characterize larval habitats in order to generate additional information valuable for an effective and targeted larval source management programme.

The four predictors associated with reduced counts of malaria vectors in housing structures were; the number of people who slept in a housing structure, housing structures with a thatched roof, the number of LLINs used the previous night and housing structures sprayed with *Fludora*® *Fusion*. However, only housing structures sprayed were found to be statistically associated with reduced counts of malaria vectors, similar to what was found in Sao Tome and Principe [40]. Individuals who sleep in sprayed houses experience a lower vector-to-host contact, which entails reduced exposure to infectious mosquito bites unlike those sleeping in unsprayed houses. Additionally, maximum benefit is derived when at least 85% of houses are sprayed with an

efficacious insecticide to kill host seeking mosquitoes that rest indoors [44]. On the other hand, number of animals in a housing structure, housing structures with mud wall surfaces and open eaves were associated with increased counts of malaria vectors but were not statistically significant. Elsewhere, a study conducted in Cameroon associated open eaves and holes in the walls to increased mosquito counts [45]. Another study in Gambia also found that closing the eaves reduces mosquitoes entering thatched houses but increases mosquito entry into metal-roofed houses [46].

Susceptibility tests in this study reveal that *An. gambiae* s.s. was fully susceptible to organophosphates (malathion and pirimiphos-methyl) and neonicotinoids (clothianidin). This was also observed in several other districts in Zambia, where *An. funestus s.s.* and *An. gambiae s.s.* was found to be susceptible to these two classes of insecticides [20,22]. In that regard, organophosphates and neonicotinoids could be effective at controlling mosquito populations of *An. gambiae s.s* in Ndola and several other districts in Zambia, with evidence of susceptibility. However, resistance of *An. gambiae* s.s. to pyrethroids (permethrin and deltamethrin) and carbamates (bendiocarb) was confirmed and this could be attributed to the extensive use of pesticides and insecticides for agriculture and public health purposes. These results align with previous studies that found extensive insecticide resistance to pyrethroids and carbamates in the Copperbelt province [9,18]. In the wake of widespread resistance to pyrethroids and carbamates, there is reduced efficacy of the malaria vector control tools used and lowered community protection where carbamate and pyrethroid-only active ingredients are used. As such, this has the potential to drive transmission in Ndola District despite implementing these interventions.

Limitations of this study

The study was conducted in the dry season and the entomological indices determined may only apply for the dry season. Additionally, due to the scarcity of *An. funestus s.s* larval habitats this study did not determine the susceptibility status of *An. funestus s.s*.

CONCLUSION

The two primary malaria vectors *An. funestus* s.s. and *An. gambiae* s.s. were found to coexist in the two ecologically distinct settings, with *An. funestus* s.s. being the dominant malaria vector. This coexistence has the potential of sustaining high malaria transmission throughout the year especially in urban areas. Urban agriculture practices created *An. gambiae s.s* breeding sites during the dry season, contributed to the high host seeking and indoor resting behaviour in the urban sites. Sprayed housing structures were associated with reduced counts of malaria vectors. *Anopheles gambiae* was found to be susceptible to organophosphates and neonicotinoids, but resistance to pyrethroids, carbamates and organochlorides was confirmed. Additional studies are needed to investigate the different mechanisms of pyrethroid resistance in the area.

REFERENCES

1. WHO. World malaria report 2022. Geneva; 2022.
2. MOH. Health Management Information System of zambia [Internet]. Lusaka; 2020. Available from: https://www.nmec.org.zm/
3. Shimaponda-Mataa NM, Tembo-Mwase E, Gebreslasie M, Achia TNO, Mukaratirwa S. Modelling the influence of temperature and rainfall on malaria incidence in four endemic provinces of Zambia using semiparametric Poisson regression. Acta Trop. 2017 Feb 1;166:81–91.
4. Sitali L, Miller JM, Mwenda MC, Bridges DJ, Hawela MB, Hamainza B, et al. Distribution of Plasmodium species and assessment of performance of diagnostic tools used during a malaria survey in Southern and Western Provinces of Zambia. Malar J [Internet]. 2019;18(1):1–9. Available from: https://doi.org/10.1186/s12936-019-2766-2
5. MOH. Zambia National Malaria Indicator Survey 2021: MINISTRY OF HEALTH. Natl Malar Elimin Centre, Minist Heal Zambia [Internet]. 2021; Available from: papers2://publication/uuid/2E7FD35E-174F-4868-8A7F-7910082D3A11
6. Chipoya MN, Shimaponda-Mataa NM. Prevalence, characteristics and risk factors of imported and

local malaria cases in North-Western Province, Zambia: a cross-sectional study. Malar J 2020 191 [Internet]. 2020 Nov 23 [cited 2021 Jul 24];19(1):1–12. Available from: https://malariajournal.biomedcentral.com/articles/10.1186/s12936-020-03504-1

7. Cross DE, Thomas C, McKeown N, Siaziyu V, Healey A, Willis T, et al. Geographically extensive larval surveys reveal an unexpected scarcity of primary vector mosquitoes in a region of persistent malaria transmission in western Zambia. Parasites and Vectors [Internet]. 2021 Dec 1 [cited 2021 Apr 17];14(1):91. Available from: https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-020-04540-1

8. Das S, Muleba M, Stevenson JC, Norris DE. Habitat partitioning of malaria vectors in nchelenge district, Zambia. Am J Trop Med Hyg. 2016;94(6):1234–44.

9. NMEC. Insecticide Resistance Management and Monitoring Plan, Ministry of Health, Government Republic of Zambia. In 2019.

10. Fornadel CM, Norris LC, Franco V, Norris DE. Unexpected Anthropophily in the Potential Secondary Malaria Vectors An coustani s.l and An squamosus in Macha, Zambia. 2011;11(8).

11. Saili K, de Jager C, Sangoro OP, Nkya TE, Masaninga F, Mwenya M, et al. Anopheles rufipes implicated in malaria transmission both indoors and outdoors alongside Anopheles funestus and Anopheles arabiensis in rural south-east Zambia. Malar J [Internet]. 2023;22(1):1–12. Available from: https://doi.org/10.1186/s12936-023-04489-3

12. Chanda E, Masaninga F, Coleman M, Sikaala C, Katebe C, MacDonald M, et al. Integrated vector management: The Zambian experience. Malar J. 2008;7:1–8.

13. Chizema-Kawesha E, Miller JM, Steketee RW, Mukonka VM, Mukuka C, Mohamed AD, et al. Scaling Up Malaria Control in Zambia: Progress and Impact 2005–2008. Am J Trop Med Hyg [Internet]. 2010 Sep 7 [cited 2023 Apr 23];83(3):480–8. Available from: https://www.ajtmh.org/view/journals/tpmd/83/3/article-p480.xml

14. Masaninga F, Chanda E, Chanda-kapata P, Hamainza B, Masendu HT, Kamuliwo M, et al. Review of the malaria epidemiology and trends in Zambia. 2013;3(2):89–94.

15. PMI USAID. The President's Malaria Initiative (PMI) / Vectorlink; Zambia 2019-2020 Entomology Annual Report. Rockville, MD PMI VectorLink Proj Abt Assoc. 2020;

16. Barnes KI, Chanda P, Barnabas GA. Impact of the large-scale deployment of artemether / lumefantrine on the malaria disease burden in Africa : case studies of South. Malar J. 2009;7(October 2005):1–7.

17. Masaninga F, Chanda E, Chanda-kapata P, Hamainza B, Masendu HT, Kamuliwo M, et al. Review of the malaria epidemiology and trends in Zambia. 2013;3(2):89–94.

18. Thomsen EK, Strode C, Hemmings K, Hughes AJ, Chanda E, Musapa M, et al. Underpinning sustainable vector control through informed insecticide resistance management. PLoS One. 2014;9(6).

19. NMEC. National Malaria Elimination Strategic Plan 2022-2026. 2022;1(1):62.

20. PMI USAID. The President's Malaria Initiative (PMI) / Vectorlink; Zambia 2018-2019 Entomology Annual Report. Rockville, MD PMI VectorLink Proj Abt Assoc. 2019;(November 2018):37.

21. Stevenson JC, Pinchoff J, Muleba M, Lupiya J, Chilusu H, Mwelwa I, et al. Spatio-temporal

heterogeneity of malaria vectors in northern Zambia : implications for vector control. Parasit Vectors [Internet]. 2016;1–15. Available from: http://dx.doi.org/10.1186/s13071-016-1786-9

22. Chanda J, Saili K, Phiri F, Stevenson JC, Mwenda M, Chishimba S, et al. Pyrethroid and Carbamate Resistance in Anopheles funestus Giles along Lake Kariba in Southern Zambia. 2020;103(Suppl 2):90–7.

23. World Health Organization(WHO). Malaria surveillance, monitoring & evaluation: a reference manual [Internet]. Licence: C. 2018. Available from: http://apps.who.int/iris.%0ASales,

24. Zhou G, Minakawa N, Githeko A, Yan G. Spatial distribution patterns of malaria vectors and sample size determination in spatially heterogeneous environments: A case study in the west Kenyan highland. J Med Entomol. 2004;41(6):1001–9.

25. World Health Organization(WHO). Test procedures for insecticide resistance monitoring in malaria vector mosquitoes Second edition. 2018;

26. Brogdon W, Chan A. Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay. 2010;1–28.

27. Coetzee M. Key to the females of Afrotropical Anopheles mosquitoes (Diptera: Culicidae). Malar J [Internet]. 2020;19(1):1–20. Available from: https://doi.org/10.1186/s12936-020-3144-9

28. Koekemoer LL, Kamau L, Hunt RH, Coetzee M. A cocktail polymerase chain reaction assay to identify members of the Anopheles funestus (Diptera: Culicidae) group. Am J Trop Med Hyg. 2002;66(6):804–11.

29. Scott JA, Brogdon WG, Collins FH. Identification of single specimens of the Anopheles gambiae complex by the polymerase chain reaction. Am J Trop Med Hyg [Internet]. 1993 [cited 2021 Apr 30];49(4):520–9. Available from: https://pubmed.ncbi.nlm.nih.gov/8214283/

30. William C. THE USE OF LOGARITHMS IN THE INTERPRETATION OF CERTAIN ENTOMOLOGICAL PROBLEMS. Ann Appl Biol [Internet]. 1937;24(March 1936):404–14. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1744-7348.1937.tb05042.x

31. Muleba M, Mbata KJ, Stevenson JC, Norris DE. Spatial–temporal vector abundance and malaria transmission dynamics in Nchelenge and Lake Mweru islands, a region with a high burden of malaria in northern Zambia. Malar J [Internet]. 2023;22(1):1–11. Available from: https://doi.org/10.1186/s12936-023-04746-5

32. Fornadel CM, Norris LC, Glass GE, Norris DE. Analysis of Anopheles arabiensis blood feeding behavior in southern zambia during the two years after introduction of insecticide-treated bed nets. Am J Trop Med Hyg. 2010;83(4):848–53.

33. Doumbe-Belisse P, Kopya E, Ngadjeu CS, Sonhafouo-Chiana N, Talipouo A, Djamouko-Djonkam L, et al. Urban malaria in sub-Saharan Africa: dynamic of the vectorial system and the entomological inoculation rate. Malar J [Internet]. 2021;20(1):1–18. Available from: https://doi.org/10.1186/s12936-021-03891-z

34. Nambunga IH, Ngowo HS, Mapua SA, Hape EE, Msugupakulya BJ, Msaky DS, et al. Aquatic habitats of the malaria vector Anopheles funestus in rural south-eastern Tanzania. Malar J [Internet]. 2020;19(1):1–11. Available from: https://doi.org/10.1186/s12936-020-03295-5

35. Lobo NF, St. Laurent B, Sikaala CH, Hamainza B, Chanda J, Chinula D, et al. Unexpected diversity of Anopheles species in Eastern Zambia: implications for evaluating vector behavior and interventions using molecular tools. Sci Rep. 2015 Dec 9;5:17952.

36. Gebhardt ME, Krizek RS, Coetzee M, Koekemoer LL, Dahan-Moss Y, Mbewe D, et al. Expanded geographic distribution and host preference of Anopheles gibbinsi (Anopheles species 6) in northern Zambia. Malar J [Internet]. 2022;21(1):1–10. Available from: https://doi.org/10.1186/s12936-022-04231-5

37. Cooke MK, Kahindi SC, Oriango RM, Owaga C, Ayoma E, Mabuka D, et al. 'A bite before bed': exposure to malaria vectors outside the times of net use in the highlands of western Kenya. Malar J. 2015 Jun 25;14(1):259.

38. Afrane YA, Bonizzoni M, Yan G. Secondary Malaria Vectors of Sub-Saharan Africa: Threat to Malaria Elimination on the Continent? Curr Top Malar. 2016;(November).

39. Cummins B, Cortez R, Foppa IM, Walbeck J, Hyman JM. A spatial model of mosquito host-seeking behavior. PLoS Comput Biol. 2012;8(5).

40. Chen YA, Lien JC, Tseng LF, Cheng CF, Lin WY, Wang HY, et al. Effects of indoor residual spraying and outdoor larval control on Anopheles coluzzii from São Tomé and Príncipe, two islands with pre-eliminated malaria. Malar J [Internet]. 2019;18(1):1–16. Available from: https://doi.org/10.1186/s12936-019-3037-y

41. Wang Y, Cheng P, Jiao B, Song X, Wang H, Wang H, et al. Investigation of mosquito larval habitats and insecticide resistance in an area with a high incidence of mosquito-borne diseases in Jining, Shandong Province. PLoS One [Internet]. 2020;15(3):1–14. Available from: http://dx.doi.org/10.1371/journal.pone.0229764

42. Wang H, Wang Y, Cheng P, Wang H, Wang H, Liu H, et al. The Larval Density of Mosquitos (Diptera: Culicidae) in Jiaxiang County, Shandong Province, China: Influence of Bacterial Diversity, Richness, and Physicochemical Factors. Front Ecol Evol. 2021;9(February):1–12.

43. Hinne IA, Attah SK, Mensah BA, Forson AO, Afrane YA. Larval habitat diversity and Anopheles mosquito species distribution in different ecological zones in Ghana. Parasit Vectors [Internet]. 2021;1–14. Available from: https://doi.org/10.1186/s13071-021-04701-w

44. World Health Organization(WHO). an Operational Manual for Indoor Residual Spraying (Irs) for Malaria Transmission Control and Elimination Second Edition Indoor Residual Spraying. 2015;(2).

45. Ngadjeu CS, Ngadjeu CS, Doumbe-Belisse P, Doumbe-Belisse P, Talipouo A, Talipouo A, et al. Influence of house characteristics on mosquito distribution and malaria transmission in the city of Yaoundé, Cameroon. Malar J [Internet]. 2020;19(1):1–14. Available from: https://doi.org/10.1186/s12936-020-3133-z

46. Jatta E, Jawara M, Bradley J, Jeffries D, Kandeh B, Knudsen JB, et al. Articles How house design affects malaria mosquito density , temperature , and relative humidity : an experimental study in rural Gambia. Lancet Planet Heal [Internet]. 2015;2(11):e498–508. Available from: http://dx.doi.org/10.1016/S2542-5196(18)30234-1

47. Syme T, Gbegbo M, Obuobi D, Fongnikin A, Agbevo A, Todjinou D, et al. Pyrethroid-piperonyl butoxide (PBO) nets reduce the efficacy of indoor residual spraying with pirimiphos-methyl against pyrethroid-resistant malaria vectors. Sci Rep [Internet]. 2022;12(1):1–13. Available from: https://doi.org/10.1038/s41598-022-10953-y

ABBREVIATIONS

ACTs : artemisinin combined therapy; B : Regression Coefficient; CDC – LT : Centres for Disease Control and Prevention light traps; CI : Confidence Interval; DDT : Dichlorodiphenyltrichloroethane; df : degrees of freedom; DNA : Deoxyribonucleic acid; IRR : Incidence rate ratio; IRS : Indoor residual spraying; ITNs : Insecticide treated bednet; LLINs : Long lasting insecticide treated nets; Mw : Williams mean; NHRA : National Health Research Authority; NMEC : National Malaria Elimination Centre; P : Probability value; PBO : piperonyl butoxide; PCR : Polymerase Chain Reaction; PSC : Pyrethrum Spray Catches; *s.l : sensu lato; s.s : sentu stricto;* SPSS : Statistical Package for Social Sciences; TDRC : Tropical Diseases Research Centre; WHO : World Health Organisation.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical clearance for the protocol (Supplementary file 1) used to undertake this study was obtained from the Tropical Diseases Research Centre Ethics Committee Reference No. TRC/C4/06/2021 (Supplementary file 2) and the National Health Research Authority Ref No: NHRA000016/29/06/2021 (Supplementary file 3). Written consent (supplementary file 4) was obtained from the head of the house prior to mosquito and larvae collection from their houses and their gardens, respectively. All data that was collected was restricted to the investigators and confidentiality was strictly maintained.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

FUNDING

This work did not receive any funding.

CONFLICTS OF INTEREST / COMPETING INTERESTS

Financial interests: The authors as well as the funders declare that they have no financial or non-financial interests to disclose and they have no conflicts of interest to declare that are relevant to the content of this manuscript.

AUTHORS' CONTRIBUTIONS

WH: Participated in study design, conducted sample collection, analysis and interpretation. Participated in drafting the manuscript and final approval of the revised manuscript. Also acted as guarantor

NMS-M: Participated in study design, supervised implementation of the project, interpretation of the results, review of the manuscript and final approval of the revised manuscript.

MM: Participated in study design, supervised implementation of the project, interpretation of the results, review of the manuscript and final approval of the revised manuscript.

VD: Participated in data analysis, review of the manuscript and final approval of the revised manuscript

MH: Participated in data analysis, drafting of the manuscript and final approval of the revised manuscript

ACKNOWLEDGEMENTS

We acknowledge the Ministry of Health Provincial Health office and the Health Centres for approval of the study and provisions towards it. Additionally, we acknowledge the support rendered by Tropical Diseases Research Centre (TDRC) and a Pan African Mosquito Control Association (PAMCA) for the laboratory supplies used in processing the samples.

AUTHORS' INFORMATION AND AFFILIATION

WH (Westone Hamwata[1,2]; phamwata@gmail.com) is a Medical Parasitologist and Entomologist based at the Tropical Diseases Research Centre and holds a MSc in Medical Parasitology and Master of Public Health.

MM (Mbanga Muleba[1]; mbangamuleba@gmail.com) is a Medical Entomologist at Tropical Diseases Research Centre and holds a PhD in Entomology

MH (Mwendalubi Hazyondo[2,3]; mwendalubihazyondo@gmail.com) is a Principal Wildlife Veterinary Officer at the Department of National Parks and Wildlife and hold a Doctor of Veterinary Medicine and Zootechnics

VD[4] (Victor Daka; dakavictorm@gmail.com) is Lecturer at The Copperbelt University, Michael Chilufya Sata School of Medicine, Department of Public Health and holds a PhD in Epidemiology.

NMS-M[2] (Nzooma M. Shimaponda-Mataa1; nzomataa@gmail.com) is a Medical Parasitologist and Lecturer at the University of Zambia and holds a PhD in Parasitology;

1. Tropical Diseases Research Centre, Department of Biomedical Sciences, Vector Biology and Parasitology Unit.

2. University of Zambia School of Health Sciences, Department of Biomedical Sciences, Ridgeway Campus, Lusaka, Zambia.

3. Department of National Parks & Wildlife, Chilanga, Zambia.

4. The Copperbelt University, Michael Chilufya Sata School of Medicine, Department of Public Health, Ndola Campus, Zambia.

LIST OF TABLES

Table 1: Entomological indices

Entomological Indices	Chipulukusu catchment		Kaniki catchment		Total
	Mapalo	Musalu	Kamalasha	Pima	
Species composition					
An. funestus s.l	45	23	15	40	123
An. gambiae s.l.	24	71	5	6	106
An. gibbinsi	0	0	1	1	2
Culex	88	181	45	78	392
Mansonia	51	63	1	6	121
Total	208	338	67	131	744
Mean densities of malaria vectors seeking a blood meal *(Mw)*					
An. funestus s.l	0.97*	0.10*	0.40*	0.40*	-
An. gambiae s.l.	1.83*	1.26*	0.10*	0.14*	-
Mean densities of malaria vectors resting indoors *(Mw)*					
An. funestus s.l	-	0.72*	-	1.2*	-
An. gambiae s.l.	-	1.31*	-	0.05*	-

**Mw* = densities using Williams mean

Table 2: Mosquito larval habitats

Site	Mosquito genera

	Type of larval habitat	No. of dips	Number of larvae collected (%)	Larval habitats identified	larval habitats with larvae	*Anopheles larvae*	*Culex larvae*
Mapalo	Foundation trenches	71	89 (3.37)	5	4	3	4
	Tyre marks	86	31 (1.17)	2	2	2	2
	Blocked drainages	18	13 (0.49)	1	1	1	1
	Subtotal	175	133 (5.03)	8	7	6	7
Musalu	irrigation canals	362	1690 (63.94)	19	11	9	11
	Tyre marks	147	258 (9.76)	3	2	2	2
	Garden ponds	203	553 (20.92)	4	3	3	3
	Blocked drainages	27	9 (0.34)	1	1	1	1
	Subtotal	739	2510 (94.97)	27	17	15	17
Kamalasha	Tyre marks	-*	0 (0.0)	5	0	0	0
	Subtotal	-*	0 (0.0)	5	0	0	0
Pima	Shallow wells	-*	0 (0.0)	2	0	0	0
	Stream	-*	0 (0.0)	1	0	0	0
	Subtotal	-*	0 (0.0)	3	0	0	0
Total from all sites		914	2643 (100)	43	24	21	24

**No larvae found after visual inspection followed by 10 dips*

Table 3: Predictors affecting Mosquito Counts of *An. funestus s.s* and *An. gambiae s.s*

Parameter	Regression coefficient (B)	Hypothesis Test			IRR**	95% Wald Confidence Interval for Exp(B)	
		Wald Chi-Square	df*	Sig.		Lower	Upper
(Intercept)	1.919	3.876	1	0.049	6.816	1.009	46.067
Number of People	-0.023	0.134	1	0.714	0.978	0.866	1.104
Number of Animals	0.004	0.006	1	0.937	1.004	0.912	1.105
Type of Roof	-0.060	0.027	1	0.870	0.942	0.462	1.921

Type of Wall	0.234	0.342	1	0.559	1.264	0.577	2.769
Type of Eaves	0.203	0.345	1	0.557	1.225	0.622	2.412
Number of LLINs***	-0.085	0.478	1	0.489	0.918	0.721	1.169
Spray Status	-0.956	10.513	1	0.001	0.384	0.216	0.685
(Scale)	1[a]						
(Negative binomial)	0.798						

Dependent Variable: No. of Malaria Vectors
Model: (Intercept), No. of People, No. of Animals, Type of Roof, Type of Wall, Type of Eaves, No. of LLINs, Spray Status
Fixed at the displayed value.
*df = degrees of freedom
**IRR = Incidence rate ratio
***LLINs = long lasting insecticide nets

Table 4: Susceptibility Status of *An. gambiae s.s* from Musalu

Insecticide tested	Number of mosquitoes exposed	Knockdown at 60min		Knockdown at 24hours		Final Mortality (24 hours)
		Dead	Alive	Dead	alive	
Bendiocarb 0.1%	108	14	94	25	83	23%
DDT 4%	113	0	113	5	108	4%
Deltamethrin 0.05%	100	13	87	18	82	18%
Permethrin 0.75%	113	6	107	16	97	14%
Pirimiphos-methyl 0.25%	110	101	9	110	0	100%**
Malathion 5%	104	94	10	104	0	100%**
Clothianidin	107	104	3	107	0	100%**

**Fully susceptible

University of Zambia
School of Health Sciences
Department of Biomedical Sciences

Malaria Vector Bionomics and Phenotypic Resistance Status to Insecticides Used in Vector Control in Ndola District.

A Research Project Proposal Submitted in Partial Fulfilment for the Master of Science Degree in Medical Parasitology

Westone Phanuel Hamwata
19000762

Name of Supervisor: Dr. N. M. Shimaponda-Mataa

Name of Co-Supervisor: Mr Mbanga Muleba (TDRC)

Lusaka
(April, 2021)

EXECUTIVE SUMMARY

Malaria is a vector-borne disease transmitted to human through the bite of an infected female *Anopheles* mosquito. It is endemic in the tropical and subtropical regions of the world with 94% of the disease burden found in Africa. Zambia contributes to 2% of the global burden of Malaria and is among the top 20 countries with the highest malaria disease burden in the world. The use of insecticide-based vector control has shown great success at controlling malaria from the 1950s to date and this has relied on a clear understanding of mosquito ecology and behaviour of the local malaria vectors.

Insecticide-based vector control is the main malaria elimination strategy identified to achieve a malaria free Zambia. However, the extensive use of insecticide-based vector control results into an increase in the population of resistant malaria vectors and behavior modification for mosquito survival. Therefore, this study will assess the malaria vector bionomics and phenotypic resistance to insecticides used in vector control in Ndola.

This will be a cross sectional study conducted in Ndola district and mosquitoes will be collected using CDC light traps, Pyrethrum Spray Catches, Aspirations and larval collection. Multiplex PCR will be used for determination of sibling species of the *An gambiae* and *An funestus* complex and for determination of blood meal sources. Data analysis will be done in Microsoft excel and stata version 14. A zero-inflated negative binomial regression model will used to analyze the mosquito count against the independent variables.

Contents

1.0 Introduction

1.1 Background

Malaria is one of the diseases of public health importance in the tropical regions. In 2019, the burden was estimated at 229 million malaria cases and 409, 000 deaths. The highest malaria disease burden (94%)of these cases and deaths) was from Africa and Zambia contributed 2% of this global malaria disease burden (WHO, 2020). This disease is caused by a protozoan parasite of the genus *Plasmodium* and is transmitted to humans through the bite of an infected *Anopheles* mosquitoes. There are about 40 species from the *Anopheles* genera known to transmit malaria (WHO, 2019). In Zambia the two main malaria vectors are *An funestus s.l* and *An gambiae s.l* (Das *et al.*, 2016). However, other *Anopheles* mosquitoes *An coustani* and *An squamosus* have shown to be more anthropophilic but tested negative for circumsporozoites (Fornadel *et al.*, 2011).

Vector control has played a significant role in reducing the global malaria disease burden (Wilson *et al.*, 2020). An estimated 1.5 billion malaria cases and 7.6 million malaria related deaths have been averted since 2000. Africa has recorded a reduction in the number of malaria cases and malaria deaths from 362.8 to 225.2 per 1000 and 121.1 to 40.3 per 100, 000 respectively (WHO, 2020). This has to a greater extent been attributed to concerted efforts in vector control (Wilson *et al.*, 2020).

To achieve a malaria free Zambia, the National Malaria Elimination Centre (NMEC) has adopted the use of Indoor Residual Spraying (IRS) and use of long-lasting insecticide treated nets (LLINs) as the main vector control strategies (Chipoya and Shimaponda-Mataa, 2020; NMEC, 2017). These interventions are aimed at reducing the human to vector interactions by creating a barrier and killing host seeking mosquitoes that rest on a sprayed wall surface or on LLINs. By doing this malaria infected host seeking mosquitoes die without making contact with the human host, thus

reducing the population of malaria vectors infected with the parasite as well as the humans getting infected.

The successful implementation of vector control strategies is hinged on a clear understanding of the ecology and behavior of malaria vectors in different geographical locations (Wilson *et al.*, 2020). Once their ecology and behaviour are known, the vector control strategies employed target one or more vulnerabilities in the behavior of the vector. Further, mosquito behaviours have been shown to change when exposed to elements that threaten their survival (Sougoufara *et al*., 2020). Therefore, routine vector surveillance studies should be conducted to ensure that malaria vector behaviours and their ecology are well understood prior to deployment of any vector control strategy. It is against this background that this study is proposed, to generate knowledge on the behavior and phenotypic resistance of malaria vectors in Zambia.

1.2 Statement of the Problem

Zambia is among the top 20 countries with a high malaria disease burden contributing 2% of the global malaria burden (WHO, 2020). These malaria cases are mainly in the provinces closer to the Democratic Republic of Congo (DRC) and in the northern parts of the country (MOH, 2019).

The government has identified vector control as the main malaria elimination strategy. This strategy heavily relies on spraying at least 80% of eligible structures through IRS and universal coverage of LLINs (NMEC, 2017). However, the extensive use of insecticide-based vector control leads to mosquito behavior modification and increase in the selection of resistant malaria vectors (WHO, 2019). Therefore, vector surveillance should be conducted to monitor the malaria vector behaviour and selected resistant malaria vectors. However, the last entomological activity conducted in Ndola was 8 years ago to determine the susceptibility status of malaria vectors and it was found that *An gambiae s.l* was resistant to carbamates (Thomsen *et al.*, 2014). As it stands,

there is no data on mosquito species composition, their behavior and insecticide resistance status of the malaria vectors. Therefore, it is uncertain whether the vector control interventions implemented target the malaria vectors and their current behavior.

1.3 Rationale of the Study

The IRS and LLINs strategy adopted for malaria control should be guided by data generated on the current malaria vector behaviour and their susceptibility status to insecticides used in vector control. Therefore, the proposed survey will identify the mosquitoes responsible for malaria transmission in Ndola to subspecies level, assess their current behavior and determine their phenotypic resistance status to the different insecticides used in malaria control.

The results generated will be useful in ensuring that vector control planning is based on updated information on the species, behavior and susceptibility status of local malaria vectors to insecticides used in malaria control. Further, the study findings will serve as baseline entomological survey to guide the possible resumption of regular surveys.

2.0 Research Questions

1. What is the species composition and density of malaria vectors in Ndola?
2. What are the biting and resting behaviours of mosquitoes in Ndola District?
3. What are the blood meal sources for mosquitoes in Ndola?
4. What is the insecticide resistance status of primary malaria vectors?

3.0 Objectives

3.1 General Objective

The main objective is to evaluate malaria vector behavior and their phenotypic resistance status to insecticides used in vector control in Ndola.

3.2 Specific Objectives

1. To determine species composition and vector density of malaria vectors in Ndola
2. To assess the biting and resting behaviour of malaria vectors in Ndola District
3. To assess host preference of blood feeding mosquitoes in Ndola
4. To evaluate the insecticide resistance status of primary malaria vectors in Ndola.

4.0 Literature Review

4.1 Global Malaria Situation

Diseases transmitted by vectors account for 17% of all infectious diseases in the world (Eder *et al.*, 2018). About 700,000 deaths occur annually from pathogens transmitted by different vectors including mosquitoes, ticks, triatome bugs, snails, fleas, sandflies, tsetse flies, lice and black flies (Benelli *et al.*, 2020). Mosquitoes alone are responsible for 8 infections affecting man and these are chikungunya, dengue, lymphatic filariasis, rift valley fever, yellow fever, malaria, Japanese encephalitis and west nile fever. In 2019, 229 million cases of malaria were recorded and there were 385,000 deaths as a result of malaria infections (WHO, 2020). The WHO African region recorded the highest malaria cases of 215 million cases accounting for 94% of the global malaria diseases burden. The majority of these cases where only in 29 countries with Nigeria (27%) and the Democratic Republic of congo (12%) having the most malaria cases in the world. The remaining WHO regions South East Asia, Eastern Mediterranean, Western Pacific and Region of the Americans contributed to 6% of the global malaria disease burden.

The implementation of malaria control interventions has seen a significant reduction in the malaria incidence from 80 per 1000 in 2000 to 58 per 1000 in 2019 (WHO, 2020). This has been to a greater extent been attributed to vector control and the World Health Organization continue to put great emphasis on the need for improving these tool in order to effectively achieve malaria control (Wilson *et al.*, 2020).

Zambia has also recorded a reduction in the malaria prevalence in under five children from 15% in 2015 to 9% in 2018. This reduction could be attributed to the increase in the number of household that have received a treated bed net and/or IRS. However, Luapula province recorded the highest malaria prevalence of 30%. Copperbelt on the other hand recorded a reduction in the

prevalence from 15.2% in 2015 to 7.7% in 2018 despite having the lowest bed net ownership rate of 55.3% (MOH, 2015)(MOH, 2019).

Further, a study showed that in the first 10 months of 2020, Zambia recorded an estimated 3.7 million cases of confirmed malaria cases. This rise in the number of confirmed malaria cases could be associated to the effects that COVID-19 had on efforts aimed at curbing malaria (Chisaya *et al.*, 2020).

4.2 Introduction to Mosquitoes

Mosquitoes taxonomically belong to the class insecta, order diptera, family Culicidae. These belong to five mosquito genera namely *Anopheles*, *Culex*, *Aedes*, *Mansonia* and *Conquillettidia* (Service, 1996; WHO, 2014). There about 3500 different species of mosquitoes worldwide, 400 of these belong to the *Anopheles* genera and only 40 of them have been implicated as malaria vectors (WHO, 2019). Mosquitoes require blood for the maturation of their eggs and its this host seeking behaviour that makes them ideal vectors of disease (O'Donnell *et al.*, 2019; Barry, 1996).

4.2.1 Life cycle of *Anopheles* Mosquito

The mosquito undergoes four distinct stages in their development; the egg, larvae, pupa and the adult stage. The first three stages of the life cycle of the mosquito are aquatic and the adult stage is the only terrestrial (Durden and Mullen, 2018). The time taken for the mosquito to metamorphose from one development stage to the next is dependent on a number of factors but most importantly is temperature, humidity and diet (Service, 2012).

a) **Egg Stage**: *Anopheles* mosquitoes lay an estimated 200 eggs in one oviposition in water. These eggs are laid singly and have floaters (Service, 2012). These eggs hatch in 2 – 3 at

optimum environmental conditions (27 °C ± 2, 80% ± 10 relative humidity (RH)) but can take as long as 14 days in cold temperatures (Mazigo *et al.*, 2019).

b) Larval Stage: The eggs hatch into larvae and the larvae will pass through 4 larval instars in this stage. This is the feeding stage of the immature stage of the mosquito and lasts for a period of 8 to 10 days at optimum environmental conditions. The larvae lay horizontal to the water surface and they breath through the spiracles along the body of the larvae. From this stage the larval will undergo metamorphosis and develop into pupa (Durden and Mullen, 2018).

c) Pupa Stage: This is the non-feeding stage in the life cycle of the mosquito and exist as a *cephalothorax.* The pupa breath through the respiratory trumpets on the apical end of the cephalothorax. The pupa speeds most of its time on the water surface taking in air and if disturbed they swim in a jerk fashion (up and down). This stage also takes 2 to 3 days at optimum environmental conditions (Service, 2012).

d) Adult Stage: The pupae then emerge into adult mosquitoes normally at dusk and then the females fly into a swam of males for mating. Once the female mosquito mate, they lay eggs throughout their lifespan. They have several blood meals depending on the length of their gonotrophic cycle (Service, 2012).

4.3 Malaria Vectors

4.3.1 Global Malaria Vectors

The distribution of malaria vectors in the world differ from continent to continent. In the Americans region *An darlingi* is the most efficient malaria vector of *P. falciparum* and *P. vivax.*. It is a highly anthropophilic malaria vector and its natural habitats are the shady areas of streams and ponds. This mosquito can breed in both clear and muddy water with floating vegetation. Other

malaria vectors in this region are *An nuneztovaris s.l, An albitarsis* found in South America. (Alimi *et al.*, 2015). In Asia, the most efficient malaria vector is *An stephensi* found to thrive most in urban areas (Alimi *et al.*, 2015). The breeding sites for this mosquito include man made water habitats such as wells, water receptacles, overhead tanks, fountains, barrels and tins and polluted water in drainages (Thomas *et al.*, 2016). Other malaria vectors include *An culicifacies s.l, An darius s.l* and *An maculatus s.l* (Sinka *et al.*, 2012).

4.3.2 Malaria Vectors in Africa

In Africa *An gambiae* and *An funestus* complex are the main malaria vectors of malaria (Mzilahowa *et al.*, 2012). *An gambiae s.l* prefers to breed in small temporal water collections such as gardens, hoof prints, tire marks and a dirty road (Ndiaye *et al.*, 2020). *Anopheles funestus s.l* on the other hand breeds in semi-permanent water bodies such as dams created from road construction, swamps, marshes and in the edges of a stream where the water is not moving (Fillinger *et al.*, 2009) (Mattah *et al.*, 2017).

There is now a threat of *An stephensi* a primary malaria vector in Asia (Balkew *et al.*, 2020). This new malaria vector poses a threat of increased urban malaria in Africa as it thrives in urban areas (Sinka *et al.*, 2020). In African countries *An. funestus* complex has shown to be abundant throughout the year and *An. gambiae* complex has shown to be most abundant in the wet season (Mzilahowa *et al.*, 2012). These two *Anopheles* complexes have been well studied and understood and this led to discovery of sibling species within these complexes. The *An. funestus* complex has 9 sibling species in Africa and these include *An funestus s.s, An rivolurum, An leesoni, An vaneedeni, An aruni, An fuscivenous, An parensis* and *An brucei*. Studies further show that *An funestus s.s* is the predominant and widely distributed malaria vector in Africa. *An leesoni, An*

revolurum, An parensis and *An vaneedeni* are the other sibling species of the *An funestus* complex in Africa (Kweka, et al, 2013).

On the other hand, there has been a reduction in the malaria vector densities and species composition of the *An gambiae* complex due to vector control interventions (Derua *et al.*, 2015). *An gambiae s.s, An arabiensis* and *An coluzzi* are the other malaria vectors in the *An gambiae* complex transmitting *Plasmodium falciparum* and *Plasmodium vivax* (Wiebe *et al.*, 2017). Other sibling species in the *An gambiae* complex are *An quadrianulatus A, An. amharicus, An merus, An melas* and *An bwambae* (Ebenezer *et al.*, 2014). *An gambiae s.s, An arabiensis, An coluzzi, An quadrianulatus A, An quadrianulatus B* are fresh water malaria vectors. *An melas* and *An merus* are salt water malaria vectors and *An bwambae* is only found in Uganda (Bartilol *et al.*, 2021).

The number of secondary malaria vectors in Africa vary with geographical locations and very little is known about these vectors (Mwangangi *et al.*, 2013b). Studies have shown that due to the lack of a proper understanding of their ecology and behavior they have contributed greatly to residual transmission in areas with low malaria transmission (Ayuya *et al* 2021). The secondary malaria vectors of public health importance in Africa include *An coustani, An Ziemani, An pharoensis, An maculipalpis* (Afrane *et al.*, 2016).

4.3.3 Malaria vectors in Zambia

An. funestus and *An. gambiae* complexes are the primary malaria vectors in Zambia (Cross *et al.*, 2021). *An. funestus s.l* is the predominant malaria vector in 8 of the 10 provinces in Zambia. The main malaria vector on the Copperbelt province is *An gambiae s.s* and *An arabiensis* in the Southern Province (NMEC, 2019). The provinces were *An funestus s.l* is the predominant malaria vector are the same provinces with the highest malaria prevalence. Other malaria vectors

potentially identified as secondary vectors have been identified through their highly anthropophilic and endophagic behaviours. *Anopheles leesoni* a zoophilic mosquito mainly found in Luapula province assumes the role of a malaria vector when its preferred blood meal source is not available (Das *et al.*, 2016). Other anopheles mosquitoes that are mainly zoophilic and exhibits a high exophagic behaviour are *An squamosus, An rufipes, An coustani, An pretoriensis* and *An quadrianuratus* (Stevenson *et al.*, 2016) (Lobo *et al.*, 2015). Further, studies have shown that other than being more zoophilic and exophagic *An arabiensis, An coustani* and *An pretoriensis* are early outdoor bitters (NMEC, 2019) (Fornadel *et al.*, 2011).

4.4 Malaria Vector Bionomics

Vector bionomics refers to the ecology of malaria vectors and their resting, biting and host preference (Massey *et al.*, 2016). Mosquitoes have been known to modify their behavior when exposed to elements that do not favour their proliferation (Sougoufara *et al*., 2020). This to a larger extent has been attributed to vector control interventions that are implemented to control mosquito borne diseases (Thomsen *et al.*, 2017). The most efficient malaria vectors in the sub-Saharan Africa have been found to be highly anthropophilic. They feed indoors and rest indoors after having their blood meals (Sougoufara, *et al*., 2020). However, in Tanzania, *An arabiensis* a zoophilic mosquito was found to prefer biting outdoors and resting outdoors (Limwagu *et al.*, 2019). The deployment of LLITNs in Senegal was associated with a change in biting time from biting in the night to biting in broad day time. Further, there was no indication of the change in their behaviour from being highly anthropophilic and endophilic (Sougoufara *et al.*, 2014). Another study in Tanzania revealed a change in biting behaviour within *An gambiae* complex from 1997 to 2009. The night biting activity of the *An. gambiae* complex was high in 1997 but in 2009 there was a shift to only *An gambiae s.s* and *An arabiensis* being the only malaria vectors

predominantly biting late in the night (Russell *et al.*, 2011). Another study in Benin revealed that there was an increase in mosquito biting in broad day light after the introduction of LLITNs (Moiroux *et al.*, 2012).

A study in the Pan American Health Organization region found that land use change, climate change, deforestation, loss of forest cover has affected distribution of malaria vectors causing a shift in behaviour thereby increasing human to vector contact (Alimi *et al.*, 2015).

4.4.1 Species Composition

A study in Kenya showed that there was a change in the predominant malaria vector from *An funestus s.s* and *An gambiae s.s* to *An arabiensis* and *An merus* over a period of 20 years. Further, a notable shift in the feeding behaviour from humans to animals of *An gambiae s.l* (99% to 16%) and *An funestus s.l* (100% to 3%). This could be attributed to the shift in the species composition recorded from *An gambiae s.s* as the predominant malaria vector to *An arabiensis* (Mwangangi *et al.*, 2013a). Further, another study in Uganda showed a change in the species composition of malaria vector after the implementation of IRS and LLITNs. Before the interventions the predominant malaria vector was *An. gambiae s.s* (76.7%) but after the intervention, 99.5% of the mosquitoes collected were *An arabiensis* and *An gambiae s.s* was 0.5% (Musiime *et al.*, 2019).

4.4.2 Malaria Vector Density

A study in Uganda revealed that the distribution of LLITNs was associated with a reduction in the *An funestus s.l* vector density from 0.07 per house per night to 0.02 per house per night but not for *An gambiae s.l*. This revealed that *An funestus s.l* was more affected as compared to *An gambiae s.l* (Mawejje *et al.*, 2021). The introduction of vector control interventions in Uganda led to the reduction in the vector density of *An. gambiae s.l* from 76.7% before the intervention to 0.5% post intervention (Musiime *et al.*, 2019). A study in Zambia revealed that there was a reduction in

vector density of *An quariannulatus* from 95.1% to 69.7% following implementation of IRS. However, there was a proportionate increase in number of *An. arabiensis* collected from 3.9% to 95.1% from the total mosquitoes collected in the *An gambiae* complex (Chinula *et al.*, 2018).

4.4.3 Resting Behaviour

Following the deployment of LLITNS in Western Kenya, a study revealed that there was a higher indoor resting density for *An. gambiae s.l* and *An. funestus s.l*. The introduction of LLITNs did not have an effect on the indoor resting behaviour of malaria vectors (Machani *et al.*, 2020). The results from this study indicate that there is need to implement an intervention that will also target indoor resting malaria vectors. A study in Western Kenya were a large proportion of the malaria vectors rest indoors revealed a reduction in the indoor resting density post IRS implementation (Abong'o *et al.*, 2020).

4.4.4 Biting Behaviour

Use of LLINs is the most appropriate vector control tool to deploy where mosquitoes predominantly endophilic. However, a study conducted in Senegal revealed that the deployment of vector control had no effect on the endophilic behaviour of the malaria vectors (Sougoufara *et al.*, 2014). Further, a study revealed that 60% of *An. Arabiensis* were able to successfully have their blood meals and an estimated 50% of these blood meals were taken from outside the house (Killeen *et al.*, 2016).

4.4.5 Host preference

Malaria vectors have their preferred hosts for their blood meals and they will only feed on another host in the event that their preferred host is unavailable. *An. arabiensis* a highly zoophilic mosquito prefers to feed on cattle but in the event that there is no cattle nearby they will feed on humans (Killeen *et al.*, 2016). Another study in conducted in Kenya were human and domestic animals

were sharing the house revealed that mosquitoes fed on any available blood meal source host human, goat or bovine. 53.1% of the *An gambiae s.s* fed on humans, 26.5% fed on goats and 18.4% fed on bovine (Ndenga *et al.*, 2016). In Honduras it was found that most of *Anopheles* mosquitoes collected had multiple blood meals and that only 24.9% had fed on a single host. The Anopheles mosquitoes preferred to feed on either chickens or bovine. The Human blood index in this study was found to be 22.1% (Escobar *et al.*, 2020).

4.5 Insecticide-Based Vector Control

Vector Control is the main method of controlling malaria and it has played a significant role in the reduction of vector-borne diseases from the 1800s. This was achieved through a clear understanding of the behaviour and ecology of the different vectors of disease. However, after the 1940s to date, malaria vector control has significantly depended on insecticide-based interventions and this has brought in the challenge of insecticide resistance (Wilson *et al.*, 2020). Historically, organophosphates, organochlorines, carbamates and pyrethroids were the four classes of insecticides approved by WHO for use in public health vector control (Corrêa *et al.*, 2019). Pyrrole (Chlorfenapyr 240 SC) and Neonicotinoids (Chlothianidin WG) have been prequalified by WHO for use in public health vector control (WHO, 2013). In Africa, the main vector control strategies employed target the adults mosquitoes and the immature stages to a lesser extent.

4.5.1 Control of Adult Mosquitoes

a) **Use of treated bed nets:** Pyrethroids and pyrroles are the only two classes of insecticides approved by WHO for use in LLINs. These classes of insecticides have shown to pose very low risk to humans yet providing the desired lethal effect to arthropods of public health importance (CDC, 2019). The use of bed nets has shown to significantly reduce malaria prevalence in areas where the malaria vectors predominantly bite indoors and late at night

(Ntonifor and Veyufambom, 2016; Steinhardt *et al.*, 2017). The treated bed net creates a barrier between the vector and the human host and produces a toxic effect to the malaria vectors that rests on the bed net (Paaijmans and Huijben, 2020).

b) Indoor Residual Spraying: Indoor Residual Spraying involves the application of a predetermined amount of insecticide with a residual effect on a wall surface (Tangena *et al.*, 2020). In this intervention organophosphates, organochlorines, carbamates, pyrethroids, pyrroles and neonicotinoid have been prequalified for use in IRS (WHO, 2013). This intervention targets host seeking mosquitoes that rest indoors after a bloodmeal. Once the mosquito rests on a sprayed wall surface, the mosquito absorbs the insecticide through its legs and the mosquito dies (Phiri *et al.*, 2015). This intervention protects the next person from getting an infectious bite by killing the infected mosquito (Pinchoff *et al.*, 2016).

c) **Use of symbionts:** The use of symbionts for the control of mosquito borne diseases still remain largely unexplored (Ricci *et al.*, 2012). Endosymbionts have been shown to interrupt transmission in their natural or engineered form. In Anopheles a Wolbachia strain (wAnga-Mali) significantly reduces the prevalence and intensity of sporozoites on field collected mosquitoes (Gomes *et al.*, 2017). Another bacteria in its engineered form *Pantoea agglomerans* successfully inhibits the development of plasmodium falciparum by 98% in *Anopheles gambiae s.l.*(Wang *et al.*, 2012) . In a more recent study, *Microsporidia MB* naturally occurring in *An arabiensis* was found to completely interrupt transmission of the malaria parasite (Herren *et al.*, 2020).

4.5.2 Control of immature stages of the malaria vectors

a) **Use of predators:** The use of predator fish in malaria control has not shown any statistical significant result on the malaria infection rates, entomological inoculation rate or the adult vector density in the areas where they have been studied (Walshe *et al.*, 2017). However, a study in western Kenya revealed that biocontrol was able to reduce the density of larvae and pupae (Howard *et al.*, 2007).

b) **Pathogens and parasites:** This is a widely practiced larval source management intervention targeting the larval stages of the mosquito (Service, 2012). *Bacillus thuringiensis var. israelensis (Bti)* and *Bacillus spharicus* have been found to be effective at controlling larvae and was well received and accepted in communities. These pathogen has been found to be ecofriendly and ease to produce in large numbers (Derua *et al.*, 2019). This intervention has been shown to be effective especially with the rise of insecticide resistance to pyrethroids (Zhou *et al.*, 2020).

c) **Use of Oils and Surface films:** This is an ancient mosquito larvae control that was mainly practiced in the Americas and India. It involves the application of a petroleum oil or isotearyl alcohol on the surface of the water so to kill the larvae through suffocation or toxic effect of the oil (Service, 2012). The use of monomolecular films is a more recent larvicidal strategy which works by forming an ultrathin film on the surface of mosquito breeding areas thus suffocating the larvae to death (Antonio-Nkondjio *et al.*, 2018).

4.6 Vector Control in Zambia

In Zambia, Indoor Residual Spraying and use of LLINs are the main malaria elimination strategies identified to achieve a malaria free Zambia (NMEC, 2017). These two vector control interventions have received massive funding from both the Government and Cooperating partners (PMI, 2019).

However, larval source management is mainly implemented by Local authorities and mining companies.

In the quest to have a malaria free Zambia, there has been wide spread scaling up of these insecticide-based vector control (LLINs and IRS) to all the Districts in the country. Some districts have received both treated bed nets and IRS whereas others have only received one of the two interventions (PMI, 2019). However, these interventions have the ability to increase selection pressure on the population of mosquitoes that have resistant alleles (Nkya *et al.*, 2013).

4.7 Insecticide Resistance

The ability mosquitoes have to withstand toxic effects of an insecticide through natural selection or mutation is referred to as insecticide resistance. Usually this arises from repeated exposure of mosquitoes to insecticides or selection of individual mosquitoes that are able to detoxify the insecticide takes place. The mosquitoes selected survive and pass their ability to detoxify insecticides to their progeny (Riveron *et al.*, 2018). The widespread in insecticide resistance world over has been attributed to the extensive use of insecticides in agriculture and public health (Mouhamadou *et al.*, 2019).

Insecticide resistance has been reported against the four different classes of insecticides used for public health vector control and these are pyrethroids, organophosphates, organochlorines and carbamates (Mint Mohamed Lemine *et al.*, 2018)(Fang *et al.*, 2019)(Ondeto *et al.*, 2017).

Insecticide resistance is widespread in Zambia and has been recorded against pyrethroids, organochlorines and carbamates. Organophosphates and neonicotinoids are the only two classes of insecticides used in public health where local malaria vectors are still susceptible to (NMEC, 2019).

4.7.1 Mechanism of resistance

Routine monitoring of the susceptibility status of malaria vectors is cardinal for the successful implementation vector control (WHO, 2018). Area earmarked for insecticide-based vector control should be preceded by resistance surveillance to help determine most appropriate insecticide for use in vector control based on the susceptibility status of local malaria vectors. This monitoring of malaria vector susceptibility should be conducted once every year and in the event that there are several malaria vectors in the area with seasonal variations, monitoring at the beginning and end of the control effort should be done (McAllister and Scott, 2020).

There are basically four mechanisms of resistance and reports in some African countries indicate that the frequency of the presence of multiple mechanism of resistance is on the rise (Kwiatkowska *et al.*, 2013).

The figure below illustrates the different mechanisms of resistance (1) Reduced Penetration- Changes to the cuticle of mosquito exoskeleton prevents absorption of the insecticide. (2) Target-site- When the target-site for the insecticide is modified, the insecticide will no longer bind. (3) Metabolic- enzymes breakdown the insecticide before they can have a toxic effect on the mosquito

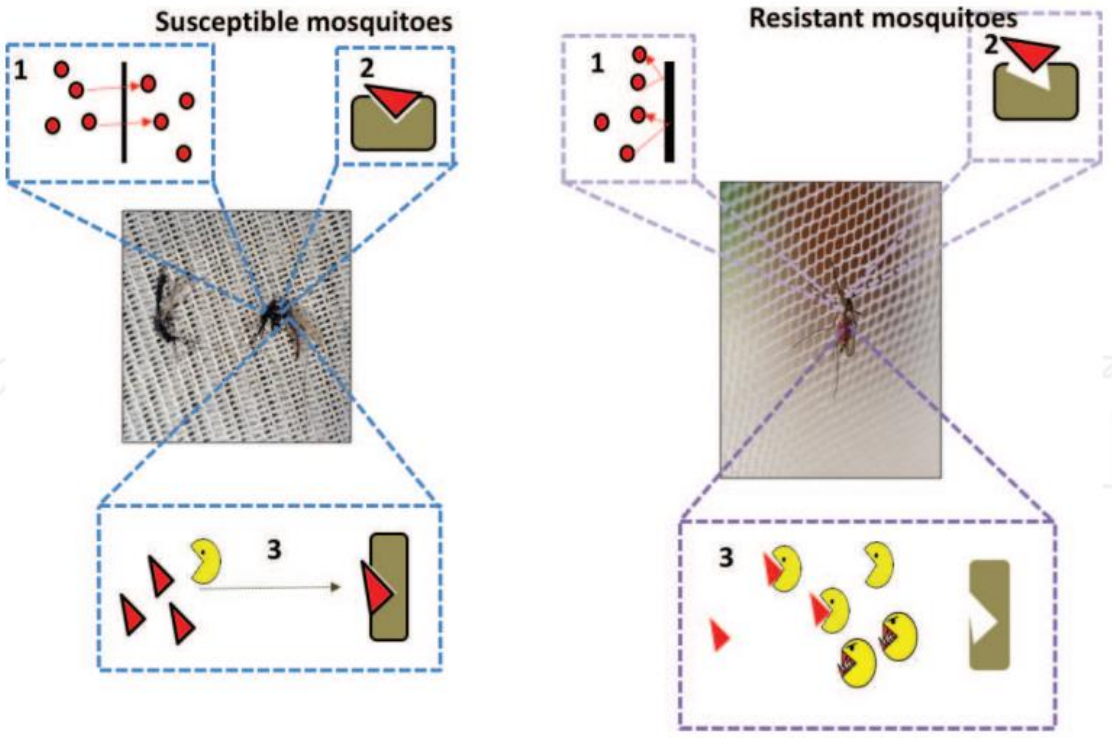

Figure 1: Summary of the 3 Main Mechanism of resistance

Source: (Riveron *et al.*, 2018)

Reduced Penetration: This type of resistance occurs when the cuticular structure composition is altered to reduce the amount of insecticide that is absorbed into the mosquito. Studies have shown that resistant mosquitoes have a thicker cuticle than susceptible mosquitoes (Riveron *et al.*, 2018). A study conducted in west Africa associated cuticle thickening to pyrethroid resistance (Yahouédo *et al.*, 2017). Additionally, the legs of resistant mosquitoes are sealed with large amounts of cuticular hydrocarbons compared to the susceptible mosquitoes (Balabanidou *et al.*, 2019). This mechanism of resistance is also one of the least studied yet it is possess a serious threat to vector control (Huang *et al.*, 2018).

Target-Site: In this mechanism of resistance, the target-site in the mosquito were the insecticide binds is modified and the insecticide can no longer bind. Insecticide molecules that enter the body

of the mosquito fail to bind to the target-site because it has been altered. This is the most common mechanism of resistance in Africa and it has contributed greatly to the malaria disease burden (Yewhalaw and Kweka, 2015).

Metabolic Resistance: This type of resistance arises when the body enzymes digest the insecticide molecules before attaching to the target-site where it will produce the desired toxic effect. This is one of the most common type of insecticide resistance report in the sub-Saharan Africa (Riveron *et al.*, 2018). This mechanism of resistance is widespread across most African countries and has been reported in at least one of the four classes of insecticides used in public health vector control (Diouf *et al.*, 2020)

Behavioural Resistance: This type of resistance if not monitored and has the potential to negatively impact the vector control strategies employed (Sokhna *et al*., 2013). Mosquitoes in this type of resistance tend to avoid surfaces that contain insecticides including sprayed wall surfaces and treated bed net surfaces (Gatton *et al.*, 2013). Other studies conducted have shown that the use of insecticidal nets has led to the change in feeding behaviours of mosquitoes with a rise in the biting times at dusk and dawn (Killeen and Chitnis, 2014).

4.7.2 Insecticide Resistance Management

To slow down development of resistance, the NMEP has provided guidelines to rotate IRS products and implement a mosaic approach. This approach is based on the data generated from mosquito susceptibility assays conducted on mosquitoes from different locations benefitting from IRS (PMI, 2019). However, the NMEP has a limited number of sites were susceptibility data is collected from and these are mostly sites that are supported by partners including PMI sites, MACEPA sites and Global Fund coordinated by NMEP. On the Copperbelt there are only two active sites operated by PMI which are used as entomological surveillance sites (PMI, 2020).

5.0 Materials and Methods

5.1 Study Design

This will be a cross sectional survey involving the collection of mosquitoes from two catchment areas (one urban and one rural) of Ndola District both with a malaria high burden.

5.2 Study Site

The study will be conducted in Ndola District (12.9726° S, 28.6265° E), the provincial capital of the Copperbelt Province. The district shares a boundary with the Democratic Republic of Congo on the eastern side.

Chipulukusu Urban Health Centre and Kaniki Rural Health Centre are the two catchment areas that will participate in this study. According to the Health Management Information System (HMIS) data for 2020, there were 19618 malaria confirmed cases in Chipulukusu and 10,679 malaria confirmed cases in Kaniki catchment area (Ndola, 2020). Chipulukusu Urban Health Centre serves a low cost area with an estimated population of 49,730 people (CSO, 2020).

Chipulukusu is one of the catchment areas found at the centre of the District. The housing structure comprise of cement block, burnt brick and mud brick walled houses with both iron and grass roofing. Most families in Chipulukusu access kitchen and toilet facilities in standalone structures apart from the main house used mostly for sleeping (CSO, 2020).

On the other hand, Kaniki Rural Health Centre shares a border post with the Democratic Republic of Congo at Sakanya border post. The majority of the housing structure in the area are mud house roofed with grass or iron sheets. The catchment has an estimated population of 11, 716 people. The sleeping house, Kitchen and toilet facilities are stand-alone structures (CSO, 2020).

5.3 Study Population

The study population will include all the housing structures occupied by people in the selected catchment areas of Chipulukusu and Kaniki. The estimated number of household in Chipulukusu is 16000 and the total number of household in Kaniki is 4412.

5.3.1 Inclusion Criteria

The houses where mosquitoes will be sampled include those houses where people sleep in and there is an adult (16 years and above)

5.3.2 Exclusion Criteria

The houses in which occupants will not consent to participate and / or cook from inside the house using firewood will be excluded from this study.

5.4 Sample Size Determination

Total Collection efforts 225 (CDC LT 56 houses, PSC 30 houses, Aspirations 80 houses and 60 Larvae collection efforts)

Sampling for this study is based on vector sampling guidelines by WHO (WHO, 2019). However, the sample size has been determined based on the minimum number of structures per square meters and the predominant malaria vector. The minimum houses to be sampled for areas where *An gambiae s.l* is the predominant vector is 17 houses per square km and 42 houses per square km where *An funestus s.l* is the predominant vector. (Zhou *et al.*, 2004). A study further revealed that sampling 120 houses (120 collection efforts) gives precision equivalent to 200 houses for mosquito surveillance (Sedda et al, 2019)

For vector susceptibility assays, a minimum of 100 adult female mosquitoes of a given species per insecticide is required. These mosquitoes will be tested in 4 replicates of 25 – 30 mosquitoes per WHO tube or CDC bottle (WHO, 2018).

5.6 Sampling Method

Purposive sampling will be used to select the two catchment areas that will participate in the study. Houses where adult mosquitoes will be collected will be randomly selected from each section maintaining at least 200 m between two participating houses. The collection tools that will be used for mosquito collection are;

5.6.1 CDC light traps

The traps will be set in randomly selected houses and mosquito collection will start at 18:00 hours and end at 06:00 hours the following morning. The traps will be set at a height of 1.5m from the ground next to a sleeping space. After every collection the mosquito collection cups will be properly labelled and transported to the laboratory for processing. At the laboratory, the mosquito collection cups will be put in a freezer at -4°C for 1 hour to kill mosquitoes that would still be alive.

5.5.2 Pyrethrum Spray Collections (PSC)

Adult mosquitoes resting indoors will be collected indoors using PSC from 05: 00 hours to 07: 00 hours in 20 houses per catchment area. The Collector will enter the house spread a 3m X 2m white linen over the floor, bed and furniture. The collector outside the house starts by spraying the eaves and other openings and once he is done the collector inside (wearing a nose mask) will saturate the house with insecticide to knockdown the mosquitoes. After the 10 minutes the collector will carefully fold the sheets towards the centre and take the sheets outside. The collector will pick the mosquitoes and place them in properly labelled petri dishes.

5.5.3 Aspirations

The live adult mosquitoes will be collected from 05:00 hours to 08:00 hours in houses where people sleep using mouth aspirator and prokopack. The live adult mosquitoes will then be put in bugdom cages and fed with a 10% sugar solution and transported to the laboratory for processing.

5.5.4 Larval collection

The larvae will be collected from breeding areas around houses where people live using dippers for larger breeding sites and Pasteur pipette for small breeding sites. The larvae will then be carefully transported to the laboratory where they will be reared into adult mosquitoes in a controlled microenvironment.

5.6 Variables

Variable type	Variable	Indicator
Outcome Variable	Mosquito Bionomics	Species Composition
		Malaria Vector Density
		Indoor Resting Density
		Man Biting Rate
Independent	Type of Wall	Mud Cement
	Roof type	Thatch Iron sheets
	Type of eaves	Open Closed
	Human Host	No. of People
	Animal Host	No. of animals
	Spray status	Sprayed Not Sprayed

	LLINs	No. of LLINs

5.7 Experimental Procedures

1. **To determine species composition and vector density of malaria vectors in Ndola:** Mosquitoes collected from houses using CDC Light traps and aspiration will be quantified by calculating total catches per household. All mosquitoes collected will be morphologically identified using a standard mosquito identification key for Afrotropical *Anopheles* mosquitoes (Coetzee, 2020). Confirmation of mosquito identification and determination of the subling species from the *An gambiae s.l* and *An funestus s.l* complexes will be done using multiplex PCR (Scott *et al.*, 1993) (Koekemoer *et al.*, 2002). DNA extraction will be done using the xygem DNA extraction kit. The PCR master mix prepared will include specific primers for sibling species. The PCR mix will then be run in the thermocycler and after amplification, the amplicons will be run on a gel.

   Malaria vector density: This is a very important indicator used in vector surveillance to assess the behaviour of malaria vectors or to assess the effectiveness of a vector control intervention. It is a mean of the malaria vectors collected from a define number of houses surveyed. It is calculated by dividing the total number of mosquitoes collected by the total number of houses surveyed.

   $$\textbf{Malaria vector density} = \frac{Number\ of\ malaria\ vectors\ collected}{Total\ number\ of\ houses\ surveyed}$$

2. **To assess the biting and resting behaviour of mosquitoes in Ndola**

The Man Biting Rate helps to estimate the potential risk of exposure to infectious bites. The formular for calculating the human biting rate or man biting rate is

$$\text{Man biting rate} = \frac{Number\ of\ mosquitoes\ collected\ using\ light\ trap}{\text{Total Number of sleepers}}\ X\ HBI$$

The Indoor Resting behaviour is indicative of the mosquitoes that rest indoors. This indicator is an important one when considering whether to implement IRS or not. This is calculated by dividing the total number of mosquitoes collected using PSC over the total number of houses surveyed.

3. **To assess host preference of blood feeding mosquitoes:** Blood fed female anopheles mosquitoes collected will be analysed using multiplex PCR for the determination of blood meal sources (Kent and Norris, 2010). Extraction of DNA will be done using a xygen DNA extraction kit and the samples will be stored at -20°C prior to running the PCR. After PCR the amplicons will be run on a gel for determination of blood meal source. The Human Blood index will be calculated using excel using the formula;

$$\text{Human Blood Index} = \frac{No.of\ female\ mosquitoes\ with\ human\ blood}{Total\ number\ of\ mosquitoes\ analysed}$$ (Escobar *et al.*, 2020). See appendi ????? for primers that will be used in multiplex PCR for determination of bloodmeal sources.

4. **To determine the insecticide resistance status of primary malaria vectors:** Live adult female anopheles mosquitoes will be exposed to various insecticides used in vector control using the using WHO Tube Bioassays and CDC bottle assays (WHO, 2018; Brogdon and Chan, 2010). The selected mosquitoes will be placed in WHO tubes lined with insecticide impregnated paper and left to sit for 60 minutes. Thereafter, knockdown will be observed

at 60 minutes, 12 hours and final mortality will be read at 24 hours post exposure. For the CDC bottle assay, mosquitoes will be put in whatton bottles coated with insecticides and knockdown will be read every 15 minutes for 2 hours and final mortality will be determined 24 hours post exposure.

5.8 Data Analysis

The data collected will entered in excel for determination of species composition, vector density, Indoor Resting Density, Human Blood Index and Man Biting Rate.

Analysis of variance will be used to determine any statistical difference in the malaria vector density, indoor resting behaviour and man biting rate between the two areas. Susceptibility status of the local malaria vectors will be determined using WHO guidelines provided (98% - 100% means susceptible; 90% - 97% means suspected resistance and less than 90% means confirmed resistance) that is if mortality in the controls is less than 5%. A corrected mortality using Abbotts formula will be computed where mortality in the controls will be between 5% and 20%. However, if the mortality in the controls will be greater than 20%, the findings will be discarded and the assay will be repeated.

6.0 Ethics Considerations

Ethics clearance to undertake this study will be obtained from the Tropical Diseases Research Centre Ethics Committee and National Health Research Authority. This study will assure protection of collectors and staff working during mosquito collection by ensuring that proper training is given to them and that they wear proper protective wear. It will also assure for

preservation of ecology by sampling without total depletion. Permission to collect mosquitoes from the District will be sought from Ndola District Health Office. Additionally, informed consent will be obtained from the head of the house before collecting mosquitoes and larvae from their houses and their gardens respectively. This study will involve invading participants personal space during mosquito collection and assurance will be given that their health, safety and privacy will be preserved.

7.0 Work Plan

Description of Activity	Apr-21				May-21				Jun-21				Jul-21				Aug-21			
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12	Week 13	Week 14	Week 15	Week 16	Week 17	Week 18	Week 19	Week 20
Proposal Writing	■	■																		
Proposal Presentation			■																	
Ethics Clearance				■	■	■														
Data Collection							■	■	■	■	■	■								
Data Analysis								■	■	■	■	■	■	■	■	■				
Report Writing										■	■	■	■	■	■	■	■	■	■	
Dissemination of Findings																				■

8.0 Activity Based Budget

Description of Activity	Amount
Communication and Internet connectivity	ZMW 2,000.00
Mosquito collection	ZMW 5,000.00
Molecular Analysis of Mosquitoes	ZMW 45,000.00
Data Analysis	ZMW 3,500.00
Ethics Clearance	ZMW 1,000.00
Publication	ZMW 2,500.00
Total Amount	ZMW 59,000.00

9.0 References

Abong'o, B., Gimnig, J. E., Torr, S. J., Longman, B., Omoke, D., Muchoki, M., *et al.* (2020) Impact of indoor residual spraying with pirimiphos-methyl (Actellic 300CS) on entomological indicators of transmission and malaria case burden in Migori County, western Kenya, *Scientific Reports*, 10 (1), pp. 1–14. DOI:10.1038/s41598-020-61350-2.

Afrane, Y. A., Bonizzoni, M. and Yan, G. (2016) Secondary Malaria Vectors of Sub-Saharan Africa: Threat to Malaria Elimination on the Continent?, *Current Topics in Malaria*, (November). DOI:10.5772/65359.

Alimi, T. O., Fuller, D. O., Qualls, W. A., Herrera, S. V., Arevalo-Herrera, M., Quinones, M. L., Lacerda, M. V. G. and Beier, J. C. (2015) Predicting potential ranges of primary malaria vectors and malaria in northern South America based on projected changes in climate, land cover and human population, *Parasites and Vectors*, 8 (1), pp. 1–16. DOI:10.1186/s13071-015-1033-9.

Antonio-Nkondjio, C., Sandjo, N. N., Awono-Ambene, P. and Wondji, C. S. (2018) Implementing a larviciding efficacy or effectiveness control intervention against malaria vectors: Key parameters for success, *Parasites and Vectors*, 11 (1), pp. 1–12. DOI:10.1186/s13071-018-2627-9.

Ayuya Stephen, Kitungulu Nicholas, Annette O. Busula, Mark Kilongosi Webale, E. O. (2021) Detection of Plasmodium sporozoites in Anopheles coustani s.l; a hindrance to malaria control strategies in highlands of western Kenya, *Biorix*, 33 (1976).

Balabanidou, V., Kefi, M., Aivaliotis, M., Koidou, V., Girotti, J. R., Mijailovsky, S. J., *et al.*

(2019) Mosquitoes cloak their legs to resist insecticides, *Proceedings of the Royal Society B: Biological Sciences*, 286 (1907). DOI:10.1098/rspb.2019.1091.

Balkew, M., Mumba, P., Dengela, D., Yohannes, G., Getachew, D., Yared, S., *et al.* (2020) Geographical distribution of Anopheles stephensi in eastern Ethiopia, *Parasites and Vectors*, 13 (1), pp. 1–8. DOI:10.1186/s13071-020-3904-y.

Bartilol, B., Omedo, I., Mbogo, C., Mwangangi, J. and Rono, M. K. (2021) Bionomics and ecology of Anopheles merus along the East and Southern Africa coast, *Parasites & Vectors*, pp. 1–11. DOI:10.1186/s13071-021-04582-z.

Benelli, G., Petrelli, R. and Canale, A. (2020) Arthropod-Borne Disease Control at a Glance: What's New on Drug Development?, *Molecules (Basel, Switzerland)*, 25 (21). DOI:10.3390/molecules25215175.

Brogdon, W. and Chan, A. (2010) Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay, pp. 1–28.

Chinula, D., Hamainza, B., Chizema, E., Kavishe, D. R., Sikaala, C. H. and Killeen, G. F. (2018) Proportional decline of Anopheles quadriannulatus and increased contribution of An. arabiensis to the An. gambiae complex following introduction of indoor residual spraying with pirimiphos-methyl: An observational, retrospective secondary analysis of pre-, *Parasites and Vectors*, 11 (1), pp. 1–7. DOI:10.1186/s13071-018-3121-0.

Chipoya, M. N. and Shimaponda-Mataa, N. M. (2020) Prevalence, characteristics and risk factors of imported and local malaria cases in North-Western Province, Zambia: a cross-sectional study, *Malaria Journal*, 19 (1), pp. 1–12. DOI:10.1186/s12936-020-03504-1.

Chisaya, M., Phiri, M. and Ngomah, M. (2020) AN UPDATE ON MALARIA TRENDS IN ZAMBIA (2019 TO 2020); A DESCRIPTIVE STUDY, *Health Press Zambia*, pp. 13–18.

Coetzee, M. (2020) Key to the females of Afrotropical Anopheles mosquitoes (Diptera: Culicidae), *Malaria Journal*, 19 (1), pp. 1–20. DOI:10.1186/s12936-020-3144-9.

Corrêa, A. P. S. A., Galardo, A. K. R., Lima, L. A., Câmara, D. C. P., Müller, J. N., Barroso, J. F. S., *et al.* (2019) Efficacy of insecticides used in indoor residual spraying for malaria control: An experimental trial on various surfaces in a 'test house', *Malaria Journal*, 18 (1), pp. 1–14.

DOI:10.1186/s12936-019-2969-6.

Cross, D. E., Thomas, C., Mckeown, N., Siaziyu, V., Healey, A., Willis, T., *et al.* (2021) Geographically extensive larval surveys reveal an unexpected scarcity of primary vector mosquitoes in a region of persistent malaria transmission in western Zambia, *Parasites & Vectors*, pp. 1–14. DOI:10.1186/s13071-020-04540-1.

Das, S., Muleba, M., Stevenson, J. C. and Norris, D. E. (2016) Habitat partitioning of malaria vectors in nchelenge district, Zambia, *American Journal of Tropical Medicine and Hygiene*, 94 (6), pp. 1234–1244. DOI:10.4269/ajtmh.15-0735.

Derua, Y. A., Alifrangis, M., Magesa, S. M., Kisinza, W. N. and Simonsen, P. E. (2015) Sibling species of the Anopheles funestus group, and their infection with malaria and lymphatic filarial parasites, in archived and newly collected specimens from northeastern Tanzania, *Malaria Journal*, 14 (1), pp. 1–8. DOI:10.1186/s12936-015-0616-4.

Derua, Y. A., Kweka, E. J., Kisinza, W. N., Githeko, A. K. and Mosha, F. W. (2019) Bacterial larvicides used for malaria vector control in sub-Saharan Africa: Review of their effectiveness and operational feasibility, *Parasites and Vectors*, 12 (1), pp. 1–18. DOI:10.1186/s13071-019-3683-5.

Diouf, E. hadji, Niang, E. hadji A., Samb, B., Diagne, C. T., Diouf, M., Konaté, A., Dia, I., Faye, O. and Konaté, L. (2020) Multiple insecticide resistance target sites in adult field strains of An. gambiae (s.l.) from southeastern Senegal, *Parasites and Vectors*, 13 (1), pp. 1–10. DOI:10.1186/s13071-020-04437-z.

Durden, L. A. and Mullen, G. R. (2018) *Medical and Veterinary Entomology*, *Medical and Veterinary Entomology*. DOI:10.1016/B978-0-12-814043-7.00001-7.

Ebenezer, A., Noutcha, A. E. M., Agi, P. I., Okiwelu, S. N. and Commander, T. (2014) Spatial distribution of the sibling species of Anopheles gambiae sensu lato (Diptera: Culicidae) and malaria prevalence in Bayelsa State, Nigeria, *Parasites and Vectors*, 7 (1), pp. 1–6. DOI:10.1186/1756-3305-7-32.

Eder, M., Cortes, F., Teixeira de Siqueira Filha, N., Araújo de França, G. V., Degroote, S., Braga, C., Ridde, V. and Turchi Martelli, C. M. (2018) Scoping review on vector-borne diseases

in urban areas: Transmission dynamics, vectorial capacity and co-infection, *Infectious Diseases of Poverty*, 7 (1), pp. 1–24. DOI:10.1186/s40249-018-0475-7.

Escobar, D., Ascencio, K., Ortiz, A., Palma, A., Sánchez, A. and Fontecha, G. (2020) Blood meal sources of anopheles spp. In malaria endemic areas of honduras, *Insects*, 11 (7), pp. 1–12. DOI:10.3390/insects11070450.

Fang, Y., Shi, W. Q., Wu, J. T., Li, Y. Y., Xue, J. B. and Zhang, Y. (2019) Resistance to pyrethroid and organophosphate insecticides, and the geographical distribution and polymorphisms of target-site mutations in voltage-gated sodium channel and acetylcholinesterase 1 genes in Anopheles sinensis populations in Shanghai, China, *Parasites and Vectors*, 12 (1), pp. 1–13. DOI:10.1186/s13071-019-3657-7.

Fillinger, U., Sombroek, H., Majambere, S., Van Loon, E., Takken, W. and Lindsay, S. W. (2009) Identifying the most productive breeding sites for malaria mosquitoes in the Gambia, *Malaria Journal*, 8 (1), pp. 1–14. DOI:10.1186/1475-2875-8-62.

Fornadel, C. M., Norris, L. C., Franco, V. and Norris, D. E. (2011) Unexpected Anthropophily in the Potential Secondary Malaria Vectors An coustani s.l and An squamosus in Macha, Zambia., 11 (8). DOI:10.1089/vbz.2010.0082.

Gatton, M. L., Chitnis, N., Churcher, T., Donnelly, M. J., Ghani, A. C., Godfray, H. C. J., *et al.* (2013) The importance of mosquito behavioural adaptations to malaria control in Africa, *Evolution*, 67 (4), pp. 1218–1230. DOI:10.1111/evo.12063.

Gomes, F. M., Hixson, B. L., Tyner, M. D. W., Ramirez, J. L., Canepa, G. E., Alves e Silva, T. L., *et al.* (2017) Effect of naturally occurring Wolbachia in Anopheles gambiae s.l. mosquitoes from Mali on Plasmodium falciparum malaria transmission, *Proceedings of the National Academy of Sciences of the United States of America*, 114 (47), pp. 12566–12571. DOI:10.1073/pnas.1716181114.

Herren, J. K., Mbaisi, L., Mararo, E., Makhulu, E. E., Mobegi, V. A., Butungi, H., *et al.* (2020) A microsporidian impairs Plasmodium falciparum transmission in Anopheles arabiensis mosquitoes, *Nature Communications*, 11 (1). DOI:10.1038/s41467-020-16121-y.

Howard, A. F. V., Zhou, G. and Omlin, F. X. (2007) Malaria mosquito control using edible fish

in western Kenya: Preliminary findings of a controlled study, *BMC Public Health*, 7, pp. 1–6. DOI:10.1186/1471-2458-7-199.

Huang, Y., Guo, Q., Sun, X., Zhang, C., Xu, N., Xu, Y., *et al.* (2018) Culex pipiens pallens cuticular protein CPLCG5 participates in pyrethroid resistance by forming a rigid matrix, *Parasites and Vectors*, 11 (1), pp. 1–10. DOI:10.1186/s13071-017-2567-9.

Kent, R. J. and Norris, D. E. (2010) Identification of Mammalian Blood Meals in Mosquitoes By a Multiplexed Polymerase Chain Reaction Targeting Cytochrome B, *Human Development*, 45 (6), pp. 1654–1668. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16103600%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4147110 [Accessed

Killeen, G. F. and Chitnis, N. (2014) Potential causes and consequences of behavioural resilience and resistance in malaria vector populations: A mathematical modelling analysis, *Malaria Journal*, 13 (1), pp. 1–16. DOI:10.1186/1475-2875-13-97.

Killeen, G. F., Govella, N. J., Lwetoijera, D. W. and Okumu, F. O. (2016) Most outdoor malaria transmission by behaviourally - resistant Anopheles arabiensis is mediated by mosquitoes that have previously been inside houses, *Malaria Journal*, pp. 1–10. DOI:10.1186/s12936-016-1280-z.

Koekemoer, L. L., Kamau, L., Hunt, R. H. and Coetzee, M. (2002) A cocktail polymerase chain reaction assay to identify members of the Anopheles funestus (Diptera: Culicidae) group, *American Journal of Tropical Medicine and Hygiene*, 66 (6), pp. 804–811. DOI:10.4269/ajtmh.2002.66.804.

Kwiatkowska, R. M., Platt, N., Poupardin, R., Irving, H., Dabire, R. K., Mitchell, S., *et al.* (2013) Dissecting the mechanisms responsible for the multiple insecticide resistance phenotype in Anopheles gambiae s.s., M form, from Vallée du Kou, Burkina Faso, *Gene*, 519 (1), pp. 98–106. DOI:10.1016/j.gene.2013.01.036.

Limwagu, A. J., Kaindoa, E. W., Ngowo, H. S., Hape, E., Finda, M., Mkandawile, G., *et al.* (2019) Using a miniaturized double-net trap (DN-Mini) to assess relationships between indoor-outdoor biting preferences and physiological ages of two malaria vectors, Anopheles arabiensis

and Anopheles funestus, *Malaria Journal*, 18 (1), pp. 1–15. DOI:10.1186/s12936-019-2913-9.

Lobo, N. F., St. Laurent, B., Sikaala, C. H., Hamainza, B., Chanda, J., Chinula, D., *et al.* (2015) Unexpected diversity of Anopheles species in Eastern Zambia: Implications for evaluating vector behavior and interventions using molecular tools, *Scientific Reports*, 5 (December), pp. 1–10. DOI:10.1038/srep17952.

Machani, M. G., Ochomo, E., Amimo, F., Kosgei, J., Munga, S., Zhou, G., Githeko, A. K., Yan, G. and Afrane, Y. A. (2020) Resting behaviour of malaria vectors in highland and lowland sites of western Kenya : Implication on malaria vector control measures, pp. 55–66. DOI:10.1371/journal.pone.0224718.

Massey, N. C., Garrod, G., Wiebe, A., Henry, A. J., Huang, Z., Moyes, C. L. and Sinka, M. E. (2016) A global bionomic database for the dominant vectors of human malaria, *Scientific Data*, 3, pp. 1–13. DOI:10.1038/sdata.2016.14.

Mattah, P. A. D., Futagbi, G., Amekudzi, L. K., Mattah, M. M., De Souza, D. K., Kartey-Attipoe, W. D., Bimi, L. and Wilson, M. D. (2017) Diversity in breeding sites and distribution of Anopheles mosquitoes in selected urban areas of southern Ghana, *Parasites and Vectors*, 10 (1), pp. 1–15. DOI:10.1186/s13071-016-1941-3.

Mawejje, H. D., Kilama, M., Kigozi, S. P., Musiime, A. K., Kamya, M., Lines, J., *et al.* (2021) Impact of seasonality and malaria control interventions on Anopheles density and species composition from three areas of Uganda with differing malaria endemicity, *Malaria Journal*, pp. 1–13. DOI:10.1186/s12936-021-03675-5.

Mazigo, E., Kidima, W., Myamba, J. and Kweka, E. J. (2019) The impact of Anopheles gambiae egg storage for mass rearing and production success, *Malaria Journal*, 18 (1), pp. 1–11. DOI:10.1186/s12936-019-2691-4.

McAllister, J. C. and Scott, M. (2020) CONUS manual for evaluating insecticide resistance in mosquitoes using the CDC bottle bioassay kit, pp. 1–19. Available from: https://www.cdc.gov/zika/pdfs/CONUS-508.pdf [Accessed

Mint Mohamed Lemine, A., Ould Lemrabott, M. A., Niang, E. H. A., Basco, L. K., Bogreau, H., Faye, O. and Ould Mohamed Salem Boukhary, A. (2018) Pyrethroid resistance in the major

malaria vector Anopheles arabiensis in Nouakchott, Mauritania, *Parasites and Vectors*, 11 (1), pp. 1–8. DOI:10.1186/s13071-018-2923-4.

MOH (2015) Zambia malaria indicator survey, *National Malaria Elimination Centre, Ministry Of Health, Zambia*. Available from: http://www.makingmalariahistory.org/wp-content/uploads/2017/06/Zambia-MIS2015_Jan20-nosigs.pdf [Accessed

MOH (2019) Zambia National Malaria Indicator Survey 2018: MINISTRY OF HEALTH, *National Malaria Elimination Centre, Ministry Of Health, Zambia*, (May), pp. 2009–2015.

Moiroux, N., Gomez, M. B., Pennetier, C., Elanga, E., Djènontin, A., Chandre, F., Djègbé, I., Guis, H. and Corbel, V. (2012) Changes in anopheles funestus biting behavior following universal coverage of long-lasting insecticidal nets in benin, *Journal of Infectious Diseases*, 206 (10), pp. 1622–1629. DOI:10.1093/infdis/jis565.

Mouhamadou, C. S., De Souza, S. S., Fodjo, B. K., Zoh, M. G., Bli, N. K. and Koudou, B. G. (2019) Evidence of insecticide resistance selection in wild Anopheles coluzzii mosquitoes due to agricultural pesticide use, *Infectious Diseases of Poverty*, 8 (1), pp. 64. DOI:10.1186/s40249-019-0572-2.

Musiime, A. K., Smith, D. L., Kilama, M., Rek, J., Arinaitwe, E., Nankabirwa, J. I., *et al.* (2019) Impact of vector control interventions on malaria transmission intensity , outdoor vector biting rates and Anopheles mosquito species composition in Tororo , Uganda, *Malaria Journal*, pp. 1–9. DOI:10.1186/s12936-019-3076-4.

Mwangangi, J. M., Mbogo, C. M., Orindi, B. O., Muturi, E. J., Midega, J. T., Nzovu, J., *et al.* (2013a) Shifts in malaria vector species composition and transmission dynamics along the Kenyan coast over the past 20 years, pp. 1–9.

Mwangangi, J. M., Muturi, E. J., Muriu, S. M., Nzovu, J., Midega, J. T. and Mbogo, C. (2013b) The role of Anopheles arabiensis and Anopheles coustani in indoor and outdoor malaria transmission in Taveta District , Kenya, pp. 1–9.

Mzilahowa, T., Hastings, I. M., Molyneux, M. E. and McCall, P. J. (2012) Entomological indices of malaria transmission in Chikhwawa district, Southern Malawi, *Malaria Journal*, 11, pp. 1–9. DOI:10.1186/1475-2875-11-380.

Ndenga, B. A., Mulaya, N. L., Musaki, S. K., Shiroko, J. N., Dongus, S. and Fillinger, U. (2016) Malaria vectors and their blood - meal sources in an area of high bed net ownership in the western Kenya highlands, *Malaria Journal*, pp. 6–8. DOI:10.1186/s12936-016-1115-y.

Ndiaye, A., Amadou Niang, E. H., Diène, A. N., Nourdine, M. A., Sarr, P. C., Konaté, L., Faye, O., Gaye, O. and Sy, O. (2020) Mapping the breeding sites of Anopheles gambiae s. l. In areas of residual malaria transmission in central western Senegal, *PLoS ONE*, 15 (12 December), pp. 1–16. DOI:10.1371/journal.pone.0236607.

Nkya, T. E., Akhouayri, I., Kisinza, W. and David, J. P. (2013) Impact of environment on mosquito response to pyrethroid insecticides: Facts, evidences and prospects, *Insect Biochemistry and Molecular Biology*, 43 (4), pp. 407–416. DOI:10.1016/j.ibmb.2012.10.006.

NMEC (2017) National Malaria Elimination Strategic Plan 2017 - 2021, Ministry of Health, Government Republic of Zambia, 70 (10), pp. 1113. Available from: https://static1.squarespace.com/static/58d002f017bffcf99fe21889/t/5b28d7f1575d1ff0942dbce1/1529403401067/National+Malaria+Elimination+Strategic+Plan+2017-Final_PRINT.pdf [Accessed

NMEC (2019) Insecticide Resistance Management and Monitoring Plan, Ministry of Health, Government Republic of Zambia.,

Ntonifor, N. H. and Veyufambom, S. (2016) Assessing the effective use of mosquito nets in the prevention of malaria in some parts of Mezam division, Northwest Region Cameroon, *Malaria Journal*, 15 (1), pp. 1–8. DOI:10.1186/s12936-016-1419-y.

O'Donnell, A. J., Rund, S. S. C. and Reece, S. E. (2019) Time-of-day of blood-feeding: Effects on mosquito life history and malaria transmission, *Parasites and Vectors*, 12 (1), pp. 1–16. DOI:10.1186/s13071-019-3513-9.

Ondeto, B. M., Nyundo, C., Kamau, L., Muriu, S. M., Mwangangi, J. M., Njagi, K., Mathenge, E. M., Ochanda, H. and Mbogo, C. M. (2017) Current status of insecticide resistance among malaria vectors in Kenya, *Parasites and Vectors*, 10 (1), pp. 1–13. DOI:10.1186/s13071-017-2361-8.

Paaijmans, K. P. and Huijben, S. (2020) Taking the 'I' out of LLINs: Using insecticides in vector

control tools other than long-lasting nets to fight malaria, *Malaria Journal*, 19 (1), pp. 1–6. DOI:10.1186/s12936-020-3151-x.

Phiri, E., Baboo, K. S. and Miller, J. (2015) Effect of Indoor Residual Spraying on the Incidence of Malaria in Kaoma District of Western Zambia, *Medical Journal of Zambia*, 42 (4), pp. 150–158.

Pinchoff, J., Larsen, D. A., Renn, S., Pollard, D., Fornadel, C., Maire, M., *et al.* (2016) Targeting indoor residual spraying for malaria using epidemiological data : a case study of the Zambia experience, *Malaria Journal*, pp. 1–6. DOI:10.1186/s12936-015-1073-9.

PMI (2019) PRESIDENT ' S MALARIA INITIATIVE; Zambia Malaria Operational Plan FY 2019, *Operational Plan*. Available from: http://www.pmi.gov/countries/mops/fy14/nigeria_mop_fy14.pdf [Accessed

Prevention, C.-C. for D. C. and (2019) CDC - Malaria - Malaria Worldwide - How Can Malaria Cases and Deaths Be Reduced? - Insecticide-Treated Bed Nets.

Ricci, I., Valzano, M., Ulissi, U., Epis, S., Cappelli, A. and Favia, G. (2012) Symbiotic control of mosquito borne disease, *Pathogens and Global Health*, 106 (7), pp. 380–385. DOI:10.1179/2047773212Y.0000000051.

Riveron, J., Tchouakui, M., Mugenzi, L., Menze, B., Chiang, M.-C. and Wondji, C. (2018) Insecticide Resistance in Malaria Vectors: An Update at Global Scale, in: *IntechOpen*.32, pp. 137–144.

Russell, T. L., Govella, N. J., Azizi, S., Drakeley, C. J., Kachur, S. P. and Killeen, G. F. (2011) Increased proportions of outdoor feeding among residual malaria vector populations following increased use of insecticide-treated nets in rural Tanzania, *Malaria Journal*, 10, pp. 1–10. DOI:10.1186/1475-2875-10-80.

Scott, J. A., Brogdon, W. G. and Collins, F. H. (1993) Identification of single specimens of the Anopheles gambiae complex by the polymerase chain reaction, *American Journal of Tropical Medicine and Hygiene*, 49 (4), pp. 520–529. DOI:10.4269/ajtmh.1993.49.520.

Service, M. (2012) *Medical entomology for students, fourth edition*, *Medical Entomology for Students, Fourth Edition*. DOI:10.1017/CBO9780511811012.

Sinka, M. E., Bangs, M. J., Manguin, S., Rubio-Palis, Y., Chareonviriyaphap, T., Coetzee, M., *et al.* (2012) A global map of dominant malaria vectors, *Parasites and Vectors*, 5 (1), pp. 69. DOI:10.1186/1756-3305-5-69.

Sinka, M. E., Pironon, S., Massey, N. C., Longbottom, J., Hemingway, J., Moyes, C. L. and Willis, K. J. (2020) A new malaria vector in Africa: Predicting the expansion range of Anopheles stephensi and identifying the urban populations at risk, *Proceedings of the National Academy of Sciences of the United States of America*, 117 (40), pp. 24900–24908. DOI:10.1073/pnas.2003976117.

Sokhna, C., Ndiath, M. O. and Rogier, C. (2013) The changes in mosquito vector behaviour and the emerging resistance to insecticides will challenge the decline of malaria, *Clinical Microbiology and Infection*, 19 (10), pp. 902–907. DOI:10.1111/1469-0691.12314.

Sougoufara, S., Diédhiou, S. M., Doucouré, S., Diagne, N., Sembène, P. M., Harry, M., Trape, J. F., Sokhna, C. and Ndiath, M. O. (2014) Biting by Anopheles funestus in broad daylight after use of long-lasting insecticidal nets: A new challenge to malaria elimination, *Malaria Journal*, 13 (1), pp. 1–7. DOI:10.1186/1475-2875-13-125.

Sougoufara, S., Ottih, E. C. and Tripet, F. (2020) The need for new vector control approaches targeting outdoor biting anopheline malaria vector communities, *Parasites & Vectors*, pp. 1–15. DOI:10.1186/s13071-020-04170-7.

Steinhardt, L. C., Jean, Y. S., Impoinvil, D., Mace, K. E., Wiegand, R., Huber, C. S., *et al.* (2017) Effectiveness of insecticide-treated bednets in malaria prevention in Haiti: a case-control study, *The Lancet Global Health*, 5 (1), pp. e96–e103. DOI:10.1016/S2214-109X(16)30238-8.

Stevenson, J. C., Pinchoff, J., Muleba, M., Lupiya, J., Chilusu, H., Mwelwa, I., *et al.* (2016) Spatio-temporal heterogeneity of malaria vectors in northern Zambia : implications for vector control, *Parasites & Vectors*, pp. 1–15. DOI:10.1186/s13071-016-1786-9.

Tangena, J. A. A., Hendriks, C. M. J., Devine, M., Tammaro, M., Trett, A. E., Williams, I., *et al.* (2020) Indoor residual spraying for malaria control in sub-Saharan Africa 1997 to 2017: An adjusted retrospective analysis, *Malaria Journal*, 19 (1), pp. 1–15. DOI:10.1186/s12936-020-03216-6.

The President's Malaria Initiative (PMI) / Vectorlink (2020) Zambia Annual Entomology Report (June 2019-August 2020), *Rockville, MD. The PMI VectorLink Project, Abt Associates*.

Thomas, S., Ravishankaran, S., Justin, J. A., Asokan, A., Mathai, M. T., Valecha, N., Thomas, M. B. and Eapen, A. (2016) Overhead tank is the potential breeding habitat of Anopheles stephensi in an urban transmission setting of Chennai, India, *Malaria Journal*, 15 (1), pp. 1–10. DOI:10.1186/s12936-016-1321-7.

Thomsen, E. K., Koimbu, G., Pulford, J., Jamea-Maiasa, S., Ura, Y., Keven, J. B., *et al.* (2017) Mosquito behavior change after distribution of bednets results in decreased protection against malaria exposure, *Journal of Infectious Diseases*, 215 (5), pp. 790–797. DOI:10.1093/infdis/jiw615.

Thomsen, E. K., Strode, C., Hemmings, K., Hughes, A. J., Chanda, E., Musapa, M., *et al.* (2014) Underpinning sustainable vector control through informed insecticide resistance management, *PLoS ONE*, 9 (6). DOI:10.1371/journal.pone.0099822.

Walshe, D. P., Garner, P., Adeel, A. A., Pyke, G. H. and Burkot, T. R. (2017) Larvivorous fish for preventing malaria transmission, *Cochrane Database of Systematic Reviews*, 2017 (12). DOI:10.1002/14651858.CD008090.pub3.

Wang, S., Ghosh, A. K., Bongio, N., Stebbings, K. A., Lampe, D. J. and Jacobs-Lorena, M. (2012) Fighting malaria with engineered symbiotic bacteria from vector mosquitoes, *Proceedings of the National Academy of Sciences of the United States of America*, 109 (31), pp. 12734–12739. DOI:10.1073/pnas.1204158109.

WHO (2019) *Guidelines for Malaria Vector Control*, *Guidelines for Malaria Vector Control*. Available from: https://apps.who.int/iris/bitstream/handle/10665/310862/9789241550499-eng.pdf?ua=1%0Ahttp://www.ncbi.nlm.nih.gov/pubmed/30844152

WHO (2020) *World malaria report 2020*.

Wiebe, A., Longbottom, J., Gleave, K., Shearer, F. M., Sinka, M. E., Massey, N. C., *et al.* (2017) Geographical distributions of African malaria vector sibling species and evidence for insecticide resistance, *Malaria Journal*, 16 (1), pp. 1–10. DOI:10.1186/s12936-017-1734-y.

Wilson, A. L., Courtenay, O., Kelly-hope, L. A., Id, T. W. S., Takken, W., Torr, S. J. and

Lindsay, S. W. (2020) *The importance of vector control for the control and elimination of vector-borne diseases*.

World Health Organization(WHO) (2014) *Malaria entomology and vector control - GUIDE FOR PARTICIPANTS, 2014 11th International Conference on Fuzzy Systems and Knowledge Discovery, FSKD 2014*. 1 st Editi. Malta: World Health Organization. DOI:10.1109/FSKD.2014.6980805.

World Health Organization(WHO) (2018) Test procedures for insecticide resistance monitoring in malaria vector mosquitoes Second edition.

World Health Organization (2013) WHO recommended insecticides for indoor residual spraying against malaria vectors, (October), pp. 2013. Available from: http://www.who.int/whopes/Insecticides_IRS_Malaria_25_Oct_2013.pdf [Accessed

World Health Organization (2019) Malaria and related entomological and vector control concepts, *Guidelines for Malaria Vector Control*. Available from: https://www.ncbi.nlm.nih.gov/books/NBK538113/ [Accessed 17 April 2021].

Yahouédo, G. A., Chandre, F., Rossignol, M., Ginibre, C., Balabanidou, V., Mendez, N. G. A., Pigeon, O., Vontas, J. and Cornelie, S. (2017) Contributions of cuticle permeability and enzyme detoxification to pyrethroid resistance in the major malaria vector Anopheles gambiae, *Scientific Reports*, 7 (1), pp. 1–10. DOI:10.1038/s41598-017-11357-z.

Yewhalaw, D. and Kweka, E. J. (2015) Insecticide Resistance in East Africa — History, Distribution and Drawbacks on Malaria Vectors and Disease Control, *Intech*, 32 (July), pp. 137–144. Available from: http://www.intechopen.com/books/trends-in-telecommunications-technologies/gps-total-electron-content-tec- prediction-at-ionosphere-layer-over-the-equatorial-region%0AInTec%0Ahttp://www.asociatiamhc.ro/wp-content/uploads/2013/11/Guide-to-Hydropower.pdf [Accessed

Zhou, G., Lo, E., Githeko, A. K., Afrane, Y. A. and Yan, G. (2020) Long-lasting microbial larvicides for controlling insecticide resistant and outdoor transmitting vectors: a cost-effective supplement for malaria interventions, *Infectious Diseases of Poverty*, 9 (1), pp. 1–8. DOI:10.1186/s40249-020-00767-3.

Zhou, G., Minakawa, N., Githeko, A. and Yan, G. (2004) Spatial distribution patterns of malaria vectors and sample size determination in spatially heterogeneous environments: A case study in the west Kenyan highland, *Journal of Medical Entomology*, 41 (6), pp. 1001–1009. DOI:10.1603/0022-2585-41.6.1001.

10.0 Appendices

Mosquito Collection Form

University of Zambia
School of Health Sciences
Department of Biomedical Sciences

Province: _____ District: _____ Study site: _____ Date of Collection: _____ Collection Type: _____ GPS Coordinates: Latitude _____ Longitude _____ Altitude (m): _____

SN	Head of Household Name	No. of People	No. of Animals	Roof Type	Wall Type	Type of eaves	No. of LLINs	Brand of LLINs	Spray status	If Sprayed Insecticide used	Mosquito Species	Abdominal Status		
												U	F	G

136/bmjopen-2024-091319 on 5 March 2025. Downloaded from

PARTICIPANT INFORMATION SHEET

University of Zambia

School of Health Sciences

Department of Biomedical Sciences

Principal Investigator: **Mr Westone Hamwata**

Co-Investigators: **Dr N.M Shimaponda-Mataa**

Mr Mbanga Muleba

Introduction

You are invited to take part in a survey titled ***Malaria Vector Bionomics and Phenotypic Resistance Status to Insecticides Used in Vector Control in Ndola District.*** You will be provided with enough information about the study which will help you decide if you will participate or not.

Purpose of the Study

This study will evaluate the behaviour of malaria vectors and their susceptibility status to insecticides used in vector control. Specifically, species composition, malaria vector density, resting behaviour, biting behaviour, host preference and mosquito susceptibility status will be determined.

What will happen if you participate in this study

You must be provided with enough information about the study to help you understand why the research is being done and what it involves. This will help you decide whether to participate or not. Please take time to read or listen as I read the document to you.

Mosquito collection will be conducted at your premises and you will be asked questions which will just take 5 minutes of your time to complete the mosquito collection form.

How long will this study last?

This study will last 5 months. However, mosquito collection at your premise will only be conducted once.

What are the risks of participating in this study?

There are no major risks to being in this study. You will only be asked to move out of the house when conducting pyrethrum spray catches in a period of about 20 minutes then you can go back in between 05:00 hrs to 07:00 hrs. If you feel uneasy leaving investigators alone, you will be provided with a face mask so that you accompany the investigator inside house during saturation of the house with the pyrethroid insecticide.

The study involves invading your personal space and as such utmost confidentiality will be adhered to by the investigators. Further, necessary protective measures against COVID-19 are put in place during our interaction with you. We will ensure physical distancing, availability of hand sanitizer, strict use of masks by both you and the investigator.

What is the study Procedure?

By consenting to this study you will allow the investigators to access your premises to collect mosquitoes. Mosquitoes will be collected using four different collection methods. CDC light traps will be set in your house around 16:00 and retrieved the following morning around 06:00hrs. Mosquito collection using pyrethrum spray catches and aspirations will be done from 05:00 hours to 08:00hours. Larval collection will be conducted in breeding sites near your house. The mosquitoes collected will then be taken to the laboratory where they will be morphologically identified and molecular identification will be conducted for determination of sibling species in the *An gambiae* and *An funestus* complex.

What are the benefits of participating in this study?

There are no direct program benefits to you for participating in the study. You may find an indirect benefit in knowing you have participated in an important study that could help generate baseline data for informed vector control programming.

Compensation

Participants in the study will not be compensated for the time taken to participate in this study. Despite this, we would be grateful if you could allow the team to collect mosquitoes from your premises.

What freedom and rights do you have in participating in the study?

The decision to participate in this study is entirely yours, and no one else should make it for you. You are free not to join this study or to stop participating in the study at any time. You will not receive any punishment now or in the future because of this. We will respect your freedom of choice. We will not share any of your information with your family, friends, or parent.

How is your confidentiality protected in this study?

The information that is collected during the study will be kept private. No one will be told that you have participated in the study or what your answers are to the questions. The study team will make every effort to protect your privacy and maintain the confidentiality of all the information that you provide. Your name or other identifiers will not be included in reports from this study. The information will be stored on a secure computer system.

At the end of the study, the report from the study will be made available to researchers or others

who are interested in using it to know more about the behaviour of malaria vectors and their phenotypic resistance status to insecticides used in vector control in Ndola. This means that others people besides the study staff will be able to see the information you provided.

What will happen to the results of the research study?

We will write a report combining all of the responses from all participants who have participated in this study. This report will be shared with The University of Zambia School of Health Sciences, Tropical Diseases Research Centre, Ndola District Health Office, National Malaria Elimination Centre and other key stakeholders.

Who has approved the research study?

This study has been reviewed and approved by the Tropical Diseases Research Centre Ethics Committee and National Health Research Authority.

Who else can you contact about the study and how do you contact them?

If you have any questions on the study or you being in the study, you or your selected relative/friend can contact Mr. Westone Hamwata, the Principal Investigator of the study on 0972790935. If you have a question about your rights as a research subject you or your selected relative/friend can contact the Secretary of the Tropical Diseases Research Centre Ethics Committee.

Declaration of Consent

I understand the contents of this Consent Form, and I agree to participate in this research study. I have had the opportunity to ask questions in an information session and all my questions have been answered to my satisfaction. I have been given sufficient time to consider the above information and to seek advice if I choose to do so. I understand that I am taking part in the study freely and

that I can stop being part of this study at any time and for any reason. By signing this consent form, I agree to participate in the research study.

I agree to participate in this study Yes ☐ No ☐

_____ _____ ____/___/_____

Head of Household Signature Date

Witness (*if the participant was not able to read and understand the Consent Information Sheet and Informed Consent Document*).

I affirm that the Informed Consent Document has been read to the participant, and she understands the study and I have witnessed her consent to study participation.

_____ _____ ____/___/_____

Name of Witness Signature Date

INFORMED CONSENT FORM

University of Zambia

School of Health Sciences

Department of Biomedical Sciences

Name of Head of Household: _____

Surveillance Activity Type: _____

House Number: _____

I can confirm that I have read (read to me) the information sheet and understand the objectives of this study. I had the opportunity to consider the information, ask questions and have had these answered fully.	Tick:
.....(name of investigator) has explained to me the nature and purpose of the activities to be undertaken. I understand fully what is to be done.	Tick:
I understand that my participation is voluntary and I am free to withdraw at any time, without giving any reason.	Tick:
I agree to take part in the above activity.	Tick:

Signature of Head of Household: _____ Date: _____

I confirm that I have explained to the person named above the nature and purpose of the activities to be undertaken.

Signature of Investigator: _____ Date: _____

1 signed copy of this form is given to the head of household;

1 signed copy of this form is kept for the Principal Investigator

8.2 PARTICIPANT INFORMATION SHEET

University of Zambia

School of Health Sciences

Department of Biomedical Sciences

Principal Investigator: **Mr Westone Hamwata**

Co-Investigators: **Dr N.M Shimaponda-Mataa**

Mr Mbanga Muleba

Introduction

You are invited to take part in a survey titled ***Malaria Vector Bionomics and Phenotypic Resistance Status to Insecticides Used in Vector Control in Ndola District.*** You will be provided with enough information about the study which will help you decide if you will participate or not.

Purpose of the Study

This study will evaluate the behaviour of malaria vectors and their susceptibility status to insecticides used in vector control. Specifically, species composition, malaria vector density, resting behaviour, biting behaviour, host preference and mosquito susceptibility status will be determined.

What will happen if you participate in this study

You must be provided with enough information about the study to help you understand why the research is being done and what it involves. This will help you decide whether to participate or not. Please take time to read or listen as I read the document to you.

Mosquito collection will be conducted at your premises and you will be asked questions which will just take 5 minutes of your time to complete the mosquito collection form.

How long will this study last?

This study will last 5 months. However, mosquito collection at your premise will only be conducted once.

What are the risks of participating in this study?

There are no major risks to being in this study. You will only be asked to move out of the house when conducting pyrethrum spray catches in a period of about 20 minutes then you can go back in between 05:00 hrs to 07:00 hrs. If you feel uneasy leaving investigators alone, you will be provided with a face mask so that you accompany the investigator inside house during saturation of the house with the pyrethroid insecticide.

The study involves invading your personal space and as such utmost confidentiality will be adhered to by the investigators. Further, necessary protective measures against COVID-19 are put in place during our interaction with you. We will ensure physical distancing, availability of hand sanitizer, strict use of masks by both you and the investigator.

What is the study Procedure?

By consenting to this study you will allow the investigators to access your premises to collect mosquitoes. Mosquitoes will be collected using four different collection methods. CDC light traps will be set in your house around 16:00 and retrieved the following morning around 06:00hrs. Mosquito collection using pyrethrum spray catches and aspirations will be done from 05:00 hours to 08:00hours. Larval collection will be conducted in breeding sites near your house. The mosquitoes collected will then be taken to the laboratory where they will be morphologically identified and molecular identification will be conducted for determination of sibling species in the *An gambiae* and *An funestus* complex.

What are the benefits of participating in this study?

There are no direct program benefits to you for participating in the study. You may find an indirect benefit in knowing you have participated in an important study that could help generate baseline data for informed vector control programming.

Compensation

Participants in the study will not be compensated for the time taken to participate in this study. Despite this, we would be grateful if you could allow the team to collect mosquitoes from your premises.

What freedom and rights do you have in participating in the study?

The decision to participate in this study is entirely yours, and no one else should make it for you. You are free not to join this study or to stop participating in the study at any time. You will not receive any punishment now or in the future because of this. We will respect your freedom of choice. We will not share any of your information with your family, friends, or parent.

How is your confidentiality protected in this study?

The information that is collected during the study will be kept private. No one will be told that you have participated in the study or what your answers are to the questions. The study team will make every effort to protect your privacy and maintain the confidentiality of all the information that you provide. Your name or other identifiers will not be included in reports from this study. The information will be stored on a secure computer system.

At the end of the study, the report from the study will be made available to researchers or others

who are interested in using it to know more about the behaviour of malaria vectors and their phenotypic resistance status to insecticides used in vector control in Ndola. This means that others people besides the study staff will be able to see the information you provided.

What will happen to the results of the research study?

We will write a report combining all of the responses from all participants who have participated in this study. This report will be shared with The University of Zambia School of Health Sciences, Tropical Diseases Research Centre, Ndola District Health Office, National Malaria Elimination Centre and other key stakeholders.

Who has approved the research study?

This study has been reviewed and approved by the Tropical Diseases Research Centre Ethics Committee and National Health Research Authority.

Who else can you contact about the study and how do you contact them?

If you have any questions on the study or you being in the study, you or your selected relative/friend can contact Mr. Westone Hamwata, the Principal Investigator of the study on 0972790935. If you have a question about your rights as a research subject you or your selected relative/friend can contact the Secretary of the Tropical Diseases Research Centre Ethics Committee.

Declaration of Consent

I understand the contents of this Consent Form, and I agree to participate in this research study. I have had the opportunity to ask questions in an information session and all my questions have been answered to my satisfaction. I have been given sufficient time to consider the above information and to seek advice if I choose to do so. I understand that I am taking part in the study freely and

that I can stop being part of this study at any time and for any reason. By signing this consent form, I agree to participate in the research study.

I agree to participate in this study Yes ☐ No ☐

_____ _____ ____/___/_____

Head of Household Signature Date

Witness (*if the participant was not able to read and understand the Consent Information Sheet and Informed Consent Document*).

I affirm that the Informed Consent Document has been read to the participant, and she understands the study and I have witnessed her consent to study participation.

_____ _____ ____/___/_____

Name of Witness Signature Date

8.3 INFORMED CONSENT FORM

University of Zambia

School of Health Sciences

Department of Biomedical Sciences

Name of Head of Household: _____

Surveillance Activity Type: _____

House Number: _____

I can confirm that I have read (read to me) the information sheet and understand the objectives of this study. I had the opportunity to consider the information, ask questions and have had these answered fully.	Tick:
.....(name of investigator) has explained to me the nature and purpose of the activities to be undertaken. I understand fully what is to be done.	Tick:
I understand that my participation is voluntary and I am free to withdraw at any time, without giving any reason.	Tick:
I agree to take part in the above activity.	Tick:

Signature of Head of Household: _____ Date: _____

I confirm that I have explained to the person named above the nature and purpose of the activities to be undertaken.

Signature of Investigator: _____ Date: _____

1 signed copy of this form is given to the head of household;

1 signed copy of this form is kept for the Principal Investigator