Anopheles species composition and insecticide resistance patterns in Uganda

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DECLARATION BY STUDENT

I, Henry Ddumba Mawejje, declare that this thesis is my own work. Where the work of others has been referred to in this thesis, this has been indicated. This work has not been submitted previously for an academic qualification.

Signature [redacted] Date: 1st May 2023

ABSTRACT

Background: Long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) are essential to malaria control, but are threatened by insecticide resistance. This thesis aimed to: 1) investigate malaria vector species collected indoor and outdoor using different methods; 2) describe species composition of *Anopheles* vectors collected from different areas; 3) determine susceptibility of *Anopheles* to pyrethroid insecticides, and 4) evaluate the association between genotypic markers of pyrethroid resistance and mosquito survival.

Methods: Between October 2011 and November 2021, mosquitoes were collected from 13 sites across Uganda – indoors: human landing catches (HLC), CDC light traps (CDC LT) and prokopack aspirators; outdoors – HLCs and pit traps. Implementation of vector control, including LLINs only and LLINs + IRS, differed across time and space. *Anopheles* species composition was assessed using PCR. Standard WHO tube assays were done for permethrin and deltamethrin, with and without piperonyl butoxide (PBO). Mosquitoes were genotyped for *Vgsc-L995S/L1014S* and *Vgsc-L995F/L1014F*, *Cyp6aa1*, *Cyp6p4, ZZB-TE, Cyp4j5*, and *Coeae1d* using locked nucleic acid (LNA) and TaqMan assays.

Results: Overall, 165,739 female *Anopheles* mosquitoes were collected from 13 sites using different collection methods. Of these, 160,657 were collected using CDC light traps (objectives 1 and 3), 349 using prokopack aspirators, 746 using human landing catches (indoor and outdoor), 1,234 using pit traps and 2,753 using larval collections.

In the assessment of the impact of different mosquito collection methods, the vector density (mosquitoes per unit collection) using CDC light traps was 4.24 compared to 2.96 using indoor HLCs (4.24 vs 2.96, density ratio [DR] 0.70, 95% CIs 0.63–0.77, $p < 0.001$) and 4.24 compared to 1.82 using prokopacks (4.24 vs 1.82, DR 0.43, 95% CIs 0.37–0.49, $p < 0.001$). Sporozoite rates were similar between indoor methods, although precision was limited. Considering outdoor collections, the vector density was 3.53 using HLCs compared to 6.43 using pit traps (3.53 vs 6.43, DR 1.82, 95% CIs 1.61–2.05, $p < 0.001$). However, the sporozoite rate using pit trap collections was significantly lower at 0.004 compared to 0.018 using outdoor HLCs (0.018 vs 0.004, rate ratio [RR] 0.23, 95% CIs 0.07–0.75, $p = 0.008$). Prokopacks collected a higher proportion of *Anopheles funestus* (75.0%) than indoor HLCs (25.8%), while pit traps collected a higher proportion of *Anopheles arabiensis* (84.3%) than outdoor HLCs (36.9%)

At least 158,095 female *Anopheles* mosquitoes were collected from 3 sites with varying malaria transmission intensities to assess the impact of control interventions. In the low transmission site, LLIN distribution was associated with a decline in *Anopheles funestus* vector density (0.07 *vs* 0.02 mosquitoes per house per night,

density ratio [DR] 0.34, 95% CI: 0.18–0.65, *p* = 0.001), but not in *Anopheles gambiae s.s. (hereafter An. gambiae)* or *Anopheles arabiensis*. In the moderate transmission site, over 98% of mosquitoes were *An. gambiae* and LLIN distribution was associated with a decline in *An. gambiae* vector density (4.00 *vs* 2.46, DR 0.68, 95% CI: 0.49–0.94, *p* = 0.02). In the high transmission site, the combination of LLINs and multiple rounds of IRS was associated with significantly lower density of *An. gambiae (*28.0 *vs* 0.17, DR 0.004, 95% CI: 0.002–0.009, *p* < 0.001), and *An. funestus* sensu lato (*s.l.*) (3.90 *vs* 0.006, DR 0.001, 95% CI: 0.0005–0.004, *p* < 0.001), with a less pronounced decline in *An. arabiensis* (9.18 *vs* 2.00, DR 0.15 95% CI: 0.07–0.33, *p* < 0.001).

In total, 2,753 *An. gambiae s.l.* were subjected to phenotype bioassays. Overall, mortality rates in *An. gambiae* and *An. arabiensis* following exposure to pyrethroids were 18.8% (148/788) and 74.6% (912/1,222) respectively. Pre-exposure to PBO resulted in higher mortality for both *An. gambiae* (permethrin: 12.9% to 56.5%; deltamethrin: 25.2% to 68.7%), and *An. arabiensis* (permethrin: 65.5% to 93.3%; deltamethrin: 82.4% to 89.8%). Most *An. gambiae* had the *Vgsc-995S/F* mutation (95% frequency) and the *Cyp6p4* resistance allele (87%), while the frequency of *Cyp4j5* and *Coeae1d* were lower (52%and 55%, respectively).

Conclusions:

The density and species of mosquitoes collected with alternative methods varied, reflecting the feeding and resting characteristics of the common vectors and the different collection approaches. LLIN distribution was associated with reductions in

An. funestus s.l. in the lowest transmission site and *An. gambiae* in the moderate transmission site. In the high transmission site, a combination of LLINs and IRS and multiple rounds of IRS was associated with the significant reduction of *An. gambiae* and *An. funestus s.l.* Following IRS, *An. arabiensis*, a behaviorally resilient vector, was the predominant species. Resistance to pyrethroids was widespread in the study area, and the mortality rate was higher in *An. arabiensis* compared to *An. gambiae*. Further surveillance of insecticide resistance and assessment of correlations between genotypic markers and phenotypic outcomes are needed to better understand mechanisms of pyrethroid resistance in conferring resistance to guide vector control.

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LIST OF ABBREVIATIONS -

LLINS	Long- lasting insecticidal nets
IRS	Indoor Residual Spraying
WHO	World Health Organization
CYP	Cytochrome p450
Vgsc	Voltage gated sodium channel
KDR	Knock Down Resistance
PBO	Piperonyl butoxide
DDT	Dichloro diphenyl trichloro ethane
DNA	Deoxyribonucleic acide
LNA	Locked Nucleic acid
TPP	Triphenyl phosphate
HLC	Human landing catch
KDR	Knock down resistance
ACT	Artemisinin based conmbination therapy
WHO	World Health Organization
PRISM	Program for Resistance, Immunology, Surveillance and Modeling of Malaria
DRC	Democratic Republic of Congo
UDHS	Uganda Demographic Health Survey
MIS	Malaria Indicator Survey
RR	Risk Ratio
IRR	Incidence Risk Ratio
DR	Density Ratio
OR	Odds Ratio
CI	Confidence Interval
UNCST	Uganda National Council of Science and Technology
SOMREC	School of Medicine Research and Ethics committee
N/A	Not applicable

PREAMBLE

This thesis was written in Research Paper Style format in accordance to guidelines provided by the London School of Hygiene and Tropical Medicine. The thesis consists of three research papers, two of which were published in the Malaria Journal and the third published in Current Research in Parasitology and Vector Borne Diseases Journal. The research papers included in this thesis were published in open access journals with retention of copyright by the authors.

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CHAPTER 1 INTRODUCTION

1.1 THESIS STRUCTURE

Chapter 1 provides the introduction, rationale, and objectives of the thesis and a summary of the study structure. **Chapter 2** presents a systematic review of the literature on malaria vectors and insecticide resistance patterns in East Africa. **Chapter 3** presents the study design and methodology including study procedures and statistical methods. **Chapter 4**, presents a manuscript reporting the impact of different mosquito collection methods on indicators of *Anopheles* malaria vectors in Uganda, which was published in the Malaria Journal. **Chapter 5** presents a manuscript entitled, Impact of seasonality and malaria control interventions on *Anopheles* density and species composition from three areas of Uganda with differing malaria endemicity; which was published in the Malaria Journal. **Chapter 6** includes a manuscript characterizing pyrethroid resistance and mechanisms in *Anopheles gambiae* s.s. and *Anopheles arabiensis* from 11 districts in Uganda, which was published in Current Research in Parasitology and Vector Borne Diseases Journal. **Chapter 7** discusses the research findings, their implications, and underscores the gaps, limitations and recommendations, concluding the thesis.

1.2 Malaria transmission

Malaria is an infectious disease caused by *Plasmodium* parasites (Figure 1.1). Five *Plasmodium* species infect humans, including *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi*. *Plasmodium falciparum* accounts for >95% of malaria infections in sub-Saharan Africa [1].

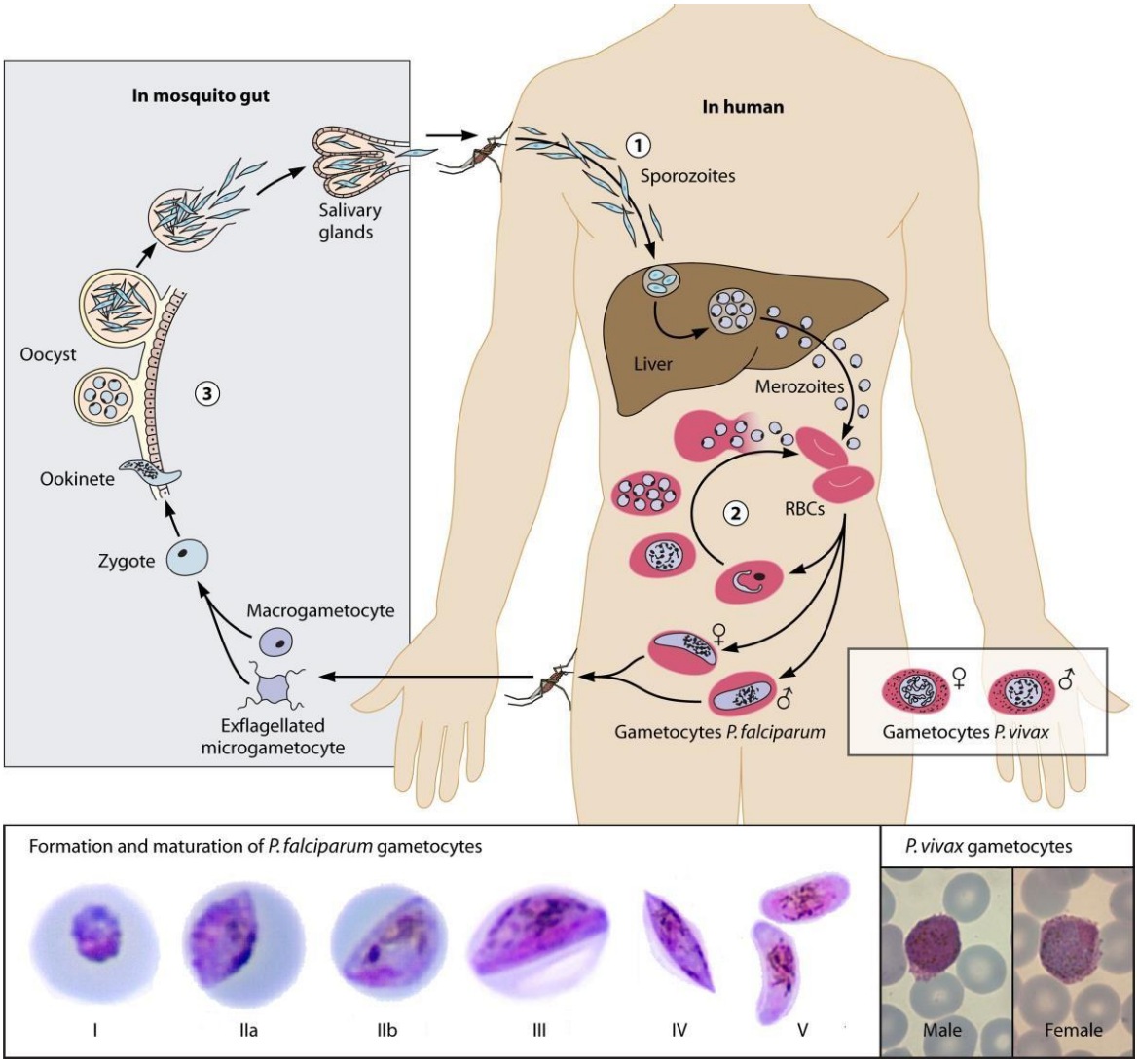


Figure 1.1: *Plasmodium falciparum* parasite life cycle *(Image credit* [2]*)*

Over 400 species of *Anopheles* mosquitoes have been identified. Among these, about 30-34 are important vectors of malaria [3] (Figure 1.2). The primary malaria vectors in sub-Saharan Africa are *Anopheles gambiae* s.s., *Anopheles arabiensis* and *Anopheles funestus* s.s. [4]. These species occur in sympatry in much of sub-Saharan Africa, and are putatively the most efficient malaria vectors worldwide [5]. Sympatric populations of *An. gambiae* s.l. and *An. funestus* have been described to be complementary, with the former maintaining malaria transmission primarily in the wet season and the latter in the dry season [5]. The distribution of *An. gambiae* s.s. and *An. funestus* (Figure 1.2) [5] mirrors the distribution of *Plasmodium falciparum* malaria in Africa (Figure 1.3) [6].

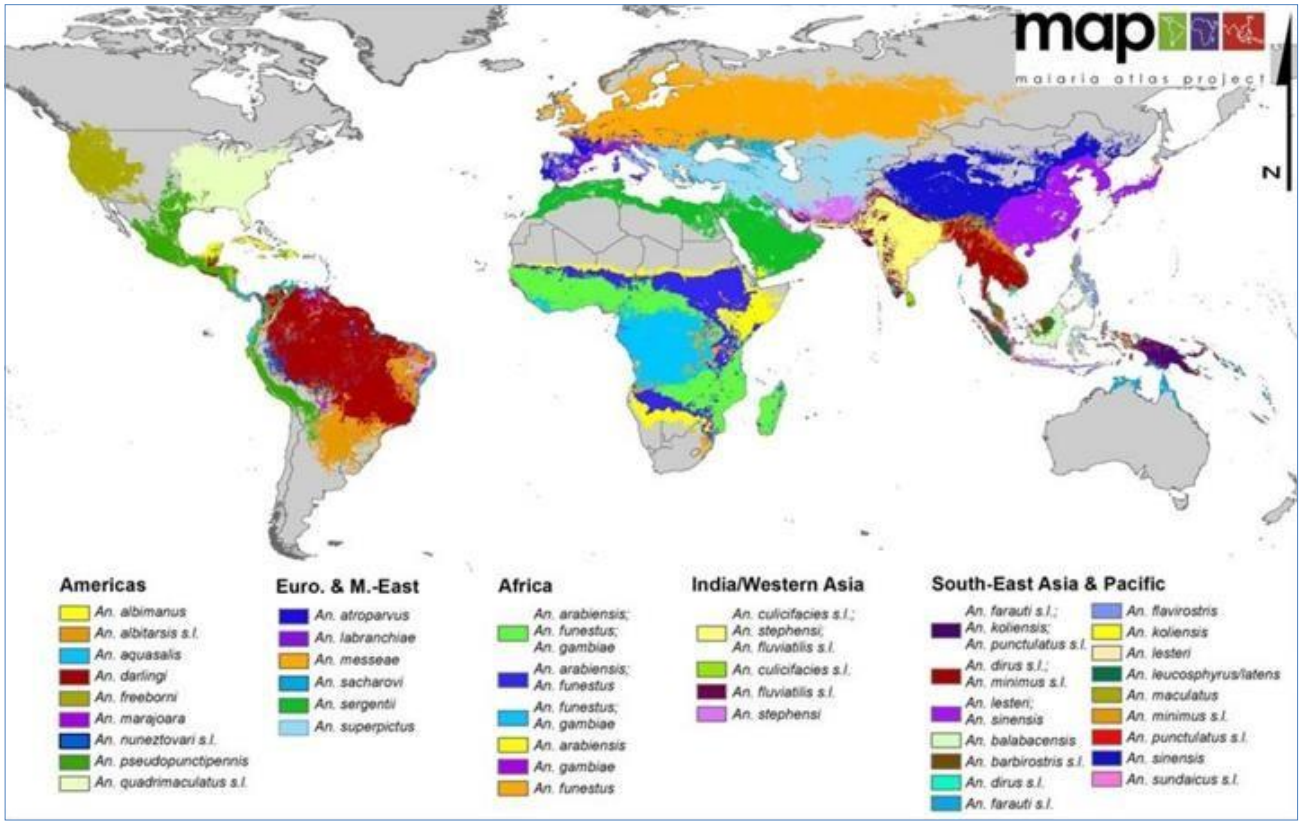


Figure 1.2: A global map of dominant malaria vector species [5]

1.3 Malaria burden

1.3.1 Global malaria burden

Malaria remains a major public health challenge with endemicity reported in 84 countries worldwide [4]. The bulk of the disease burden is in sub-Saharan Africa where more than 50% of malaria cases occur (Figure 1.3) [4]. In 2021, a slight increase of 2 million malaria cases compared to 2020 estimates was realized from the reported global estimate of 247 million cases [4]. Most malaria cases (96%) occurred in sub-Saharan Africa, with four countries, including Nigeria (26.6%), the Democratic Republic of the Congo (12.3%), Uganda (5.1%) and Mozambique (4.1%), accounting for nearly half of the global burden [4]. In addition, four countries including Nigeria, the Democratic republic of the Congo, the United Republic of Tanzania and the Niger accounted for at least 50% of all malaria deaths, with Nigeria alone accounting for nearly 40% (38.4%) of the global malaria deaths in children under 5 years of age [4]. The WHO has identified 11 high burden countries, including Uganda, Burkina Faso, Cameroon, the Democratic Republic of the Congo, Ghana, India, Mali, Mozambique, the Niger, Nigeria and the United Republic of Tanzania, which account for 70% of the global malaria case burden and 71% of global malaria deaths [7]. These countries have been prioritized, for the High Burden to High Impact approach which is intended to catalyze a targeted, country-led response to malaria control [7]. In addition to standard malaria control tools (LLINs, IRS, treatment with ACTs and intermittent preventive treatment in pregnancy-IPTp), newer interventions have been deployed or recommended, including, seasonal malaria

chemoprevention and the RTS,S malaria vaccine [8].

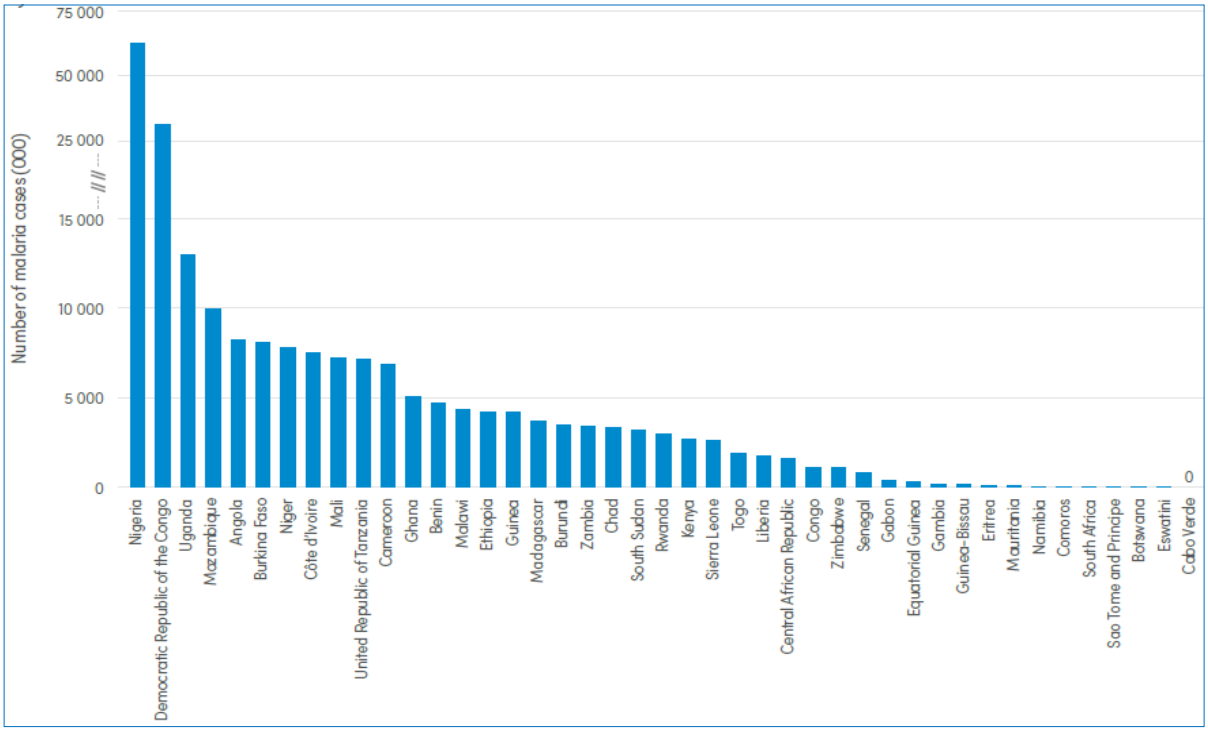


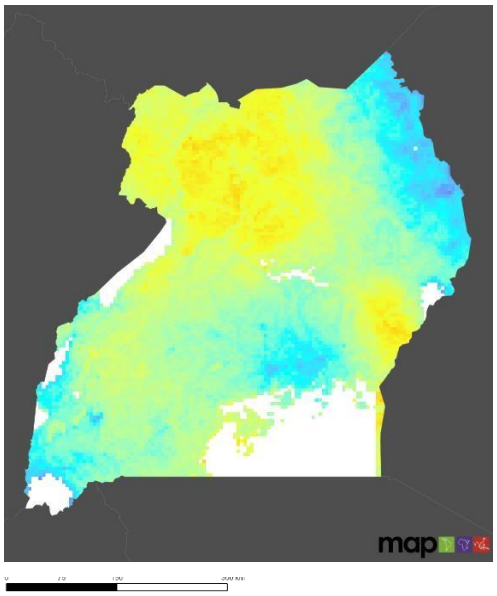
Figure 1.3: Number of malaria cases by country in the WHO African Region, 2021 showing the high burden countries in which Uganda ranks 3rd. *Source: WHO estimates; World Malaria Report, 2022* [4].

Between the years 2000 and 2015 rapid declines in malaria morbidity were observed, however by the end of 2017, progress in malaria control had stalled [9], with reports of loss of gains in some areas [6]. Considerable increase in the number of malaria cases (14 million) was observed in 2020 [1]. Scale up of primary vector control tools, particularly LLINs was implemented under the universal coverage campaign model, although the coverage of IRS declined in 2020 [1]. Malaria control in sub-Saharan Africa, has seen increased engagement from local governments evidenced by sustained domestic funding [1]. Funding and logistical bottlenecks remain in realizing malaria control and elimination targets.

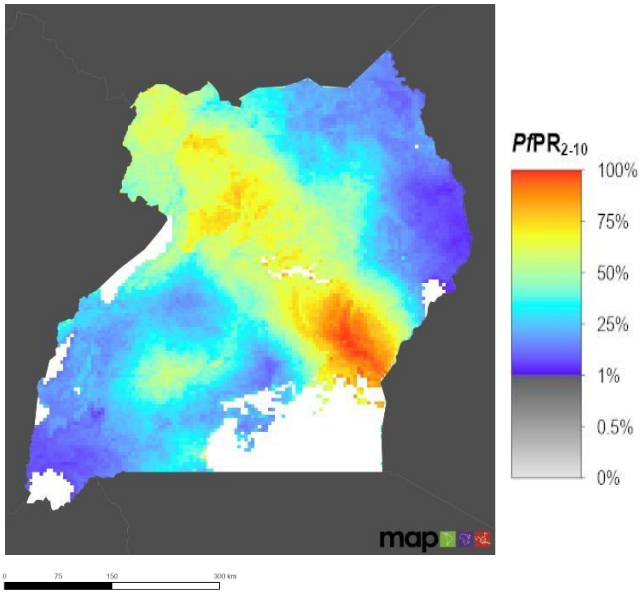
1.3.2 Malaria burden and control in Uganda

Malaria is endemic in approximately 95% of Uganda, accounting for 30-50% of outpatient care, and the entire population of about 41 million people is at risk [10] (Uganda malaria reduction and elimination plan 2020-2025, unpublished). Malaria transmission is stable in most of the country and is perennial with two major peaks aligned to rainfall patterns [10] (Uganda malaria reduction and elimination plan 2020-2025, unpublished). Nearly all malaria infections are caused by *P. falciparum parasites,* with *An. gambiae*, *An. arabiensis* and *An. funestus* s.s. as the main vectors [10]. Like elsewhere in Africa, the primary malaria control strategies inUganda include LLINs, IRS and ACTs (Uganda malaria reduction and elimination plan 2020-2025, unpublished). LLINs are distributed nationwide every 3 years since 2013-2014 [11], 2017-2018 [12] and 2020-2021 [1]. Although considerable progress in malaria control has been made in Uganda (Figure 1.4), with parasite prevalence in children underfive decreasing from 40% in 2009 [11] to less than 10% in 2019 [13]. This control has been tenuous and punctuated by malaria resurgence [14, 15] mainly in areas where IRSwas withdrawn [15]. In some districts, a recent change in the IRS insecticide from the organophosphate pirimiphos methyl to clothianidin, a neonicotinoid, either alone or in combination with deltamethrin, was associated with surprising resurgence of malaria beyond pre-IRS levels, despite active deployment of IRS and LLINs [16, 17]. Significant changes in *Anopheles* species composition have also been observed in areas where IRS has been deployed with *An. arabiensis* becoming predominant and replacing both *An. gambiae* and *An. funestus* following sustained IRS [18].

P. falciparum parasite rate in 2-10 years old children in 2000



P. falciparum parasite rate in 2-10 years old children in 2010



P. falciparum parasite rate in 2-10 years old children in 2015

P. falciparum parasite rate in 2-10 years old children in 2020

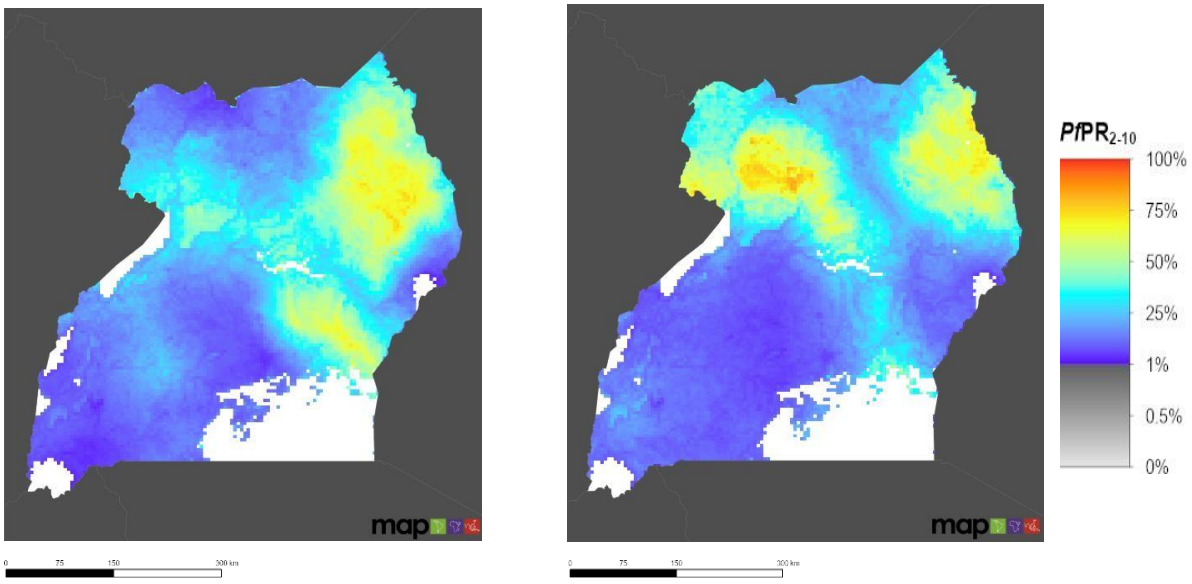


Figure 1.4: Prevalence of *Plasmodium falciparum* malaria in 2000, 2010, 2015 and 2020 (Source: https://malariaatlas.org/trends/country/UGA-Malaria Atlas Project-Accessed 19th July 2022)

1.3.3 Malaria vector control in Uganda

Early vector control efforts in the 1950s and 1960s in Uganda were conducted using indoor residual spraying initially with the organochlorine DDT [19, 20] and later with the organophosphate malathion [21]. Subsequent vector control was sporadic and limited in scope particularly after cessation of the WHO's, Global Malaria Eradication Program in 1969 [22] and the political instability in Uganda, during the decade that followed [23]. Malaria vector control was revived in Uganda in the early 2000s following the established framework from WHO's Global Plan of Action for Malaria Control 1993-2000 [23, 24]. Insecticide treated bed nets (ITNs) were introduced in Uganda between 2000-2002 [23]. Information on the ownership of ITNs in Uganda was first reported in the 2000-2001 Demographic Health Survey which documented 13% ITN ownership (defined as the percentage of households with at least one insecticide treated net) [25]. ITN usage (defined as the percentage of children who slept under a mosquito net the night before

the survey) was recorded at only 7% [25]. The subsequent Demographic Health Survey in 2006 recorded a slight increase in ITN ownership to 16% [26]. However, increase in ITN ownership was reported in 2011 at 60% [27], which was likely catalyzed by availability of new funding for malaria control through the Global Fund [28]. This Fund enabled investment in new technologies leading to the gradual shift from insecticide treated nets which required periodic re-treatment with insecticide [29] to long lasting insecticidal nets (LLINs) which had a useful life under field conditions of at least 3 years [30]. The first Malaria Indicator Survey (2009) in Uganda [31], showed a gradual adoption of LLINs at 46% ownership. In 2013-2014, Uganda launched its first universal LLIN distribution campaign in line with the Uganda Malaria Reduction Strategic Plan [10] aiming to achieve universal coverage of LLINs, defined as one net for every two persons. The Ministry of Health is committed to distributing LLINs nationwide in Uganda every 3-4 years. New generation LLINs, particularly with the synergist piperonyl butoxide (PBO) to counteract pyrethroid resistance, were adopted and first distributed in the 2017/2018 LLIN campaign [32] and are now incorporated in the Uganda Malaria Reduction and Elimination Strategy 2021-2025 (Uganda Malaria Reduction and Elimination plan 2020-2025, unpublished). In the 2019 Malaria Indicator Survey, LLIN coverage was recorded at 83% [12]. The latest LLIN distribution campaign 2020/2021 occurred during the COVID-19 pandemic, and was largely successful, despite some delays and disruptions [1].

After a nearly 5-decade absence as a malaria vector control tool, indoor residual spraying was reintroduced in mid-Northern Uganda using the pyrethroid lambda-cyhalothrin, which was later replaced with DDT and alpha-cypermethrin between 2007-2010 [11, 33].

Following reports of increasing resistance to both DDT and pyrethroids [11, 33], the IRS insecticide was changed to the carbamate bendiocarb in 2010. Between 2009-2014, IRS was scaled up to 10 high-malaria burden districts in Northern Uganda using bendiocarb [11]. In 2015, the IRS programme was shifted to 14 high malaria burden districts in Northern and Eastern regions. The insecticides used for IRS were changed from bendiocarb to an organophosphate pirimiphos-methyl (Actellic) between 2015-2019, and thereafter to a neonicotinoid clothianidin, with or without deltamethrin [15, 16].

1.4 History of malaria control

1.4.1 Early malaria control efforts: the malaria eradication era

Global efforts to eliminate and eradicate malaria in the mid twentieth century were driven by the discovery of DDT [34, 35]; a synthetic insecticide effective against indoor resting mosquitoes [34]. Initially, the perceived cost of malaria control deterred the implementation of control programs in many areas [36]. However, the combination of indoor residual spraying (IRS) with DDT plus treatment with chloroquine, at that time a highly effective and inexpensive drug, strengthened the resolve to interrupt malaria transmission and expand the malaria eradication campaign led by WHO Global Malaria Eradication Program (GMEP) [37, 38]. Through these efforts, malaria was eradicated in Europe and much of the Americas, but control efforts were not sustained in sub-Saharan Africa, due to the failure to interrupt malaria transmission in Garki, Nigeria [35]. Malaria eradication in Europe was also supported by additional factors including industrialization, drainage of potential mosquito breeding sites such as canals for

agriculture, increased urbanization, improved house construction and screening of windows [39]. Successful vector control in the GMEP campaign targeted endophilic (indoor resting), endophagic (indoor feeding) and anthropophagic (attracted to human host) *Anopheles* mosquitoes [35]. However, a non-targeted mosquito population of indoor biting, but outdoor resting mosquitoes continued to propagate malaria transmission [39]. Elimination efforts were further challenged by the development of resistance to DDT, emergence of chloroquine resistance [40, 41], social and cultural barriers which limited coverage in remote regions, limited health infrastructure and a shift in priorities, which all contributed to the abandonment of the malaria eradication campaign in 1969 [39]. Subsequently the malaria burden increased dramatically [38, 42].

Sub-Saharan Africa was generally excluded from the GMEP campaign due to the scale of malaria transmission, highly efficient malaria vectors with very high human biting rates (over 100 infective bites per person per year), and poor infrastructure [43, 44]. However, some success was achieved in the Garki project, coordinated by the WHO in the late 1960s and 1970s as a pilot to assess the impact of IRS and mass drug administration on malaria burden in Nigeria [35, 39]. Although the IRS campaign with the carbamate propoxur in combination with mass drug administration resulted in significant reductions in parasite prevalence, it was not sufficient to interrupt malaria transmission [35, 39]. As a result, the WHO's focus shifted from eradication/elimination to control in Africa [45] but the programme was eventually abandoned, and malaria control in Africa and elsewhere collapsed and remained quiescent from the 1970s to the 1990s [39]. In addition, the primary antimalarial treatment available was chloroquine, to which

Plasmodium parasites were increasingly resistant [46, 47]. The burden of malaria increased with reports of resurgences and epidemics in several countries [38, 48]. Eventually, the malaria control agenda was revived in the mid-1990s with the addition of insecticide treated nets (ITNs) as a vector control measure [39]. Since then, substantial investments have been made in supporting increased coverage of key interventions including, long lasting insecticidal nets (LLINs), Indoor Residual Spraying (IRS) and case management with artemisinin-based combination therapies (ACTs) [1]. Whilst, the call for malaria elimination and global eradication was revisited in 2007 (African Union) following initial successes, it is widely recognized that new tools will be required to attain this goal [42].

1.5 Vector control measures

1.5.1 Long lasting insecticidal nets (LLINs)

Insecticide treated bed nets are the primary malaria vector control tools in sub-Saharan Africa and are credited with at least 68% of all clinical malaria cases averted between 2000 and 2015 [49]. Insecticide treated nets provide protection through two mechanisms, with the net acting as a physical barrier between malaria vectors and the human host while the insecticide kills mosquitoes upon contact. The excito-repellency effect of the insecticide [50] contributes to community wide protection [51]. Early versions of the insecticide treated nets [52-56], required regular retreatment of the net fabric with insecticide [57, 58]. Studies of these older nets in the 1990s [59] indicated that they were highly effective as measured by mosquito survival [60], blood feeding success, and protection of vulnerable groups particularly pregnant mothers and children

under five years [56, 58]. However, the requirement for net retreatment was labor intensive and challenged the sustainability and acceptability of the strategy [29]. The utility of nets has been improved by technology enabling the impregnation of pyrethroid and other insecticides into synthetic fibers, thereby permitting the slow release of the active ingredients over a projected period of at least 3 years [57, 61, 62]. Such nets are referred to as long lasting insecticidal nets (LLINs) [30, 63]. The transition to LLINs eliminated the need for net retreatment, catalyzing the scale-up of this tool [64]. The WHO recommends universal coverage of LLINs; which is defined as the universal access to and distribution of at least one net for every two persons at risk of malaria [65, 66]. The compact nature of LLINs as a commodity that can be purchased in bulk and distributed individually to households has enabled the rapid scale-up of this effective vector control tool [67-69] across sub-Saharan Africa.

Until recently [70], all LLINs were impregnated only with pyrethroid insecticides due to their effectiveness against susceptible mosquitoes, a good safety record [71] and relatively lower cost of production compared to alternatives [72]. However, resistance to pyrethroids has increased substantially from a few sites in the 1990s [73] to widespread in several areas in less than two decades [74], likely driven by the scale up of vector control since 2000 [75]. Resistance to pyrethroids presents a major threat to the efficacy of LLINs in sub-Saharan Africa [47, 67, 74, 76-78]. To combat insecticide resistance, new formulations that combine pyrethroids with additional chemicals have been developed, including the synergist piperonyl butoxide (PBO) [32, 79], chlorfenapyr (a pyrrole insecticide) [80, 81] and pyriproxyfen (an insect growth regulator) [82, 83]. These new generation LLINs have been shown to be more effective than standard pyrethroid only LLINs in reducing malaria morbidity as measured by epidemiological and entomological

outcomes [32, 79, 80, 83, 84]. In areas with active malaria transmission where the primary malaria vectors are resistant to pyrethroids, the WHO recommends deployment of PBO-based LLINs as an alternative to pyrethroid only LLINs [8]. Table 1.1 summarizes WHO prequalified LLINs.

Table 1.1: WHO Prequalified list of LLINs as of 26th August 2020 [70]

Classification	Product Name	Active Ingredient	Date of Prequalification
	Interceptor	Alpha-cypermethrin	08/12/17
	Royal Sentry	Alpha-cypermethrin	07/12/17
	Royal Sentry 2.0	Alpha-cypermethrin	06/02/19
	Duranet LLIN	Alpha-cypermethrin	07/12/17
	MiraNet	Alpha-cypermethrin	21/02/18
Classification	**Product Name**	**Active Ingredient**	**Date of Prequalification**
Standard pyrethroid only LLINs	MAGNet	Alpha-cypermethrin	19/02/18
	SafeNet	Alpha-cypermethrin	19/02/18
	PermaNet 2.0	Deltamethrin	08/12/17
	Yahe LN	Deltamethrin	19/02/18
	Yorkool LN	Deltamethrin	19/02/18
	Panda Net 2.0	Deltamethrin	03/05/18
	Tsara	Deltamethrin	14/8/20
	OLYSET Net	Permethrin	07/12/17
Dual active ingredient	Interceptor G2	Alpha-cypermethrin; chlorfenapyr	29/01/18
Combination with insect growth regulator	Royal Guard	Alpha-cypermethrin; Pyriproxyfen	29/03/19
Combination with PBO	DuraNet Plus	Alpha-cypermethrin; PBO	13/8/20
	VEERALIN	Alpha-cypermethrin; PBO	29/01/18
	PermaNet 3.0	Deltamethrin, PBO	29/01/18
	Tsara Boost	Deltamethrin, PBO	29/01/18
	Tsara Plus	Deltamethrin, PBO	29/01/18
	OLYSET PLUS	Permethrin; PBO	29/01/18

1.5.2 Indoor residual spraying (IRS)

IRS is the application of a residual insecticide to potential malaria vector resting surfaces, such as internal walls, eaves and ceilings of houses or structures (including domestic animal shelters), where such vectors are likely to come into contact with the insecticide [8]. IRS has been instrumental in malaria elimination and eradication efforts, effectively

lowering the longevity and density of malaria vectors that rest and feed indoors, thus reducing sporozoite rates [18] and clinical malaria episodes [15, 85]. Unlike LLINs, which have traditionally relied on only one class of insecticides (pyrethroids) [50], multiple insecticide classes are available for indoor residual spraying including pyrethroids, carbamates, organophosphates and neonicotinoids [8, 70]. Insecticide combinations such as clothianidin + deltamethrin (Fludora Fusion) have also been deployed for IRS [16]. The use of DDT (organochlorine) for public health has largely been discontinued with current WHO recommendations advocating for a total ban of the persistent organic pollutant [8].

Despite the potency of IRS in reducing malaria morbidity and mortality, the high operational costs and financing required for deployment of this tool have limited the scale-up of this intervention [86]. Notably, the population protected by IRS in sub-Saharan Africa in the last decade halved from 11.2% in 2010 to 5.3% in 2020 [1]. Overall, IRS coverage declined from 5.8% in 2010 to 2.6% in 2020 [1]. Although IRS is highly effective, the discontinuation of spraying has been associated with resurgence of malaria to pre- IRS levels in some environments including parts of Uganda [14, 15, 87]. Current WHO guidelines for IRS [8] recommend rotation of insecticides based on susceptibility outcomes of local malaria vectors as a strategy to mitigate insecticide resistance. The co-deployment of LLINs with non-pyrethroid-based IRS has also been recommended by the WHO under certain conditions [8].

1.5.3 Insecticides, compounds and their properties

1.5.3.1 DDT and Pyrethroids

DDT (organochlorine) and pyrethroids share the same target site in the voltage gated sodium channel [78], which is associated with cross-resistance between the two insecticide classes. Carbamates and organophosphates also share the same target site, where point mutations leading to insensitive acetylcholinesterase are associated with cross resistance between these insecticide classes [88, 89]. Insecticides with alternative modes of action such as clothianidin (neonicotinoid) [90], chlorfenapyr (pyrrole) [80], and pyriproxyfen [82] are recommended for deployment in malaria vector control programs to counteract pyrethroid resistance [8]. The specific mechanisms of resistance are expounded in section 1.6.

1.5.3.2 Neonicotinoids

'Neonicotinoids are selective agonists of the insect nicotinic acetylcholine receptor (*nAChR*), a pentameric cys-loop ligand- gated ion channel located in the central nervous system of insects' [91]. Neonicotinoids have been used extensively in agriculture since the 1990s [92]. However, new evidence shows resistance to clothianidin, a new active ingredient in IRS formulations, in areas with extensive agricultural application of neonicotinoids [93].

1.5.3.3 Pyrroles

Pyrroles such as chlorfenapyr are new generation insecticides without cross resistance to other insecticide classes [94, 95]. The activity of pyrroles against malaria vectors is through disruption of pathways that enable cellular respiration and production of

energy in the mitochondria [95]. For disruption of metabolic pathways in *Anopheles* to occur, pyrroles like chlorfenapyr require activation by cytochrome p450 monooxygenases into active metabolites [96], characterized by slow acting toxicity [94]. Given the alternative mode of action and absence of cross resistance with other insecticide classes, chlorfenapyr has shown strong insecticidal properties with high mortalities in *Anopheles gambiae* s.l. mosquito populations regardless of resistance status [94, 97-100].

1.5.3.4 Piperonyl butoxide

Piperonyl butoxide (PBO) is a synergist which by definition is 'a substance which does not itself have insecticidal properties, but which, when mixed or applied with insecticides of a particular class, considerably enhances their potency, for example by inhibiting an enzyme that normally has detoxifying activity against the insecticide' [50]. PBO is a methylenedioxyphenyl compound whose synergistic properties have been exploited to create a viable combination with pyrethroid insecticides [101]. PBO blocks the activity of monooxygenase enzymes associated with insecticide metabolism [102], particularly to pyrethroids. Insecticide resistance to pyrethroids is widespread and remains a major threat to malaria vector control intervention efficacy, particularly LLINs [74]. The addition of the synergist PBO to pyrethroids has been associated with increased mortality to pyrethroid exposure in both *An.* gambiae s.l. [103] and *An. funestus* [104]. PBO has been used in combination with pyrethroids, especially, type I (permethrin) and type II (deltamethrin) in areas with high pyrethroid resistance as a

resistance management strategy in sub-Saharan Africa [8]. The economic attraction of PBO for deployment in LLINs and its broad spectrum (non-specific nature to particular p450s) [105] has enabled the development of PBO-based long lasting insecticidal nets which show significantly lower morbidity due to malaria compared to pyrethroid only LLINs [32, 79].

1.5.3.5 Insect-growth regulator-Pyriproxyfen

Pyriproxyfen is juvenile hormone that disrupts mosquito reproduction and physical development through inhibition of embryogenesis, egg production and the process of metamorphosis [83, 106, 107], leading to sterilization of females and mosquito population control [107, 108]. Pyriproxyfen is a newly repurposed insecticide class combined with alpha-cypermethrin on LLINs for pyrethroid resistance management [83]. This new generation tool induces insecticidal activity and blood feeding inhibition with alpha-cypermethrin and inhibits egg production in adult females for population reduction [109, 110]. Pyriproxyfen treated nets present an alternative to standard LLINs in controlling pyrethroid resistant *An. gambiae* mosquitoes [111, 112]. However, work by Yunta et al, [113] shows that pyriproxyfen can be metabolized by a number of cytochrome p450 enzymes including *Cyp6p4,* which is associated with pyrethroid metabolism in *An. gambiae* [113] and *An. arabiensis* [114].

1.6 Insecticide resistance

1.6.1 Overview

Insecticide resistance in *Anopheles* vectors refers to the inate and/or acquired ability of mosquitoes to survive insecticide exposure and the underlying genetic mechanisms driving this ability [103].

Aerobic organisms in nature, have the innate ability to adapt to changes in their environment through the natural selection process and genetic recombination. Through this process, heritable characteristics such as insecticide resistance can progressively increase in a naïve *Anopheles* vector population as a consequence of prolonged insecticide selection pressure [115, 116]. Insecticide resistance tests are conducted using both phenotypic and genotypic methods to characterize the resistance profile of the target population.

1.6.2 Measuring resistance

1.6.2.1 Phenotypic resistance

Phenotypic resistance refers to the observed response of malaria vectors to diagnostic insecticide exposure measured as a function of knock down rate or mosquito mortality. The resistant phenotype in malaria vectors is a physical manifestation of underlying resistance genes or mutations and is determined using established protocols including the WHO diagnostic bioassay tube tests, the recently developed WHO bottle assay [117] and the established CDC bottle assay [118]. The key differences between the two bottle assays include the test end-points, insecticide concentrations used and exposure time to the mosquitoes [117]. Whilst the WHO bottle assay measures mosquito mortality at 24 hours post exposure for standard insecticides and at 72 hours post insecticide exposure for chlorfenapyr, the CDC bottle assay measures mosquito mortality

immediately after the exposure period of either 30 minutes or 45 minutes (for DDT) [117]. Description of the assay used for measuring phenotypic resistance is expanded in the methods section (section 3). Briefly, mosquitoes of a known age range (3-5 days) were exposed to pre-determined diagnostic concentrations of insecticides for a predetermined diagnostic time of 60 minutes [117]. The end point measured was mosquito mortality. All tests were performed with non-insecticide exposed mosquito controls for quality assurance purposes.

1.6.2.2 Genotypic resistance

Genotypic resistance constitutes genetic changes and / or amino acid responses of mosquito genes to insecticide exposure [103]. The major molecular resistance mechanisms have been described, including target site and metabolic resistance [74]. Behavioral resistance and reduced cuticular penetration have also been described [103, 116, 119, 120]. Target site mutations alone or in combination with metabolic resistance determinants confer resistance to several insecticide classes, occasionally with very strong phenotypes [103]. Target site mutations in particular, may only partly explain the heritable variation in the resistance phenotype [121]. Resistance mechanisms are discussed in greater detail in the subsequent sections.

1.6.3 Fitness cost associated with insecticide resistance

Insecticide resistance is characterized by heritable changes in insect genes [103], which

may confer a fitness advantage in the presence of insecticide exposure, but potentially confer a 'fitness cost' in absence of the insecticide [122]. Random mutations in insecticide target genes usually occur below detectable thresholds, unless insecticide selection pressure is maintained [122]. Given that these changes are not naturally occurring, genes from wild-type insect populations are considered to be at equilibrium and in the absence of insecticide selection, it is presumed that inherited changes would be reversed [123]. Fitness cost arising from metabolic resistance has been reported in the malaria vector, *An. funestus*, where mosquitoes possessing the resistant allele oviposited significantly lower numbers of eggs and developed at a slower rate than susceptible individuals [124]. However, the life span of resistant mosquitoes was not affected [124]. Removal of insecticide selection pressure has in some cases been followed by reversal of the resistant genotype to wild type after a number of generations [124]. Insecticide resistance management strategies that advocate for insecticide rotations target delayed onset or reversal of resistance by removing insecticide selection pressure [122].

1.6.4 Physiological basis of insecticide resistance

For insecticidal compounds to be effective, target insect species must come into contact with the insecticide [116]. Killing of naïve insects can be achieved through cuticle penetration, ingestion or digestion of the insecticide at the target site of action (Figure 1.5) [116]. However, any alteration that obstructs or limits these insecticidal properties may signal development of resistance (Figure 1.5) [103, 116]. Mechanisms of resistance in malaria vectors include i) cuticle thickening or alterations in cuticle composition to

reduce or slow down insecticide penetration [119, 120, 125, 126], ii) modification of behavior to avoid or limit contact with the insecticide [127], iii) metabolism or detoxification of the active ingredients [103] and iv) modification of insecticide target sites arising from genetic mutations giving rise to target site resistance [88, 128-131] (Figure 1.5). Overall, insecticide resistance can be complex and multifactorial with more than one mechanism mediating resistance to several compounds in a single individual [74, 103, 132] (Figure 1.5).

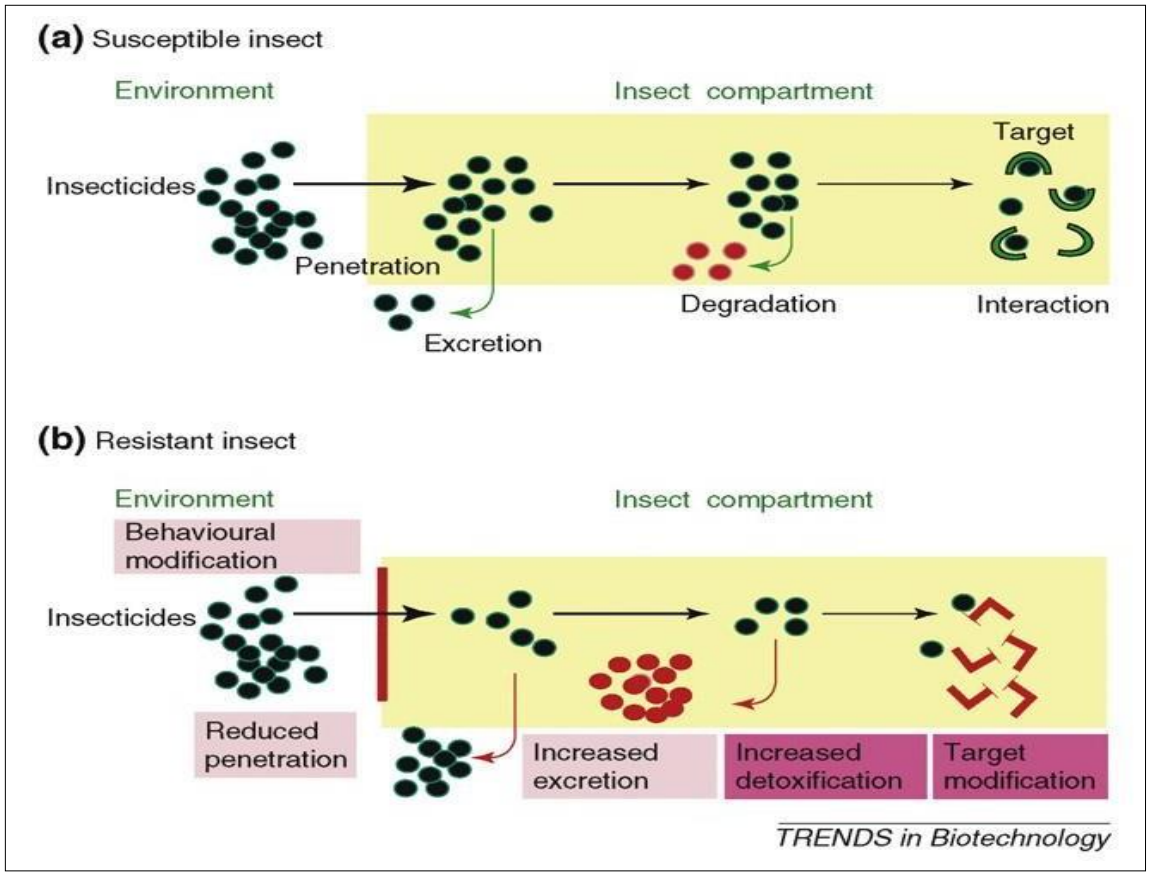


Figure 1.5: Insecticide physiology in (a) susceptible (naïve) and (b) resistant insects. Image credit: Lapied et al, [116].

1.6.5 Target site mutations/resistance

Target site resistance refers to modification of the protein receptor regarded as the insecticidal target subsequently nullifying or reducing the effectsof the insecticide [117]. As a consequence of target site mutations, the resistant insect may remain unaffected, or may be less affected, by the insecticidal effects of the chemical formulation [122]. Pyrethroid and organochlorine insecticides share the same target site in the voltage-gated sodium channel (*Vgsc*) while carbamates and organophosphates target acetylcholinesterase [78, 88, 128, 129] (Figure 1.6). The voltage-gated sodium channel, nicotinic acetylcholine ligand-gated ion channel and acetylcholinesterase are essential in mosquitoes, and their inhibition or repeated firing is fatal [88, 91]. Mutations in the *Vgsc* gene have been found in at least 13 species of *Anopheles,* with strong linkage to pyrethroid resistance [133].

1.6.6 *Voltage gated sodium channel (Vgsc)*

Two-point mutations in the *Vgsc* gene of *An. gambiae* s.s. are associated with knockdown resistance (*kdr*) (Figure 1.6) including i) a leucine to phenylalanine substitution at position 1014 (*L1014F*) initially described in West Africa [128] and ii) a leucine to serine substitution (*L1014S*) at the same codon, first described in East Africa [129]. An additional mutation in the voltage gated sodium channel (*Vgsc-N1575Y*) only found on the *L1014F* haplotype background has been shown to synergize the *L1014F* mutation increasing pyrethroid resistance [134]. Recent developments from the *Anopheles gambiae* 1000 genomes project have led to new nomenclature for the *kdr*

mutation in *An. gambiae* replacing 1014 with 995; the new nomenclature for *An. gambiae* s.s. is *Vgsc-L995F* and *Vgsc-L995S* [135]. This nomenclature distinguishes the description of the target site mutation between *An. gambiae* and *An. arabiensis* (which retains the *L1014S/F* numbering) because the *An. gambiae Vgsc* codon *995* is orthologous to the *Vgsc* codon *1014* [135]. Mutations in the voltage-gated sodium channel are associated with cross resistance between pyrethroids and organochlorines (DDT) [78].

1.6.7 Insensitive *acetylcholinesterase*

A second target site mutation is associated with insensitive acetylcholinesterase (*AChE*) arising from a single point mutation in the *Ace-1* gene (Figure 1.6). This mutation is due to glycine to serine substitution at position 119 (*G119S*) [136, 137]. Insensitive acetylcholinesterase is associated with cross resistance between carbamates and organophosphates [88, 89, 130].

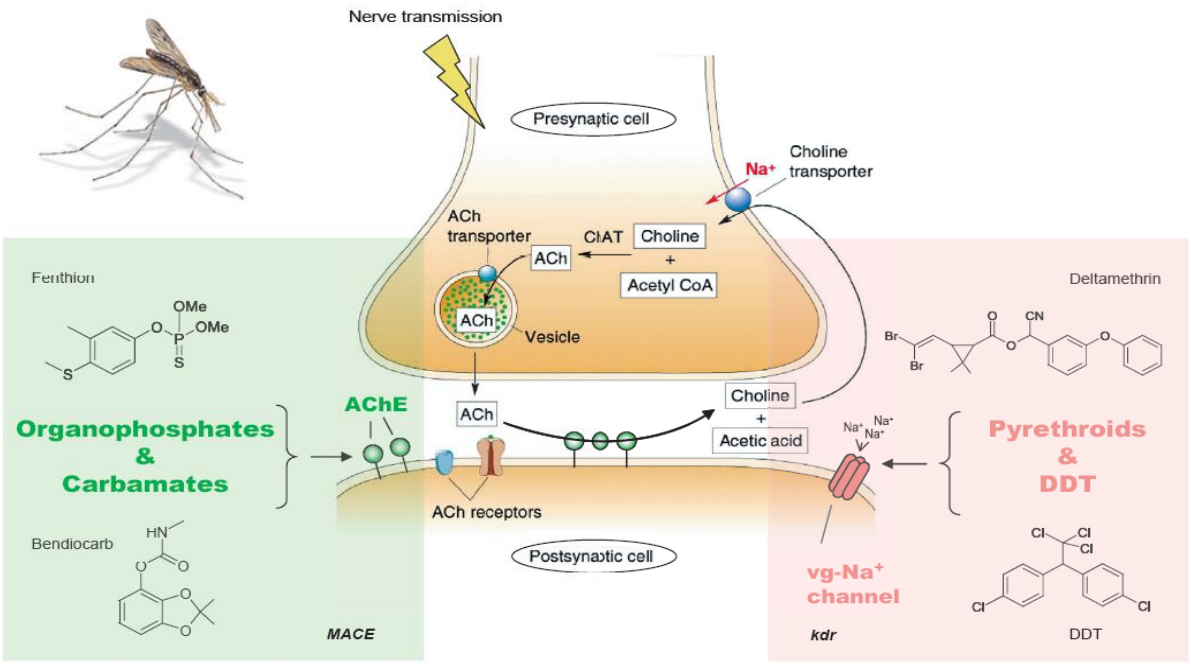


Figure 1.6: Illustration of target site resistance mechanisms showing cross-resistance between pyrethroids and DDT and cross-resistance between carbamates and organophosphates (*MACE*-Modified Acetylcholinesterase; *Kdr*-knock down resistance).Image credit: Liverpool School of Tropical Medicine.

1.7 Metabolic resistance

Metabolic resistance is a consequence of changes in the activity of enzyme systems that detoxify xenobiotic compounds in *Anopheles* vectors resulting in reduced or cancelled out effects of insecticide exposure [122]. Whilst target site mutations are a result of specific modifications in target genes, usually involving single nucleotide polymorphisms, metabolic resistance is mediated by a broad spectrum of enzymes, primarily from 3 enzyme systems, namely: esterases, monooxygenases and glutathione-S-transferases [102].

1.7.1 Cytochrome P450 monooxygenases (p450s)

Cytochrome p450s comprise a diverse and complex family of hydrophobic enzymes capable of metabolizing several compounds [103, 138]. P450s are the only enzyme system capable of mediating resistance to all insecticide classes [102, 138, 139]. This group of enzymes has wide ranging substrate specificity and catalytic properties [102] that are critical for mediating resistance to insecticides and synthesis of hydrocarbons which limit cuticle penetration [140]. Monooxygenases are particularly associated with metabolism of pyrethroids and detoxification and/or activation of organophosphates, but have limited activity in resistance to carbamates [138]. Upregulated, monooxygenase activity is associated with resistance to pyrethroids in the primary malaria vectors *An. gambiae* s.s. [141, 142]; *An. arabiensis* [114, 141, 143-146] and *An. funestus* s.s. [104, 147-150].

Knowledge of cytochrome p450 activity in metabolism of insecticides, particularly pyrethroids, has generated interest in the use of substances referred to as synergists [101], which enhance the potency of the insecticide [50]. P450 enzymes are the target of the synergist, piperonyl butoxide (PBO) [105, 151], which is equally used as a pyrethroid resistance management strategy [8]. Whilst PBO is non-specific in target, its mode of action is reliant on the level of metabolic activity from the cytochrome p450s and its strength of inhibition is dependent on the active p450 enzymes, suggesting that PBO does not inhibit all p450 enzymes equally [105]. In addition, whereas the activity of PBO is to inhibit monooxygenase enzymes [103], cytochrome p450s are required to bio activate phosphorothioate insecticides (such as organophosphates) through an oxidative process [105, 138] to make the insecticide toxic. This counteractive mode of action between PBO and cytochrome p450 enzymes suggests antagonism between PBO LLINs and organophosphate (pirimiphos methyl) based indoor residual spraying [152]. For instance, a cluster randomized trial comparing PBO LLINs and IRS with pirimiphos methyl conducted in Tanzania found no additional benefit in the reduction of parasite prevalence when the combination of PBO LLINs and IRS with pirimiphos methyl was compared to PBO LLINs only [79].

Candidate cytochrome p450 single nucleotide polymorphisms, such as *Cyp4j5* [153] and *Cyp6p4* have been associated with pyrethroid resistance. In a genome wide association study, a single nucleotide polymorphism (SNP) in the *Cyp4j5* gene represented by a leucine to phenylalanine substitution at position 43 (*Cyp4j5-L43F*) was found to be associated with metabolism of lambda-cyhalothrin and deltamethrin [153].

Another cytochrome p450 gene of interest, *Cyp6p4* has been associated with metabolism of pyrethroids in *An. gambiae* [154], *An. arabiensis* [114] and *An. funestus* [147] as well as metabolism of the insect growth regulator pyriproxyfen [113]. Recently, a triple mutant haplotype involving the cytochrome p450 duplication *Cyp6aa1,* a nonsynonymous point mutation in *Cyp6p4* and a Zanzibar-like transposable element [154] has been found to be strongly associated with the metabolism of pyrethroid insecticides, particularly deltamethrin. The nonsynonymous (SNP) point mutation in the *Cyp6p4* gene represented by an isoleucine to methionine substitution at position 236 (*Cyp6p4-I236M*) was found to be associated with metabolism of deltamethrin [154].

1.7.2 Glutathione-S-transferases (GSTs)

Glutathione-S-transferases (GSTs), are a group of multifunctional enzymes capable of metabolizing three classes of insecticides namely; organochlorines, organophosphates and pyrethroids [102]. GST based resistance is mainly through amplification or up regulation of target genes [138]. GSTs, including *GSTE2* and *GSTE4* have been identified in *An. gambiae* [155, 156] and implicated in resistance to organochlorines (DDT) [156, 157]; *An. arabiensis* [158] and in *An. funestus* [150, 159].

1.7.3 Esterases

Esterases are a group of metabolic enzymes implicated in organophosphate, carbamate and pyrethroid resistance in insects [102]. Esterase based resistance is associated with sequestration of insecticides, which involves rapid binding and inhibition of the compounds [138]. In *An. gambiae*, a carboxylesterase gene, *Coeae1d*, has been

implicated in metabolism of pyrethroids [153]. Several malaria vectors have more than one insecticide resistance mechanism giving rise to multimodal insecticide resistance [148]. Table 1.2 Provides a summary of insecticide modes of action and resistance mechanisms.

Table 1.2: Insecticide modes of action and resistance mechanisms

INSECTICIDE CLASS	TARGET SITE	MODE OF ACTION	INSECTICIDE RESISTANCE MECHANISM		REFERENCES
			TARGET SITE	METABOLIC	
Class I pyrethroid (e.g. Permethrin)	Nerve and Muscle	Voltage-gated sodium channel modulators	*Kdr* mutations	Monoxygenases, Esterases	[103, 128, 129, 156, 160]
Class II pyrethroid (e.g. Deltamethrin)					
Organochlorines (e.g. DDT)					
Carbamates (e.g. Bendiocarb)	Nerve and Muscle	Acetylcholinesterase (*AChE*) inhibitor	$Ace1^{R}$ mutation	Esterases, GSTs, Monooxygenases	[103, 130, 156, 160-162]
Organophosphates (e.g. Pirimiphos methyl)					
Neonicotinoids (e.g. clothianidin)	Nerve and Muscle	Nicotinic acetylcholine receptor (*nAChR*)-competitive modulators	None reported	Monooxygenases	[92, 93]
Pyrrole (e.g. chlorfenapyr)	Respiration	Oxidative phosphorylation-uncouplers	None reported	None reported	
Insect growth regulator (e.g. pyriproxyfen)	Growth and Development	Juvenile hormone receptor agonists	None reported	Monoxygenases	[113]

Insecticide modes of action and resistance targets table modified using excerpts from two sources including the Insecticide Resistance Action Committee (IRAC) Mode of Action classification scheme 2020 [163] and mechanisms of insect resistance [116].

1.8 Evolution of insecticide resistance

In *Anopheles*, the development of resistance to insecticides is driven by a number of factors including insecticide selection pressure, the fecundity and longevity of the target vector, and the inherent ability of the vector to propagate resistant genes [138]. The

prolonged application of insecticides with similar modes of action has been associated with development of resistance (Figure 1.7) [78, 122].

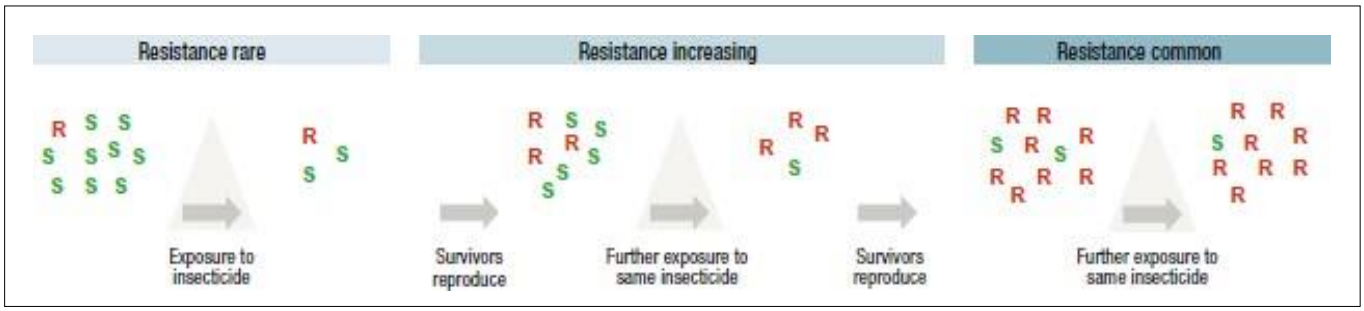


Figure 1.7: Insecticide resistance selection [122].

In *Anopheles* mosquito populations, resistant genes may remain undetected for some time until a particular threshold or 'tipping point' (Figure 1.8) is reached. Once this threshold is crossed, insecticide resistance genes have been shown to spread rapidly [78, 122, 164, 165]. The removal/reduction of insecticide selection pressure driving the increase in resistance variants may not at this stage result in reversal to susceptibility or wild type status in the vector population [122, 166].

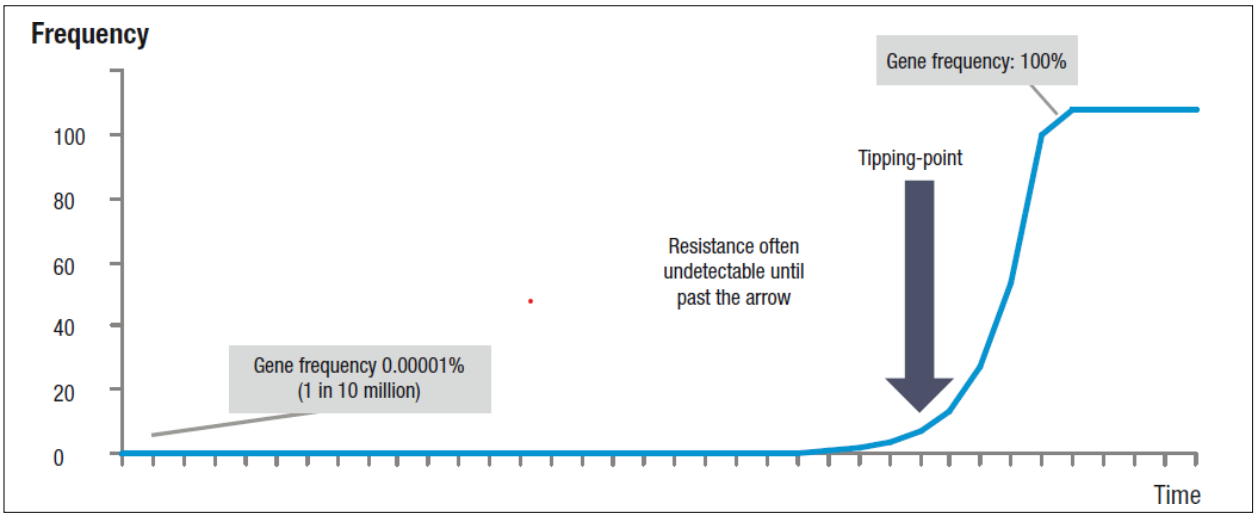


Figure 1.8: Concept of tipping point, beyond which selected resistance genes will remain in the population [122].

The WHO recommends rotating insecticides in IRS programs to slow down the development of resistance [8]. However, use of insecticides in agriculture contributes significantly to insecticide pressure on the larval stages of mosquitoes over large geographical areas [167]. For instance, resistance to clothianidin in local mosquito populations prior to its deployment in public health was observed in Cameroon and associated with the unregulated use of neonicotinoids in agriculture [93]. Historical evidence also indicates a cumulative increase in the number of insecticide resistance cases reported, coincident with increased insecticide use [163].

1.8.1 Current status of insecticide resistance

Resistance to insecticides commonly used for vector control remains a major threat to malaria vector control and consolidation of gains in elimination efforts [47]. In the last decade (2010-2020), up to 88 countries have reported resistance to at least one insecticide with approximately 90% of this resistance confirmed in 85 countries (Figure 1.9) [1]. Resistance to primary insecticides (pyrethroids) used on LLINs is widespread in sub-Saharan Africa (Figure 1.10) whilst countries reporting resistance to carbamates and organophosphates used for IRS is increasing (Figure 1.11). The spread of resistance to the four insecticide classes including pyrethroids, organophosphates, carbamates and organochlorines has been reported in at least 30% of malaria endemic countries [1].

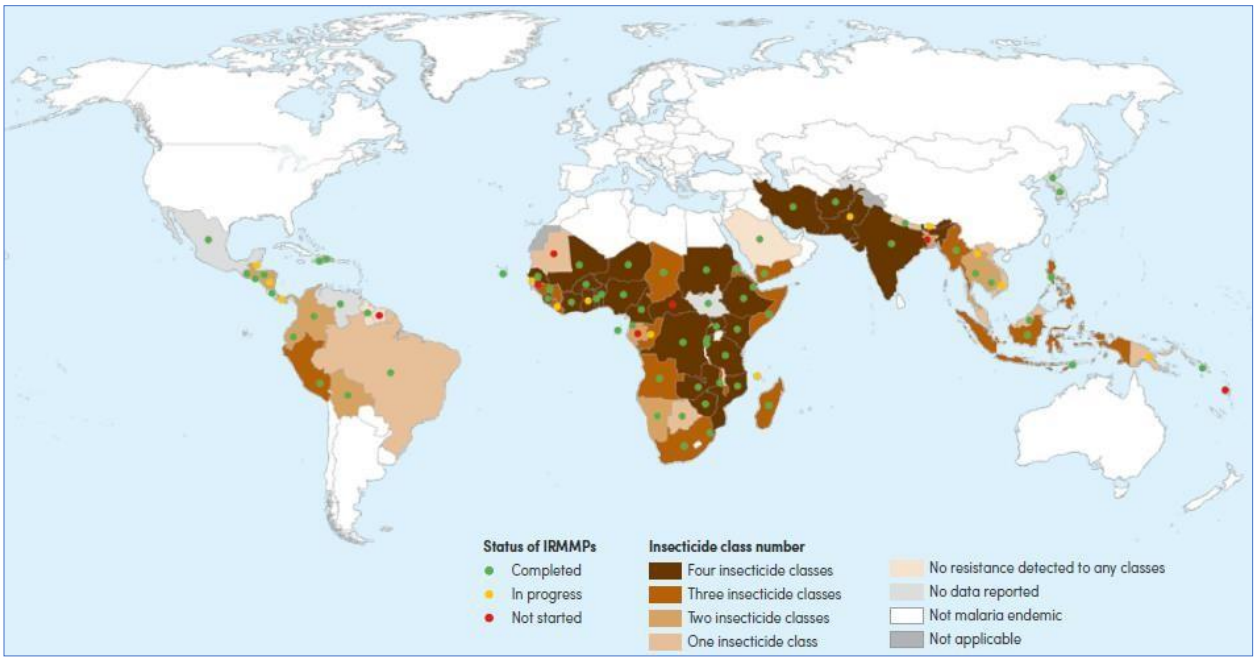


Figure 1.9: Number of insecticide classes to which resistance was confirmed in at least one malaria vector species in at least one monitoring site, 2010–2020 [1].

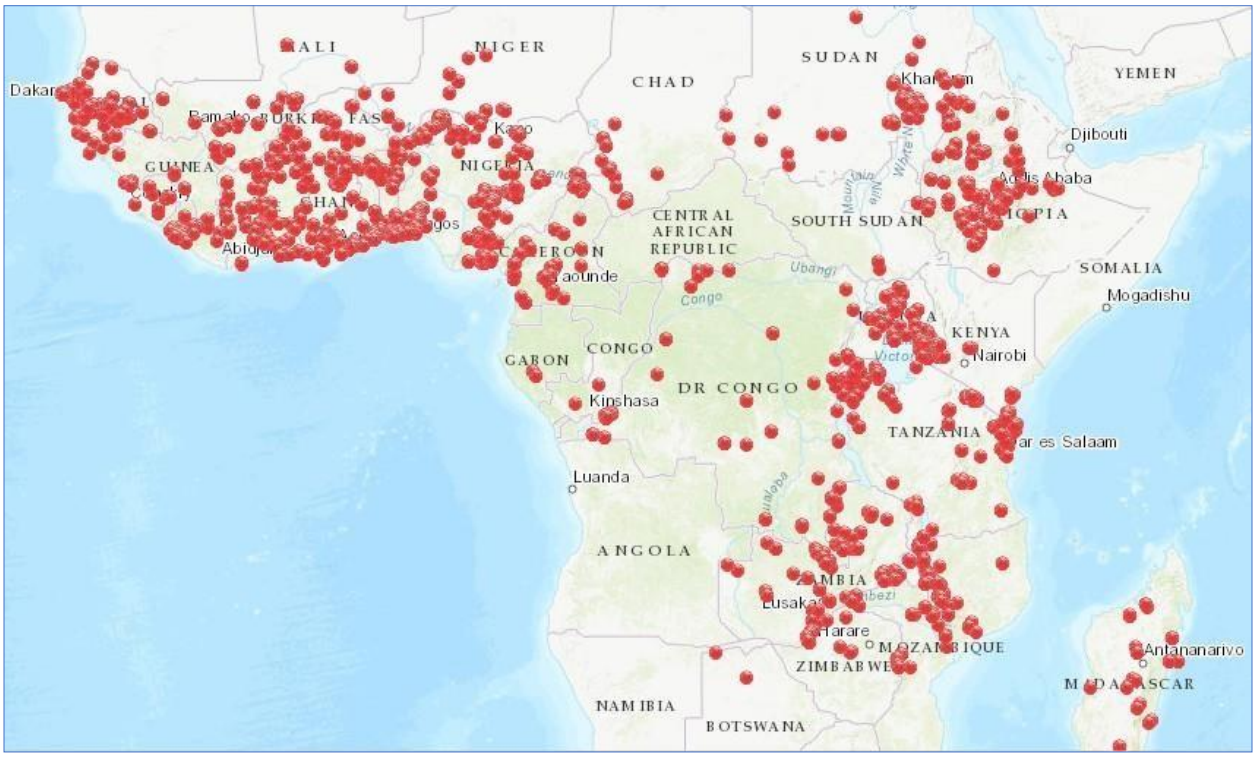


Figure 1.10: Confirmed resistance to pyrethroids in sub-Saharan Africa from 2000-2022 (each red dot is a confirmed report of pyrethroid resistance in major malaria vectors; *An. gambiae* s.s., *An. arabiensis* or *An. funestus*. IRMapper-https://anopheles.irmapper.com/accessed 6th July 2022.

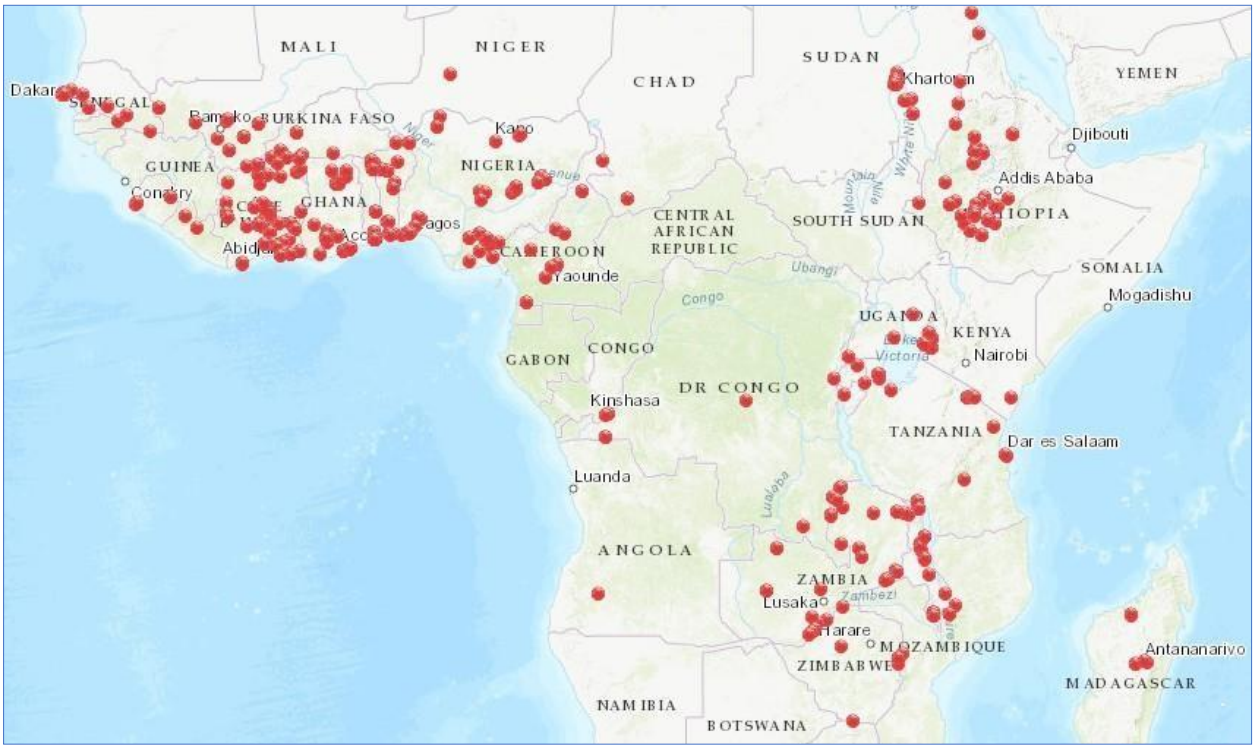


Figure 1.11: Confirmed resistance to carbamates and organophosphates in sub- Saharan Africa from 2000-2022. (each red dot is a confirmed report of either carbamate or organophosphate resistance in major malaria vectors; *An. gambiae*s.s., *An. arabiensis* or *An. funestus* IRMapper-https://anopheles.irmapper.com/ accessed 6th July 2022.

1.8.2 Impact of insecticide resistance

Widespread insecticide resistance and the nearly pervasive presence of resistance genes are major limitations to defining the epidemiological impact of insecticide resistance [168]. Notably, high levels of resistance have been associated with less than optimal declines in malaria prevalence in some areas [169]. Experimental studies show reduced blood feeding inhibition in areas with high pyrethroid resistance [170, 171]. A clear example of vector control failure arising from resistance in local malaria vectors was reported in Kwazulu Natal where deltamethrin resistant *An. funestus* were associated with increased malaria burden after prolonged control [172]. Whilst the implications of

insecticide resistance may be unclear, primarily due to study design limitations [67, 173], it is widely accepted that insecticide resistance has the potential to derail progress made in malaria control [74].

1.8.3 Monitoring and management of insecticide resistance

The World Malaria Report 2021 [1] recognized that consistent reporting of insecticide resistance patterns across malaria endemic countries remains an obstacle to evaluating progress in managing insecticide resistance. For instance, of the 85 malaria endemic countries, only 33% (29 countries) consistently reported insecticide monitoring results annually since 2019 and only 19% (16 countries) consistently provided insecticide resistance data annually since 2010 [1]. Insecticide resistance monitoring therefore remains a critical, but underutilized tool in resistance management [174]. Insecticide resistance management strategies call for increased resistance monitoring and tracking, with data management and deployment of vector control interventions based on the evidence of insecticide susceptibility [122]. To prolongthe efficacy of available tools, insecticide management strategies (use of sequences, mixtures, mosaics and combinations of insecticides with different modes of action) have been recommended to delay the onset of resistance; in addition to the use of PBO LLINs in areas with established pyrethroid resistance mediated by monooxygenase enzymes [8, 174]. Additionally, resistance management strategies ought to take into consideration potential insecticide interactions arising from cross resistance between compounds, mainly arising from similar modes of action [103, 116, 174].

1.9 Thesis rationale

Monitoring the impact of vector control tools through entomologic surveillance is essential to guide policy and programs, but different sampling methods may influence mosquito measures due to species-specific differences in the behaviors of *Anopheles* vectors. Moreover, the precision of the different collection methods varies, which may influence results [175-177].

Behavioral differences in malaria vector species include feeding and resting behavior, host preferences and breeding preferences, differences also exist in vectorial capacity and insecticide resistance patterns. As a result, the effectiveness of indoor based vector control, including LLINs and IRS, on different *Anopheles* vectors may vary [43, 178-181]. The impact of vector control, particularly IRS on *Anopheles* species composition has been shown to result in the gradual decline of highly anthropophilic species such as *An. gambiae* s.s. and *An. funestus*, being replaced by a behaviorally resilient *An. arabiensis* [182]. These studies however, rarely quantify the species-specific impact of vector control interventions on mosquito density using the absolute number of mosquitoes collected and rely on the use of proportions. The utilization of absolute numbers of mosquitoes collected to examine the impact of vector control interventions on mosquito density provides better precision and estimates for observed changes [183].

Resistance to insecticides, particularly pyrethroids, is widespread and identification of underlying genotypes driving resistance is vital to guide deployment of vector control interventions. Target site resistance mutations including *Vgsc-L995S* and *Vgsc-L995F*

have been associated with pyrethroid resistance [184], with the former (*Vgsc-995S*) at very high frequency in many areas within Uganda, but the latter (*Vgsc-L995F*) at low frequency [185]. These target site mutations conferring knockdown resistance to pyrethroids in *An. gambiae* s.s. and *An. arabiensis* may occur in combination with other resistance variants [153]. Differences in insecticide resistance profiles between *An. gambiae* s.s. and *An. arabiensis*, are well described with generally higher levels of resistance reported in the more endophilic *An. gambiae* s.s., than in *An. arabiensis* [171]. The deployment of new generation PBO LLINs requires a robust understanding of metabolic resistance mechanism in local populations. The recently described triple mutation (*Cyp6p4, Cyp6aa1* and *ZZB-TE*) in samples collected from Uganda, Kenya and DRC is associated with resistance to deltamethrin [154] and provides a DNA based diagnostic for monitoring metabolic resistance variants. Examining both target site and metabolic resistance mechanisms is essential in defining the resistance profile of malaria vector species given that target site mutations do not represent all the variation observed in phenotypic assays [153], are absent in some resistant mosquito populations [146, 186] or absent in some mosquito species like *An. funestus* [104, 150, 187].

This thesis aims to address the gaps in the evidence, as follows: First, examine the impact of alternative mosquito collection methods including, CDC light traps and prokopacks indoors and pit traps outdoors on *Anopheles* vector density, species composition and sporozoite rates as compared to human landing catch collections to provide insight into the sampling differences of each mosquito collection method. Second, the impact of seasonality and vector control interventions on *Anopheles* density and species composition is examined in three sites, assessing both the absolute

numbers (actual counts of mosquitoes collected) and relative proportions to provide precise estimates in species-specific differences. Thirdly, pyrethroid resistance in areas with differing vector control is characterized, examining the underlying mechanisms and measures of association to establish resistance genotype-phenotype relationships, which are essential for guiding insecticide resistance monitoring and management programs.

1.10 Study objectives and hypotheses

1.10.1 Study objectives

Objective 1

To compare the proportion of *An. gambiae*, *An. arabiensis and An. funestus* s.l. mosquitoes collected using different sampling methods.

- **Hypothesis**: The proportion of *An. gambiae* s.s., *An. arabiensis* and *An. funestus* s.l. mosquitoes collected from the same study area using different sampling methods will differ significantly indoor or outdoor assuming differences in host seeking, biting and resting behavior of mosquitoes

Objective 2

To describe malaria vector species composition in areas with differing vector control interventions.

Objective 3

To determine insecticide susceptibility level of malaria vectors in areas with differing

vector control interventions

- **Hypothesis**: Prolonged application of a single insecticide class (pyrethroids) in vector control increases insecticide selection pressure promoting development of resistance.

Objective 4

To evaluate the association between genetic polymorphisms *(Cyp6aa1, Cyp6p4, ZZB-TE, Cyp4j5, Coeae1d, Vgsc-L995S/L1014S and Vgsc-L995F/L1014F)* andmosquito survival in insecticide exposed *An. gambiae* s.l. adults

Hypothesis: Mosquitoes, which contain genetic polymorphisms associated with insecticide resistance, will be less susceptible when exposed to specific insecticides under bioassay conditions

1.11 Publications from this thesis

1. Mawejje HD, Asiimwe JR, Kyagamba P, Kamya MR, Rosenthal PJ, Lines J, Dorsey G, Staedke SG: **Impact of different mosquito collection methods on indicators of Anopheles malaria vectors in Uganda.** *Malaria Journal* 2022, **21:**1-12.

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3. Mawejje HD, Weetman D, Epstein A, Lynd A, Opigo J, Maiteki-Sebuguzi C, Lines J, Kamya MR, Rosenthal PJ, Donnelly MJ: **Characterizing pyrethroid resistance and mechanisms in Anopheles gambiae (ss) and Anopheles arabiensis from 11 districts in Uganda.** *Current Research in Parasitology & Vector-borne Diseases* 2023, **3:**100106.

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CHAPTER 2 SYSTEMATIC REVIEW

2.1 Introduction

This chapter presents a systematic review on patterns of insecticide resistance in East Africa. Data from 6 countries including Uganda, Kenya, Tanzania, Democratic Republic of Congo, Rwanda and Burundi is presented from a synthesis of 40 studies.

2.2 Aims and objectives

This systematic literature review was conducted to examine the patterns of insecticide resistance in *Anopheles* vectors from East Africa. The objectives of the review were 1) to summarize studies of phenotypic resistance in common *Anopheles* vectors following exposure to common insecticides, with and without synergists, 2) to characterize genotypic markers of insecticide resistance, and 3) to examine associations between genotypic markers of resistance and phenotypic resistance. This review, aimed to investigate the existing evidence on insecticide resistance patterns in malaria vectors from East Africa.

2.3 Methods

2.3.1 Information sources

Three electronic databases were searched, including: MEDLINE, EMBASE and Global Health. Reference lists from target review articles that met the inclusion criteria were

also used to examine additional literature from publications that may have been missed in the wider electronic database search.

2.3.2 Search strategy

The search strategy used the key search terms, study limitations, and selection criteria, outlined in Table 2.0

2.3.3 Study design

The study took into consideration longitudinal and cross-sectional studies describing insecticide resistance patterns in common malaria vectors. Longitudinal studies were based on annual assessment of insecticide resistance for at least 3 years within the same geographical area. Cross-sectional studies were based on a single time point assessment of insecticide resistance, sometimes with sampling repeated but in non-consecutive time periods such as Stump et al, [1], Verhaeghen et al, [2], and Njoroge et al, [3] or across wet and dry seasons such as Philbert et al, [4] and Matowo et al, [5].

2.3.4 Selection of articles for review

Lists of titles and abstracts retrieved by the search were exported to Endnote library and all duplicates were removed. Full article titles and abstracts identified as potentially relevant for inclusion in the systematic literature review and those where there was uncertainty about exclusion or inclusion were retrieved. Retrieved articles were

screened further to establish whether they met the selection criteria. For all articles that were excluded, reasons for exclusion were recorded.

Table 2.0: Systematic review search strategy

#	Databases	Medline Embase Global Health
	Key Search Terms	
1	Broad terms	Malaria OR *Plasmodium falciparum* OR *falciparum* malaria
2	Anopheles vectors	Anopheles OR *Anopheles gambiae* OR OR *Anopheles arabiensis* OR *Anopheles funestus* OR *Anopheles coluzzii*
3	Species	Combine #1 (Broad terms) and #2 (Anopheles vector)
4	Insecticides	Pyrethroid OR Carbamate OR Organophosphate OR Organochlorine OR Permethrin OR Deltamethrin OR Bendiocarb OR Pirimiphos-methyl OR Actellic OR SumiShield OR Piperonyl butoxide OR PBO
5	Insecticide resistance	Target site resistance OR metabolic resistance OR pyrethroid resistance OR voltage-gated sodium channel OR knock down resistance OR *kdr* OR *Vgsc* OR *GSTE* OR *ACHE* OR *L1014S* OR *L1014F* OR *L995S* OR *L995F* OR *Cyp4j5* OR *Coeae1d* OR *Cyp6p4* OR *Cyp6aa1* OR *ZZB-TE* OR *Ace-1R* OR *G119S*
6	Insecticide resistance Patterns	Combine #3 (species), # 4 (Insecticides) and #5 (Insecticide resistance)
Limits		
Date		1990-2022
Language		English
Location		*1* Sub-Saharan Africa- *Countries considered in the review include: Angola, Benin, Botswana, Burkina Faso, Burundi, Cameroon, Central African Republic, Chad, Congo, Cote d'Ivoire, Djbouti, Eritrea, Ethiopia, Gabon, Gambia, Ghana, Guinea, Guinea-Bissau, Kenya, Lesotho, Liberia, Madagascar, Malawi, Mali, Mauritania, Mauritius, Mozambique, Namibia, Niger, Nigeria, Rwanda, Sudan, South Sudan, Senegal, Sierra Leone, Somalia, South Africa, United Republic of Tanzania, Togo, Uganda, Democratic Republic of Congo (Zaire), Zambia, Zimbabwe.* 2 Studies conducted outside sub-Saharan Africa were excluded

Type of publication	Peer reviewed research articles
Selection Criteria	
Species composition	1 Species determined from field collections of local *Anopheles* mosquitoes 2 *Anopheles* species defined by species-specific PCR for *An. gambiae* s.l. and/or *An. funestus* s.l. 3 Studies without PCR identification of *Anopheles* species, or those that only identified malaria vectors using morphological criteria, were excluded.
Insecticide resistance	*1* *Anopheles* species defined by species-specific PCR for *An. gambiae* s.l. and/or *An. funestus* s.l. 2 Studies without PCR identification of *Anopheles* species, or studies that identified malaria vectors using morphological characteristics only, were excluded 3 Phenotypic insecticide resistance patterns as measured by WHO tube assays or CDC Bottle assays 4 Genotypic insecticide resistance as measured by molecular analysis tools such as PCR 5 Studies describing laboratory assays were excluded 6 Experimental hut studies that only used laboratory reared mosquitoes and did not include the use of wild mosquito populations were excluded 7 Studies that did not include insecticide resistance measurements and only included species composition were excluded. 8 Studies comparing effectiveness of interventions were excluded. 9 Studies that did not have insecticide resistance as the primary outcome were excluded.
Location – focus on East Africa	1 East African countries included: Uganda, Kenya, Tanzania, Rwanda, Burundi, Democratic Republic of Congo and The Republic of South Sudan 2 Countries outside East Africa were excluded from final selection.
Date search conducted	1st -31st August 2022

2.4 Results

2.4.1 Research study inclusion

Overall, 2,172 records were retrieved (Figure 2.1). Of these, 1,261 (58%) were duplicates and were excluded. The remaining 911 papers were screened using titles and abstracts; of these 645 did not meet the selection criteria, and were excluded for the following reasons: (1) did not address the research question (n=328), (2) described laboratory assays/experimental research studies (n=109), and (3) conducted outside sub-Saharan Africa (n=103). Full text articles were retrieved for 266 articles, which were screened to identify those carried out within East Africa. An additional 228 were conducted outside of East Africa and were excluded. Two additional records were identified from the reference lists of selected articles. In total, 40 full text articles fulfilled the eligibility criteria were included in the systematic literature review (Figure 2.1).

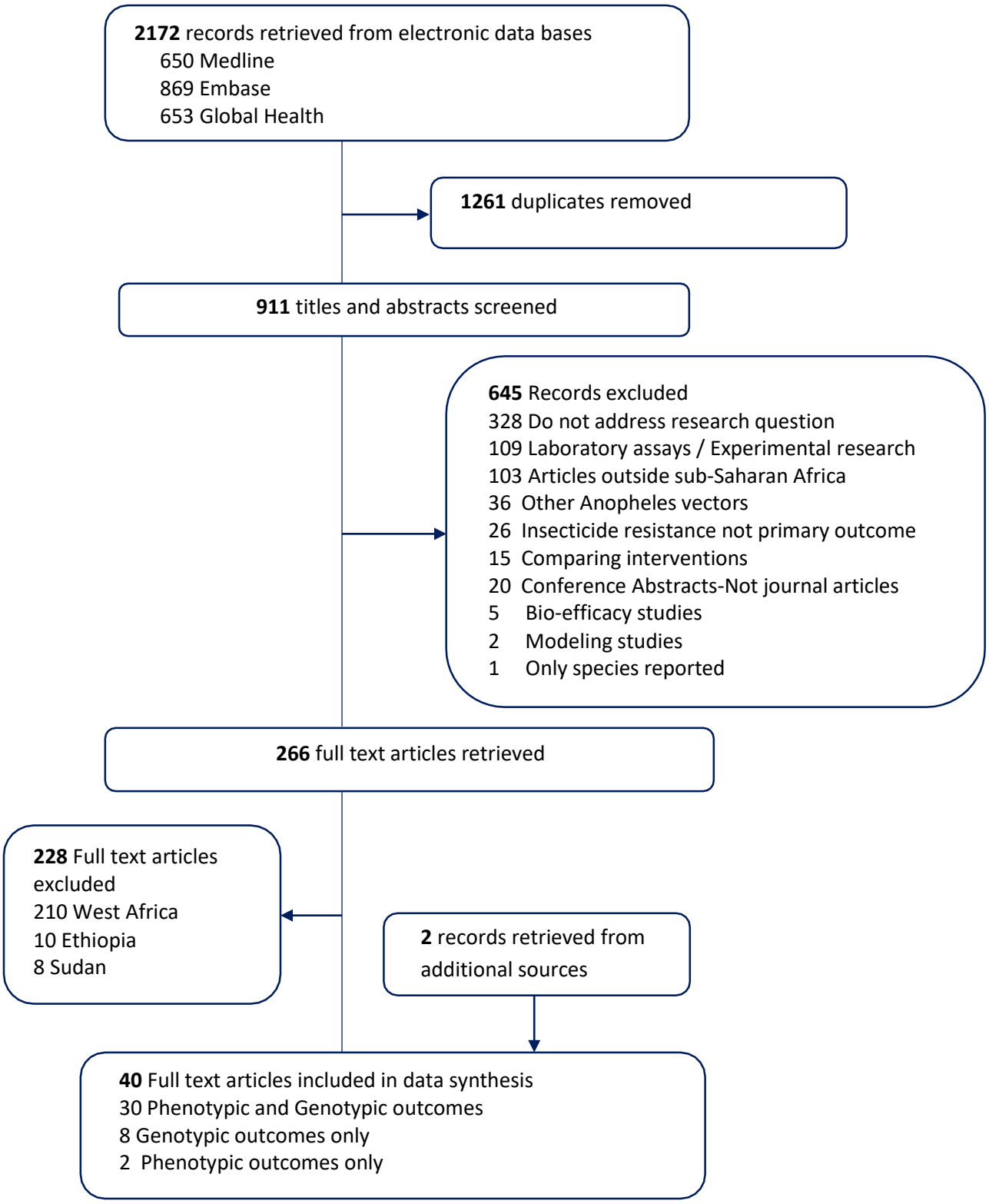

Figure 2.1: Systematic review profile of selected studies.

2.4.2 Characteristics of selected studies

The 40 papers described studies conducted in 6 of the 7 target countries in East Africa, including 11 from Uganda (n=11), Kenya (n=12), Tanzania (n=10), Democratic Republic of the Congo (n=5), Rwanda (n=1) and Burundi (n=1); no studies were conducted in South Sudan (Table 1). Most studies (95%) were of cross-sectional study design; only two studies including Mathias et al, [6] and Protopopoff et al, [7] were longitudinal. Two studies (Pinda et al, [8] and Ochomo et al, [9]) reported phenotypic outcomes only, while 8 studies focused only on genotypic outcomes of insecticide resistance (Stump et al, [1]; Verhaeghen et al, [10]; Chen et al,[11]; Matowo et al, [12]; Ochomo et al, [13]; Weetman et al, [14]; Lynd et al, [15]and Njoroge et al, [3]. Thirty studies (75%) assessed both phenotypic and genotypic outcomes. The phenotypic outcomes were assessed using WHO tube bioassays to evaluate for the resistance phenotype; only 5 studies used CDC bottle assays (Ochomo et al, [16]; Okia et al, [17]; Watsenga et al, [18]; Owuor et al, [19]; and Matowo et al, [20]). For phenotypic studies, a variety of sampling procedures were utilized, including larval collections only (16 studies), a combination of larval and mixed adults (4 studies; Mathias et al, [6]; Abeku et al, [21]; Lynd et al, [22] and Okiaet al, [17]), using the F1 progeny from blood fed adults (4 studies; Morgan et al, [23]; Mulamba et al, [24]; Owuor et al, [19]; Matowo et al, [20]) or using mixed physiologicalage adults (6 studies; Pinda et al, [8]; Verhaeghen et al, [2]; Kabula et al, [25] Protopopoffet al, [26]; Nardini et al, [27] and Protopopoff et al, [28]).

All studies assessed pyrethroid and/or DDT resistance except Kitungulu et al, [29], which only assessed pirimiphos methyl (Table 1). Carbamate (bendiocarb, propoxur) and organophosphate (malathion, fenitrothion, pirimiphos methyl) resistance were assessed in 25 studies (Table 1). Primary malaria vectors evaluated in these studies included *An. gambiae* s.s. (30 studies), *An. arabiensis* (31 studies) and *An. funestus* (12 studies). There was considerable co-occurence in species composition across the studies. Only two studies reported the presence of *Anopheles coluzzii* (Lynd et al, [22] and Watsenga et al, [18]).

Synergist assays were performed using piperonyl butoxide (PBO) an inhibitor of cytochrome p450 (monooxygenase) enzymes and triphenyl phosphate (TPP) an inhibitor of esterase enzymes. Overall, synergist assays were conducted in nearly half of the studies (16 total). PBO was the most commonly used synergist (14 studies), while TPP was assessed in only two studies (Table 2.1); Matowo et al, [5] and Nardini et al, [27]. The resistance genotype was evaluated using target site and metabolic resistance measurements. Up to 24 studies recorded data on target site resistance mechanisms, only 3 studies reported exclusively on metabolic resistance mechanisms (Kisinza et al, [30]; Matowo et al, [12] and Njoroge et al, [3]) and 11 studies reported outputs from both target site and metabolic mechanisms (Lynd et al, [15]; Weetman et al, [14]; Morgan et al, [31]; Mulamba et al, [24]; Chen et al, [11]; Ochomo et al, [16]; Owuor et al, [19]; Jones et al, [32]; Nkya et al, [33]; Matowo et al, [20]; Nardini et al, [27])

Table 2.1: Summary of insecticticide resistance and mechanisms conducted in East Africa

Country	Author, year of publication, (reference)	Study design	Study type	Insecticides tested	Anopheles Species			Synergists		Molecular Analysis		
					An. gambiae s.s.	*An. arabiensis*	*An. funestus*	PBO	TPP	Target site	Metabolic	Target site & Metabolic
Uganda	Verhaeghen et al, 2006 [10]	Cross-sectional	Genotype	N/A	√	√	_	N/A	N/A	√	_	_
Uganda	Lynd et al, 2019 [15]	Cross-sectional	Genotype	N/A	√	√	√	N/A	N/A	_	_	√
Uganda, Kenya	Weetman et al, 2018 [14]	Cross-sectional	Genotype	N/A	√	_	_	N/A	N/A	_	_	√
Uganda, Kenya, DRC	Njoroge et al, 2022 [3]	Cross-sectional	Genotype	N/A	√	_	_	N/A	N/A	_	√	_
Uganda	Ramphul et al, 2009 [34]	Cross-sectional	Phenotype & Genotype	DDT, permethrin, deltamethrin, bendiocarb, malathion	√	√	_	_	_	√	_	_
Uganda	Morgan et al, 2010 [31]	Cross-sectional	Phenotype & Genotype	DDT, permethrin, deltamethrin, bendiocarb, malathion, dieldrin	_	_	√	√	_	_	√	_
Uganda	Verhaeghen et al, 2010 [2]	Cross-sectional	Phenotype & Genotype	DDT, permethrin, deltamethrin	√	√	√	_	_	√	_	_
Uganda	Mawejje et al, 2013 [35]	Cross-sectional	Phenotype & Genotype	DDT, permethrin, deltamethrin, bendiocarb, fenitrothion	√	√		√		√	_	_
Uganda	Mulamba et al, 2014 [24]	Cross-sectional	Phenotype & Genotype	DDT, permethrin, deltamethrin, bendiocarb, malathion, fenitrothion	_	_	√	√	_	_	_	√
Uganda	Abeku et al, 2017 [21]	Cross-sectional	Phenotype & Genotype	permethrin, deltamethrin, bendiocarb	√	√	_	_	_	√	_	_
Uganda	Okia et al, 2018 [17]	Cross-sectional	Phenotype & Genotype	permethrin, deltamethrin, bendiocarb	√	√	√	√	_	√	_	_
Kenya	Ochomo et al, 2014 [9]	Cross-sectional	Phenotype	permethrin, deltamethrin	√	√	_	_	_	N/A	N/A	N/A

Country	Author, year of publication, (reference)	Study design	Study type	Insecticides tested	Anopheles Species			Synergists		Molecular Analysis		
					An. gambiae s.s.	*An. arabiensis*	*An. funestus*	PBO	TPP	Target site	Metabolic	Target site & Metabolic
Kenya	Stump et al, 2004 [1]	Cross-sectional	Genotype	N/A	√	√	_	_	_	√	_	_
Kenya	Chen et al, 2008 [11]	Cross-sectional	Genotype	N/A	√	√	_	N/A	N/A	_	_	√
Kenya	Ochomo et al, 2015 [13]	Cross-sectional	Genotype	N/A	√	√	_	N/A	N/A	√	_	_
Kenya	Hemming-Schroeder et al, 2018 [36]	Cross-sectional	Phenotype & Genotype	Deltamethrin	_	√	_	_	_	√	_	_
Kenya	*Mathias et al, 2011 [6]	Longitudinal	Phenotype & Genotype	DDT, permethrin, deltamethrin, bendiocarb	√	√	_	_	_	√	_	_
Kenya	Ochomo et al, 2013 [16]	Cross-sectional	Phenotype & Genotype	permethrin, deltamethrin, bendiocarb	√	√	_	_	_	_	_	√
Kenya	Kiuru et al, 2018 [37]	Cross-sectional	Phenotype & Genotype	permethrin, deltamethrin	√	√	√	_	_	√	_	_
Kenya	Munywoki et al, 2021 [38]	Cross-sectional	Phenotype & Genotype	DDT, permethrin, deltamethrin, bendiocarb, fenitrothion	_	√	_	_	_	√	_	_
Kenya	Orondo et al, 2021 [39]	Cross-sectional	Phenotype & Genotype	DDT, deltamethrin, malathion	_	√	_	√	_	√	_	_
Kenya	Owuor et al, 2021 [19]	Cross-sectional	Phenotype & Genotype	permethrin, deltamethrin, alphacypermethrin, malathion	√	√	√	√	_	_	_	√
Kenya	Kitungulu et al, 2022 [29]	Cross-sectional	Phenotype & Genotype	Pirimiphos-methyl	√	√	_	_	_	√	_	_
Tanzania	Pinda et al, 2020 [8]	Cross-sectional	Phenotype	permethrin, deltamethrin, bendiocarb, pirimiphos methyl	_	√	√	√	_	N/A	N/A	N/A
Tanzania	Matowo et al, 2014 [12]	Cross-sectional	Genotype	N/A	_	√	_	N/A	_	_	√	_
Tanzania	Kabula et al, 2014 [26]	Cross-sectional	Phenotype & Genotype	lambdacyhalothrin	√	√	_	_	_	√	_	_
Tanzania	Jones et al, 2013 [32]	Cross-sectional	Phenotype & Genotype	DDT, permethrin, lambdacyhalothrin, bendiocarb	_	√	_	√	_	_	_	√

Country	Author, year of publication, (reference)	Study design	Study type	Insecticides tested	Anopheles Species			Synergists		Molecular Analysis		
					An. gambiae s.s.	*An. arabiensis*	*An. funestus*	PBO	TPP	Target site	Metabolic	Target site & Metabolic
Tanzania	Protopopoff et al, 2013 [25]	Cross-sectional	Phenotype & Genotype	DDT, permethrin, deltamethrin, lambdacyhalothrin, bendiocarb	√	√	–	–	–	√	–	–
Tanzania	Nkya et al, 2014 [33]	Cross-sectional	Phenotype & Genotype	DDT, deltamethrin, bendiocarb	√	√	–	–	–	–	–	√
Tanzania	Kisinza et al, 2017 [30]	Cross-sectional	Phenotype & Genotype	DDT, permethrin, deltamethrin, bendiocarb, pirimiphos-methyl	√	√	–	–	–	–	√	–
Tanzania	Matowo et al, 2017 [5]	Cross-sectional	Phenotype & Genotype	DDT, dieldrin, permethrin, deltamethrin, lambdacyhalothrin, bendiocarb, propoxur, malathion, pirimiphos-methyl	–	√	–	√	√	√	–	–
Tanzania	Philbert et al, 2017 [4]	Longitudinal	Phenotype & Genotype	DDT, permethrin, deltamethrin, lambdacyhalothrin, etofenprox, cyfluthrin	–	√	–	–	–	√	–	–
Tanzania	Matowo et al, 2022 [20]	Cross-sectional	Phenotype & Genotype	permethrin, lambdacyhalothrin, bendiocarb, pirimiphos-methyl	√	√	√	√	–	–	–	√
DRC	Kanza et al, 2013 [40]	Cross-sectional	Phenotype & Genotype	DDT, permethrin, deltamethrin, lambdacyhalothrin, malathion	√	–	–	–	–	√	–	–
DRC	Nardini et al, 2017 [27]	Cross-sectional	Phenotype & Genotype	DDT, dieldrin, deltamethrin, bendiocarb, propoxur, malathion, fenitrothion	√	–	√	√	√	–	–	√
DRC	Lynd et al, 2018 [22]	Cross-sectional	Phenotype & Genotype	Permethrin, deltamethrin, bendiocarb	√	–	–	√	–	√	–	–
DRC	Watsenga et al, 2020 [18]	Cross-sectional	Phenotype & Genotype	Permethrin, deltamethrin, alpha-cypermethrin, bendiocarb	√	–	–	–	–	√	–	–
DRC	N'do et al, 2021 [41]	Cross-sectional	Phenotype & Genotype	DDT, Permethrin, deltamethrin, alpha-cypermethrin, etofenprox, bendiocarb, propoxur	√	–	√	√	–	√	–	–
Rwanda	Hakizimana et al, 2016 [42]	Cross-sectional	Phenotype & Genotype	DDT, permethrin, deltamethrin, lambdacyhalothrin, bendiocarb, fenitrothion	√	√	–	√	–	√	–	–

Country	Author, year of publication, (reference)	Study design	Study type	Insecticides tested	Anopheles Species			Synergists		Molecular Analysis		
					An. gambiae s.s.	*An. arabiensis*	*An. funestus*	PBO	TPP	Target site	Metabolic	Target site & Metabolic
Burundi	*Protopopoff et al, 2008 [7]	Longitudinal	Phenotype & Genotype	DDT, permethrin, deltamethrin	√	√	√	_	_	√	_	_

*Longitudinal studies; DRC-Democratic Republic of Congo; N/A-Not applicable; PBO-Piperonyl butoxide; TPP-Triphenyl phosphate; √=means the measure of interest was reported; hyphen (-) means the measure of interest was not done.

2.4.3 Phenotypic resistance

The results of phenotypic assays were reported in 32 studies for four classes of insecticides, including organochlorines, pyrethroids, carbamates and organophosphates, (Table 2.1). Organochlorines tested included DDT and dieldrin. A variety of pyrethroids were assessed including permethrin, deltamethrin, alphacypermethrin, lambdacyhalothrin, etofenprox and cyfluthrin. Carbamates included bendiocarb and propoxur, while pirimiphos methyl, fenitrothion and malathion were the organophosphates examined. Overall, most assays were conducted on pyrethroids, primarily permethrin and deltamethrin, while approximately 50% of the studies assessed carbamates, mainly bendiocarb. Only a few studies evaluated organophosphates.

2.4.3.1 Organochlorine resistance

Resistance to DDT in *An. gambiae* s.s. was generally high with mortality below 70% in 9 out of 12 studies (Table 2). Resistance to DDT was greatest in DRC where mortality was below 6% (Lynd et al, [22]). In *An. gambiae* s.s., susceptibility to DDT was observed in several areas, including Arua, Uganda (100% mortality in 2005) (Verhaeghen et al, [2]), Tanzania (94.5% mortality in 2011) (Nkya et al, [33]), Rwanda (95% mortality in 2011/2013) (Hakizimana et al, [42]) and Burundi (98% mortality in 2005/2006) (Protopopoff et al, [7]). In *An. arabiensis*, mortality to DDT was much higher ranging from 80%-100%, suggesting lower resistance. Only 4 studies out of 12 showed resistance to DDT in *An. arabiensis* including in Uganda (90.2% mortality in 2008) Ramphul et al, [34],

Tanzania (80% mortality in 2018/2019) Pinda et al, [8], Tanzania (83.5% mortality in 2015) Matowo et al, [5], and Rwanda (89% mortality in 2011) Hakizimana et al, [42]. In *An. funestus*, high levels of resistance to DDT were reported in Tanzania in 2018/2019, with mortality below 25% (Pinda et al, [8]), in Uganda with mortality ranging from 40%-70% in 2011/2012 (Mulamba et al, [24]). Mortality to dieldrin ranged from 97%-100% in *An. gambiae* s.s. (Nardini et al, [27]), in *An. arabiensis* (Matowo et al [5]) and *An. funestus* (Morgan et al, [31]) suggesting susceptibility to this insecticide.

2.4.3.2 Pyrethroid resistance

Permethrin and deltamethrin were the most studied pyrethroids, and were assessed in *An. gambiae* s.s., *An. arabiensis* and *An. funestus* (Table 2a, 2b and 2c). Alphacypermethrin and lambdacyhalothrin were assessed in 8 studies each, while etofenprox was evaluated in only 3 studies (N'do et al, [41]; Philbert et al, [4]; Mulamba et al, [24]) and cyfluthrin in only one study (Philbert et al, [4]). In *An. gambiae* s.s., outcomes of phenotypic assays were heterogeneous (Table 2a), with mortality ranging from as low as 2% in Kakoma-Tanzania, 2016/2017 (Matowo et al, [20]) to 100% in Kenya (Kiuru et al, [37]), Tanzania (Nkya et al, [33]), DRC (Kanza et al, [40]), Rwanda (Hakizimana et al, [42]) and Burundi (Protopopoff et al, [7]).

In *An. arabiensis*, results of phenotypic assays were heterogeneous but overall mortality appeared to be higher (>60% in most studies) compared to *An. gambiae* s.s. (Table 2b),

with full susceptibility recorded in some study areas (100% mortality), including Uganda, Kenya, Rwanda, Tanzania and Burundi. However, some studies reported very low mortality in *An. arabiensis*, suggesting high resistance to pyrethroids. In Kenya, Ochomo et al, [9] reported 5.9% mortality to permethrin in sampled sites in 2011, and Owuor et al, [19] reported mortality to permethrin, deltamethrin and alphacypermethrin in 2019 of 31%, 37% and 30%, respectively. In Tanzania, Jones et al [32] found 19% mortality to lambdacyhalothrin in 2011, and Matowo et al, [5] in Tanzania recorded mortality to lambdacyhalothrin of 21%. Mortality to both etofenprox and cyfluthrin in *An. arabiensis* ranged from 79.5% to 98.3%.

In *An. funestus* results were also heterogeneous (Table 2c) with low mortality following exposure to permethrin, suggesting high-level resistance, ranging from 5% mortality in Uganda, (Mulamba et al, [24]), 9% mortality in DRC (N'do et al, [41]) and 20% mortality in Tanzania (Pinda et al, [8]). Mortality to deltamethrin was also very low in Uganda at 2% (Mulamba et al, [24]), but was slightly higher following exposure to alphacypermethrin at 28% in DRC (N'do et al, [41]). High levels of resistance to etofenprox were reported by N'do et al, [41] in DRC at 6.7% mortality. However, areas susceptible to permethrin and deltamethrin were reported in Kenya by Kiuru et al, [37] and in Burundi by Protopopoff et al, [7]). Overall, resistance in *An. funestus* was recorded to permethrin, deltamethrin, alphacypermethrin, lambdacyhalothrin, etofenprox and cyfluthrin.

2.4.3.3 Carbamate resistance

Susceptibility to carbamates was assessed using bendiocarb (in 20 studies) and propoxur (in 1 study) (Nardini et al, [27]) (Table 2). In *An. gambiae* s.s. resistance to bendiocarb was reported in 7 out of 12 studies including 2 from Kenya: Mathias et al, [6] (mortality 79%-96%), Ochomo et al, [16] (mortality 78%), 3 in Tanzania: Protopopoff et al, [25] (70%-90%), Nkya et al, [33] (mortality 20%-33%), Matowo et al, [20] (mortality 62%-95%) and 2 studies in DRC: Nardini et al, [27] (mortality 53%-97%), N'do et al, [41](mortality 68%). For *An. arabiensis*, resistance to bendiocarb was reported in 5 out of 12 studies including 1 in Uganda: Abeku et al, [21] (mortality 84%), 2 in Kenya: Mathias et al, [6] (mortality 82%), Munywoki et al, [38] (mortality 93%) and 2 in Tanzania: Nkya et al, [33] (mortality 46%-63%), Matowo et al, [5] (mortality 24.6%). In *An. funestus*, resistance to bendiocarb is reported in 2 out of 8 studies with 1 in Tanzania: Matowo et al, [20] (mortality 80%) and another in DRC: N'do et al, [41](mortality 48.7%). Overall resistance to bendiocarb was recorded in at least 50% of the studies that assessed for this. Resistance to propoxur was only reported in *An. gambiae* s.s. from DRC by Nardini et al, [27] (mortality 86%).

2.4.3.4 Organophosphate resistance

Overall, 15 studies reported phenotypic assay results for organophosphates (Table 2). Resistance to organophosphates was assessed using pirimiphos methyl, fenitrothion and malathion. In *An. gambiae* s.s., resistance to pirimiphos methyl was reported in 1 study in Kenya: Kitungulu et al, [29] (mortality 86.9%) and resistance to malathion reported in DRC: Kanza et al, [40] (mortality 80%). No resistance to fenitrothion was reported in *An.*

gambiae s.s. For *An. arabiensis*, results of phenotypic assays following exposure to pirimiphos methyl was reported in Tanzania: Kisinza et al, [30] (mortality 87%) and to following exposure to fenitrothion in 2 studies from Kenya by Munywoki et al, [38] (mortality 88%) and Kitungulu et al, [29] (mortality 80.6%-89%). No resistance to malathion was reported in this species. For *An. funestus*, suspected resistance to pirimiphos methyl is reported in DRC by N'do et al, [41] (mortality 95.5%), but no resistance is observed to fenitrothion or malathion in this species.

Table 2.2a: Results of phenotypic bioassays in *An. gambiae*

Country; Author, year of publication, (reference)	Phenotypic resistance in *An. gambiae* (Mortality %)														
	Date of Mosquito collection	Organochlorines		Pyrethroids						Carbamates		Organophosphates			
		DDT (4%)	Dieldrin (0.4%)	Permethrin (0.75%)	Deltamethrin (0.05%)	Alphacypermethrin (0.05%	Lambdacyhalothrin (0.05%)	Etofenprox (0.5%)	Cyfluthrin (0.15%)	Bendiocarb (0.1%)	Propoxur (0.1%)	Pirimiphos-methyl (0.25%)	Fenitrothion (1%)	Malathion	
Uganda Ramphul et al, 2009 [34]	April 2008	14.6% - 55.8%	_	61.5% - 89.6%	74.7-89.7%	_	_	_	_	98%	_	_	_	100%	
Uganda Verhaeghen et al, 2010 [2]	2004 to 2006	60%-100%	_	54%-100%	87%-100%	_	_	_	_	_	_	_	_	_	
Uganda Mawejje et al, 2013 [35]	July to October 2011	48.8%	_	24.7%	33%	_	_	_	_	100%	_	_	100 %	_	
Uganda Abeku et al, 2017 [21]	September to October 2012	_	_	15.2%-92.3%	37.5%-100%	_	_	_	_	100%	_	_	_	_	
Uganda Okia et al, 2018 [17]	June 2015	_	_	14%-67.3%	9.7%-87%	_	_	_	_	100%	_	_	_	_	
Kenya Ochomo et al, 2014 [9]	July-September 2011	_	_	35.1%-100%	28%-100%	_	_	_	_	_	_	_	_	_	
Kenya *Mathias et al, 2011 [6]	Asembo: 1996 to 2010 and Seme 2000 to 2008	33%-62%	_	16%-74%	42%-48%	_	_	_	_	79%-96%	_	_	_	_	
Kenya Ochomo et al, 2013 [16]	July to December 2010	_	_	44.5%	78.5%	_	_	_	_	78%	_	_	_	_	
Kenya Kiuru et al, 2018 [37]	July to August 2015	_	_	100%	75%	_	_	_	_	_	_	_	_	_	
Kenya Owuor et al, 2021 [19]	May to July 2019	_	_	7%-51%	49%-53%	70%-80%	_	_	_	_	_	_	_	100%	

Kenya Kitungulu et al, 2022[29]	October 2018 to March 2019	–	–	–	–	–	–	–	–	–	–	86.9%-100%	–	–

Country; Author, year of publication, (reference)	**Phenotypic resistance in *An. gambiae* (Mortality %)**													
	Date of Mosquito collection	**Organochlorines**		**Pyrethroids**						**Carbamates**		**Organophosphates**		
		DDT (4%)	**Dieldrin (0.4%)**	**Permethrin (0.75%)**	**Deltamethrin (0.05%)**	**Alphacypermethrin (0.05%**	**Lambdacyhalothrin (0.05%)**	**Etofenprox (0.5%)**	**Cyfluthrin (0.15%)**	**Bendiocarb (0.1%)**	**Propoxur (0.1%)**	**Pirimiphos-methyl (0.25%)**	**Fenitrothion (1%)**	**Malathion**
Tanzania Protopopoff et al, 2013 [25]	April to December 2011	13%-40%	–	11%	28%-70%	8%-40%	–	–	–	70%-90%	–	–	–	–
Tanzania Nkya et al, 2014 [33]	Not mentioned	94.5%	–	–	98%-100%	–	–	–	–	20%-33%	–	–	–	–
Tanzania Matowo et al, 2022 [20]	April to May 2016 and 2017	–	–	4%-20%	–	–	2%-8%	–	–	62%-95%	–	100%	–	–
DRC Kanza et al, 2013 [40]	September 2008 to November 2009	13%-66%	–	44%-100%	73%-100%	–	36%-94%		–	–	–	–	–	80%-100%
DRC Nardini et al, 2017 [27]	March 2011 and July 2012	15%-60%	97%-100%	–	44%-51%	–	–	–	–	53%-97%	86%-100%	–	–	–
DRC Lynd et al, 2018 [22]	March to April 2016	0%-5%	–	28%-69%	63%-83%	–	–	–	–	100%	–	–	–	–
DRC Watsenga et al, 2020[18]	June 2016 and February 2017-October 2017	–	–	2%-50%	20%-98%	10%-83%	–	–	–	–	–	–	–	–
DRC N'do et al, 2021 [41]	April to November 2018	39.6%	–	5%	10%	15%	–	13.3 %	–	66.8%	–	97.8 %	99. 9%	–

Rwanda Hakizimana et al, 2016[42]	2011 and 2013	76%-95%	_	66%-86%	81%-98%	_	57%-100%	_	_	_	_	_	_	_
Burundi *Protopopoff et al, 2008 [7]	2002, 2005 and 2007	98%	_	58%-100%	94%-100%	_	_	_	_	_	_	_	_	_

*Longitudinal studies; DRC-Democratic Republic of Congo; hyphen (-) means there is no record at all for the measurement of interest

Table 2.2b: Results of phenotypic bioassays in *An. arabiensis*

Country; Author, year of publication, (reference)	Phenotypic resistance in *An. arabiensis* (Mortality %)														
	Date of Mosquito collection	Organochlorines		Pyrethroids						Carbamates		Organophosphates		
		DDT (4%)	Dieldrin (4%)	Permethrin (0.75%)	Deltamethrin (0.05%)	Alphacypermethrin (0.05%	Lambdacyhalothrin (0.05%)	Etofen-prox (0.5%)	Cyfluthrin (0.15%)	Bendiocarb (0.1%)	Propoxur (0.1%)	Pirimiphos-methyl (0.25%)	Fenitro-thion (1%)	Malathion (5%)
Uganda Ramphul et al, 2009 [34]	April 2008	90.2%-100%	_	100%	100%	_	_	_	_	100%	_	_	_	_
Uganda Mawejje et al, 2013 [35]	July to October 2011	100%	_	56.9%	84.6%	_	_	_	_	100%	_	_	100%	_
Uganda Abeku et al, 2017 [21]	September to October 2012	_	_	94.6%	38.5%-90.0%	_	_	_	_	84.6%-100%	_	_	_	_
Kenya Ochomo et al, 2014 [9]	July-September 2011	_	_	5.9%-95%	44%-95.4%	_	_	_	_	_	_	_	_	_
Kenya Hemming-Schroeder et al, 2018 [36]	May to October 2014	_	_	_	73.7%-82.8%	_	_	_	_	_	_	_	_	_
Kenya *Mathias et al, 2011 [6]	Asembo: 1996 to 2010 and Seme 2000 to 2008	98%-100%	_	82%-97%	83%-100%	_	_	_	_	82%-100%	_	_	_	_
Kenya Ochomo et al, 2013 [16]	July to December 2010	_	_	82.1%-90.5%	88.9%-91.2%	_	_	_	_	97.3%-97.9%	_	_	_	_
Kenya Kiuru et al, 2018 [37]	July to August 2015	_	_	69.9%	61.1%	_	_	_	_	_	_	_	_	_
Kenya Munywoki et al, 2021 [38]	August to	99%-100%	_	48%-100%	45.5%-100%	_	_	_	_	93%-100%	_	_	88%-100%	_

	November 2013 and July 2014													
Kenya Orondo et al, 2021 [39]	February to July 2018 and 2019	99.0%-100%	–	–	78.2%-97.8%	–	–	–	–	–	–	–	–	100%
Kenya Owuor et al, 2021 [19]	May to July and October to November 2019	–	–	31%-51%	37%-51%	30%-60%	–	–	–	–	–	–	–	100%
Kenya Kitungulu et al, 2022 [29]	October 2018 to March 2019	–	–	–	–	–	–	–	–	–	–	–	80.6%-89.0%	–

Country; Author, year of publication, (reference)	**Phenotypic resistance in *An. arabiensis* (Mortality %)**														
	Date of Mosquito collection	**Organochlorines**		**Pyrethroids**						**Carbamates**		**Organophosphates**		
		DDT (4%)	**Dieldrin (4%)**	**Permethrin (0.75%)**	**Deltamethrin (0.05%)**	**Alphacypermethrin (0.05%**	**Lambdacyhalothrin (0.05%)**	**Etofen-prox (0.5%)**	**Cyfluthrin (0.15%)**	**Bendiocarb (0.1%)**	**Propoxur (0.1%)**	**Pirimiphos-methyl (0.25%)**	**Fenitro-thion (1%)**	**Malathion (5%)**
Tanzania Protopopoff et al, 2013 [25]	April to December 2011	100%	–	50%	100%	100%	–	–	–	100%	–	–	–	–
Tanzania Pinda et al, 2020 [8]	September 2018 to November 2019	80%-100%	–	60%-80%	70%-88%	–	–	–	–	100%	–	100%	–	–
Tanzania Jones et al, 2013 [32]	April/May 2011 and April/May 2013	–	–	–	–	–	19%-88.4%	–	–	100%	–	–	–	–
Tanzania Nkya et al, 2014 [33]	Not mentioned	95%-100%	–	–	86%-100%	–	–	–	–	46%-63%	–	–	–	
Tanzania Kisinza et al, 2017 [30]	May and June 2015	99%	–	57%	63%	–	–	–	–	98%		87%		

Tanzania Matowo et al, 2017 [5]	June to December 2015	83.5%-100%	98.8%-100%	37.7%-80.6%	56.3%-90.3%	_	21.6%-87.4%	_	_	24.6%-100%	_	99.0-100%	_	100%
Tanzania Philbert et al, 2017 [4]	January to September 2014	90%-100%	_	73%-100%	76.3%-96.3%	_	59.5%-93.4%	79.5%-98.3%	85.3%-98.3%	_	_	_	_	_
Rwanda Hakizimana et al, 2016 [42]	2011 and 2013	89%-100%	_	95%-100%	88%-100%	_	72%-100%	_	_	_	_	_	_	_

*Longitudinal studies; DRC-Democratic Republic of Congo; hyphen (-) means there is no record at all for the measurement of interest

Table 2.2c: Results of phenotypic bioassays in *An. funestus*

Country, Author, year of publication, (reference)	Phenotypic resistance in *An. funestus*														
	Date of Mosquito collection	Organochlorines		Pyrethroids						Carbamates		Organophosphates		
		DDT (4%)	Dieldrin (4%)	Permethrin (0.75%)	Deltamethrin (0.05%)	Alphacypermethrin (0.05%	Lambdacyhalothrin (0.05%)	Etofenprox (0.5%)	Cyfluthrin (0.15%)	Bendiocarb (0.1%)	Propoxur (0.1%)	Pirimiphos methyl (0.25%)	Fenitrothion (1%)	Malathion (5%)
Uganda Morgan et al, 2010 [31]	April to November 2009	82%	100%	62%	28%	_	_	_	_	100%	_	_	_	100%
Uganda Verhaeghen et al, 2010 [2]	2004 to 2006	81%-100%	_	92%-99%	_	_	_	_	_	_	_	_	_	_
Uganda Mulamba et al, 2014 [24]	December 2011 to June 2012	40%-70%	_	5%-70%	2%-55%	_	10%-22%	65%		98%-100%	_	_	_	98%-100%
Uganda Okia et al, 2018 [17]	June 2015	_	_	20.8%	82.9%	_	_	_	_	100%	_	_	_	_
Kenya Kiuru et al, 2018 [37]	July and August 2015	_	_	100%	100%	_	_	_	_	_	_	_	_	_
Kenya Owuor et al, 2021 [19]	May to July; October to November 2019	_	_	74%	68%	77%	_	_	_	_	_	_	_	_
Tanzania Pinda et al, 2020 [8]	September 2018 to November 2019	10%-22%	_	20%-40%	50%-80%	_	_	_	_	100%	_	100%	_	_
Tanzania Matowo et al, 2022 [20]	April to May 2016 and 2017	_	_	31%	_	_	_	_	_	80%	_	_	_	_
DRC Nardini et al, 2017 [27]	March 2011 and July 2012	95%-99%	100%	_	69%-93%	_	_	_	_	98%	97%-100%	_	100%	100%
DRC N'do et al, 2021 [41]	April to November 2018	58.4%	_	9%	19%	28%	_	6.7%	_	48.7%	_	95.5%	98.6%	_

Burundi Protopopoff et al, 2008 [7]	2002, 2005 and 2007	95%-98%	_	97%-100%	100%	_	_	_	_	_	_	_	_	_

*Longitudinal studies; DRC-Democratic Republic of Congo; hyphen (-) means there is no record at all for the measurement of interest

2.4.4 Synergist assays

Metabolic resistance mediated by cytochrome p450 monooxygenase enzymes in pyrethroids was investigated in 14 studies using the synergist piperonyl butoxide (PBO) which has been shown to inhibit monooxygenase enzyme activity [43]. The enzyme activity of esterases associated with metabolism of carbamate and organophosphate insecticides was investigated in 2 studies using triphenyl phosphate (TPP) an inhibitor of esterase enzymes [44]. In *An. gambiae* (Table 2.3a), PBO increased mortality to permethrin, deltamethrin, alphacypermethrin and lambdacyhalothrin in 7 out of 7 studies. However, in one study (Matowo et al), [20], the increase in mortality to permethrin was marginal from 1.4% (95%CI: 0.1–3.6%) to 18.1% (95%CI: 9.5–26.7%). PBO restored full susceptibility (100% mortality) to deltamethrin in 2 of 7 studies from 87% to 100% (Okia et al), [17] and from 53% to 100%, (Owuor et al), [19] and to alphacypermethrin from 80% to 100% (Owuor et al), [19]. The addition of TPP to deltamethrin did not increase mortality in *An. gambiae* s.s. (Nardini et al), [27].

Similar patterns were observed for *An. arabiensis* (Table 2.3b), with PBO increasing mortality to permethrin, deltamethrin, alphacypermethrin and lambdacyhalothrin. PBO restored susceptibility (100% mortality) in 6 out of 7 studies in *An. arabiensis*, while TPP increased mortality to bendiocarb from 55.5% (95% CI 46.4-64.6) to 72% (95%CI 62.9-81.0) in observations by Matowo et al, [5]. However, TPP did not increase mortality to permethrin or deltamethrin in *An. arabiensis* (Matowo et al), [5].

In *An. funestus*, PBO increased mortality to permethrin, deltamethrin, alphacypermethrin and lambdacyhalothrin to varying degrees in 7 out of 7 studies (Table 23c). Full susceptibility (100% mortality) was restored in 3 out 7 studies from 74% in permethrin, 68% in deltamethrin and 77% in alphacypermethrin (Owuor et al), [19]; from 40% in permethrin,50% in deltamethrin (Pinda et al), [8] and from 69%-93% in deltamethrin (Nardini et al), [27]. TPP did not increase mortality to deltamethrin in *An. funestus* (Nardini et al, [27]). Overall, PBO increased mortality to pyrethroids tested and TPP only increased mortality to bendiocarb.

Table 2.3a: Synergist assays in *An. gambiae* using piperonyl butoxide and triphenyl phosphate

Author, year of publication, (reference)	Insecticide exposure without synergist				Mortality with PBO (4%)						Mortality with TPP (10%/20%)		
	Permethrin (0.75%)	Deltamethrin (0.05%)	Alphacypermethrin (0.05%	Lambdacyhalothrin (0.05%)	Permethrin (0.75%)	Synergism ratio	Deltamethrin (0.05%)	Synergism ratio	Alphacypermethrin (0.05%)	Synergism ratio	Lambdacyhalothrin (0.05%)	Permethrin (0.75%)	Deltamethrin (0.05%)	Bendiocarb (0.1%)
Mawejje et al, 2013 [35]	24.7%	33%	–	–	89%	3.6	95%	2.9	–	–	–	–	–	–
Okia et al, 2018 [17]	14%-67.3%	9.7%-87%	–	–	78.7%-98.9%	1.5-5.6	95.3%-100%	1.1-9.8	–	–	–	–	–	–
Owuor et al, 2021 [19]	7%-51%	49%-53%	70%-80%	–	92%-95%	1.9-13.1	100%	1.9-2.0	98%-100%	1.3-1.4	–	–	–	–
Matowo et al, 2022 [20]	1.4%-20%	–	–	2%-8%	18.1	12.9	–	–	–	–	–	–	–	–
Nardini et al, 2017 [27]	–	44%-51%	–	–	–	–	92%	2.1	–	–	–	–	28%	–
N'do et al, 2021 [41]	5%	10%	15%	–	50%	10.0	90%	9.0	90%	6.0	–	–	–	–
Hakizimana et al, 2016 [42]	66%-86%	81%-98%	–	57%-100%	98%	1.5	–	–	–	–	–	–	–	–

Hyphen (-) means there is no record at all for the measurement of interest; PBO: Piperonyl butoxide; TPP- Triphenyl phosphate; synergism ratio = mortality without PBO vs mortality with PBO

Table 2.3b: Synergist Assays in *An. arabiensis* using piperonyl butoxide and triphenyl phosphate

Author, year of publication, (reference)	Insecticide exposure without synergist				Mortality with PBO (4%)								Mortality with TPP (10%/20%)		
	Permethrin (0.75%)	Deltamethrin (0.05%)	Alphacypermethrin (0.05%	Lambdacyhalothrin (0.05%)	Permethrin (0.75%)	Synergism ratio	Deltamethrin (0.05%)	Synergism ratio	Alphacypermethrin (0.05%	Synergism ratio	Lambdacyhalothrin (0.05%)	Synergism ratio	Permethrin (0.75%)	Deltamethrin (0.05%)	Bendiocarb (0.1%)
Mawejje et al, 2013 [35]	56.9%	84.6%	_	_	100%	1.8	98%	1.2	_	_	_	_	_	_	_
Orondo et al, 2021 [39]	_	78.2% - 97.8%	_	_	_	_	100%	1.3	_	_	_	_	_	_	_
Owuor et al, 2021 [19]	31%-51%	37%-51%	30% - 60%	_	80%-100%	2.0-2.6	97%-100%	2.0-2.6	90%-98%	1.6-3.0	_	_	_	_	_
Pinda et al, 2020 [8]	60%-80%	70%-88%	_	_	98%-100%	1.3-1.6	100%	1.1-1.4	_	_	_	_	_	_	_
Jones et al, 2013 [32]	_	_	_	19%-88.4%	_	_	_	_	_	_	100%	1.1-5.3	_	_	_
Matowo et al, 2017 [5]	37.7%-80.6%	56.3% - 90.3%	_	21.6%-87.4%	56.8% - 91.3%	1.1-1.5	73%-92.5 %	1.0-1.3	_	_	85.2%-97.5%	1.1- 3.9	29.5%	27.0%	72% (from 55.5%)
Hakizimana et al, 2016 [42]	95%-100%	88%-100%	_	72%-100%	100%	1.0-1.1	98%-100%	1.0-1.1	_	_	_	_	_	_	_

Hyphen (-) means there is no record at all for the measurement of interest; PBO: Piperonyl butoxide; TPP- Triphenyl phosphate; synergism ratio = mortality without PBO vs mortality with PBO

Table 2.3c: Synergist Assays in *An. funestus* using piperonyl butoxide and triphenyl phosphate

Author, year of publication, (reference)	Insecticide exposure without synergist				Mortality with PBO							Mortality with TPP (10%/20%)		
	Permethrin (0.75%)	Deltamethrin (0.05%)	Alphacypermethrin (0.05%	Lambda-cyhalothrin (0.05%)	Permethrin (0.75%)	Synergism ratio	Deltamethrin (0.05%)	Synergism ratio	Alphacypermethrin (0.05%	Synergism ratio	Lambda-cyhalothrin (0.05%)	Permethrin (0.75%)	Deltamethrin (0.05%)	Bendiocarb (0.1%)
Morgan et al, 2010 [31]	62%	28%	–	–	90%	1.5	–	–	–	–	–	–	–	–
Mulamba et al, 2014 [24]	5%-70%	2%-55%	–	10%-22%	90-98%	1.4-18	95%-98%	1.8-47.5	–	–	–	–	–	–
Owuor et al, 2021 [19]	74%	68%	77%	–	100 %	1.4	100%	1.5	100%	1.3	–	–	–	–
Pinda et al, 2020 [8]	20%-40%	50%-80%	–	–	98%-100 %	2.5-4.9	100%	1.3-2.0	–	–	–	–	–	–
Matowo et al, 2022 [20]	6.5%-31%	–	–	–	53.2 %	8.2	–	–	–	–	–	–	–	–
Nardini et al, 2017 [27]	–	69%-93%	–	–	–	–	100%	1.1-1.4	–		–	–	51%	–
N'do et al, 2021 [41]	9%	19%	28%	–	70%	7.8	95%	5	95%	3.4	–	–	–	–

Hyphen (-) means there is no record at all for the measurement of interest; PBO: Piperonyl butoxide; TPP- Triphenyl phosphate; synergism ratio = mortality without PBO vs mortality with PBO

2.4.5 Target site resistance

Of the 40 studies, 35 examined target site resistance, making this the most investigated genotypic mechanism in *An. gambiae* s.s. and *An. arabiensis* in East Africa (Table 4). In *An. gambiae* s.s. (Table 4a) allele frequencies between 70%-100% were reported for Vgsc-L1014S, however, the mutation was heterogeneous with variability within and between studies. Mathias et al, [6] reported frequencies of the a *Vgsc-1014S* allele ranging from 3% to 100%, noting that whilst some study areas had very low kdr, others had extremely high frequencies. . Conversely, Kiuru et al, [37], reports a *Vgsc-1014S* allele frequency of only 1.3% in *An. gambiae* s.s. in coastal Kenya despite high levels of pyrethroid resistance. *Vgsc-L1014F* in *An. gambiae* s.s. was found at frequencies below 10% with a few exceptions including Owuor et al, [19] (33%) in Kenya, Kanza et al, [40] (78%), and in DRC whereLynd et al, [22] reported that *Vgsc-L1014F* frequency is very high (93%-99%).. Another mutation in the sodium gated channel, Vgsc-N1575Y (only found on the *Vgsc-L1014F* haplotype), [45] was also found at very low frequency (0.011-.0.02%) in DRC, recognized as the first report of this mutation in *An. gambiae* s.s. in East Africa (Lynd et al), [22]. The *Ace-1R* mutation was reported in 2 studies from Kenya by Owuor et al, [19] with an allele frequency between 2%-5% and by Kitungulu et al, [29] with an allele frequency between 0.0% to 3.1%.

In *An. arabiensis* (Table 4b), the*Vgsc* gene was predominantly wild type (>90% homozygous for *Vgsc-L101L);* with some areas remaining 100% wild type (Ochomo et al, [16]; Kiuru et al, [37]; Matowo et al, [5]; Protopopoff et al, [7]). However, *Vgsc-L1014S* was reported with relatively high frequencies in *An. arabiensis* including in studies from

Uganda: Abeku et al, [21] at 59.5%; Kenya: Hemming-Schroeder et al, [36] at 54.7% andOwuor et al, [19] at 60%, and Rwanda: Hakizimana et al, [42] at 27.1%. *Vgsc-L1014F* was not found in 9 out of 15 studies and the remaining 6 studies reported frequencies below 10%. In two studies however, *Vgsc-1014F* was found at higher frequency in some areas, at 19% frequency in Kenya (Owuor et al, [19]) and at 40.6% in Tanzania (Kabula et al), [26]. *Vgsc-N1575Y* was not found in *An. arabiensis*. The *Ace-1R* mutation in *An. arabiensis* was reported by Owuor et al, [19] at a frequency of 12%-23% (higher than what was observed in *An. gambiae* s.s. at 2%-5%). Similarly, Kitungulu et al, [29] reports the *Ace-1R* mutation in *An. arabiensis* at 3.0%-8.9% frequency (higher than observed in *An. gambiae* s.s. at 0.0%-3.1%). *An. coluzzii* was assessed in 2 studies from DRC (Table 4c). In Lynd et al, [22] only four *An. coluzzii* were collected and *Vgsc-1014S* was absent, *Vgsc-1014F* resistant allele was found at 75% frequency and the wildtype allele *Vgsc-1014L* was found at 25% frequency, *Vgsc-N1575Y* at 0.25% and *Ace-1R* was absent in this species. In Watsenga et al, [18], 17 *An. coluzzii* were collected and *Vgsc-1014S* was reported at 94% frequency and *Vgsc-1014F* was found at 6% frequency, the wild type allele was absent.

No *Vgsc* target site mutations were found in *An. funestus* (0%) in 6 out 6 studies that assessed this species (Mulamba et al, [24]; Owuor et al, [19]; Matowo et al, [20]; Nardini et al, [27] ; N'do et al, [41]; Morgan et al, [31]).

Table 2.4a: Target site resistance in pyrethroid exposed *An. gambiae*

Country	Author, year of publication, (reference)	TARGET SITE RESISTANCE-*Anopheles gambiae*				
		Target site resistance mutation-allele frequencies				
		Vgsc-L1014L (wild type)	***Vgsc-L1014S (kdr-east)***	***Vgsc-L1014F (kdr-west)***	***Vgsc-N1575Y***	***Ace-1R (G119S)***
Uganda	Ramphul et al, 2009 [34]	4%-46%	54%-96%	0%	_	0%
	Verhaeghen et al, 2010 [2]	52%-97%	3%-48%	0.3%-3%	_	_
	Mawejje et al, 2013 [35]	5%	95.04%	0.33%	_	_
	Abeku et al, 2017 [21]	4%-26.2%	73.8%-96%	11.9%	_	0%
	Weetman et al, 2018 [14]	6%	94%	_	_	_
	Okia et al, 2018 [17]	1%	99%-100%	0%	_	_
	Lynd et al, 2019 [15]	<0.01	94%	6%	0%	_
Kenya	Stump et al, 2004 [1]	52.5%-97.5%	2.5%-47.5%	0%	_	_
	Chen et al, 2008 [11]	85%-94.7%	5.3%-15.0%	_	_	_
	*Mathias et al, 2011 [6]	1%-96.2%	3.8%-100%	0%	_	_
	Ochomo et al, 2013 [16]	0%-0.1%	99.8%-100%	0%	_	_
	Ochomo et al, 2015 [13]	6%-32%	68%-94%	2.5%-5.4%	_	_
	Kiuru et al, 2018 [37]	98.67%	1.33%	0%	_	_
	Owuor et al, 2021 [19]	25%-33%	67%-75%	3%-33%	0%	2%-5%
	Kitungulu et al, 2022 [29]	_	_	_	_	0.0%-3.1%
Tanzania	Protopopoff et al, 2013 [25]	0%-6%	94%-100%	_	_	_
	Kabula et al, 2014 [26]	66.7-100%	0%-33.3%	7.1%	_	_
	Nkya et al, 2014 [33]	16%-41%	59%-84%	0%	_	_
DRC	Kanza et al, 2013 [40]	22%-97.6%	_	2.4%-78%	_	_
	Nardini et al, 2017 [27]	48%	52%	9%	_	_
	Lynd et al, 2018 [22]	0%	18%-36%	93%-99%	0.011-0.02%	0%
	Watsenga et al, 2020 [18]	_	83%	3%	_	_
	N'do et al, 2021 [41]	2%	98%	_	_	0%
Rwanda	Hakizimana et al, 2016 [42]	50%	50%	_	_	_
Burundi	*Protopopoff et al, 2008 [7]	2.4%-77.4%	22.6%-97.6%	_	_	_

DRC=Democratic Republic of Congo; *Longitudinal studies; hyphen (-) means no record for measurement of interest

Table 2.4b: Target site resistance in pyrethroid exposed *An. arabiensis*

Country	Author, year of publication, (reference)	TARGET SITE RESISTANCE-*Anopheles arabiensis*				
		Target site resistance mutation-allele frequencies				
		Vgsc-L1014L (Wild type)	***Vgsc-L1014S (kdr-east)***	***Vgsc-L1014F (kdr-west)***	***Vgsc-N1575Y***	***Ace-1R***
Uganda	Ramphul et al, 2009 [34]	64%-95%	6%-36%	0%	_	0%
	Mawejje et al, 2013 [35]	93%	7%	0%	_	_
	Abeku et al, 2017 [21]	40.6%-90.7%	9.3%-59.4%	0%	_	0%
	Okia et al, 2018 [17]	100%	0%	0.7%	_	_
	Lynd et al, 2019 [15]	96%	1%	3%	0%	_
Kenya	Stump et al, 2004 [1]	99.8%	0.17%	0%	_	_
	Chen et al, 2008 [11]	99.1%	0.9%	_	_	_
	*Mathias et al, 2011 [6]	97.7%	2.3%	0%	_	_
	Ochomo et al, 2013 [16]	100%	0%	0%	_	_
	Ochomo et al, 2015 [13]	99.2%-94.5%	0.8%-5.5%	1.1%-4.7%	_	_
	Hemming-Schroeder et al, 2018 [36]	45.3%-99.4%	0.6%-54.7%	1.7%-10.5%	_	_
	Kiuru et al, 2018 [37]	100%	0%	0%	_	_
	Munywoki et al, 2021 [38]	_	_	_	_	_
	Orondo et al, 2021 [39]	_	_	_	_	_
	Owuor et al, 2021 [19]	_	1%-60%	6%-19%	0%	12%-23%
	Kitungulu et al, 2022 [29]	_	_	_	_	3.0%-8.9%
Tanzania	Protopopoff et al, 2013 [25]	_	_	_	_	_
	Jones et al, 2013 [32]	_	_	_	_	_
	Kabula et al, 2014 [26]	95.5%-100%	4.2%	6%-40.6%	_	_
	Matowo et al, 2014 [12]	_	_	_	_	_
	Nkya et al, 2014 [33]	99.4%	0.6%	0%	_	_
	Matowo et al, 2017 [5]	100%	0%	0%	_	_
	*Philbert et al, 2017 [4]	_	_	_	_	_
	Pinda et al, 2020 [8]	_	_	_	_	_
Rwanda	Hakizimana et al, 2016 [42]	71.9%	27.1%	_	_	_
Burundi	Protopopoff et al, 2008 [7]	100%	0%	_	_	_

DRC=Democratic Republic of Congo; *Longitudinal studies; hyphen (-) means no record for measurement of interest

Table 2.4c: Target site resistance in pyrethroid exposed *Anopheles coluzzii*

Country	Author, year of publication, (reference)	TARGET SITE RESISTANCE-*Anopheles coluzzii*				
		Target site resistance mutation-allele frequencies				
		Vgsc-L1014L ***(Wild type)***	***Vgsc-L1014S*** ***(kdr-east)***	***Vgsc-L1014F*** ***(kdr-west)***	***Vgsc-N1575Y***	***Ace-1R***
DRC	Lynd et al, 2018 [22]	25%	0%	75%	0.25%	0%
DRC	Watsenga et al, 2020 [18]	_	94%	6%	_	_

DRC=Democratic Republic of Congo; hyphen (-) means no record for measurement of interest

2.4.6 Metabolic resistance

Overall, 14 out of 40 studies (35%) evaluated metabolic resistance (Table 5). Broadly, three enzyme systems were assessed including cytochrome p450 monooxygenases (*Cyp450*), esterases and glutathione-s-transferases (GSTs). Metabolic resistance was investigated using two mechanisms, including DNA (deoxyribonucleic acid) based markers in 3 studies (Table 5a: Weetman et al, [14]; Lynd et al, [15]; Njoroge et al, [3]) and elevated metabolic enzyme activity (Table 5b, 5c & 5d). All DNA based metabolic markers, with the exception of the carboxylesterase gene (*Coeae1d*) and the Zanzibar-like transposable element (ZZB-TE), were cytochrome p450 genes including *Cyp4j5*-L43F, *Cyp6p4*-1236M and *Cyp6aa1,* all associated with pyrethroid metabolism. The frequency of *Cyp4j5* marker frequency ranged from 37% [14] in Uganda to 60% in DRC [22]. The carboxylesterase marker, *Coeae1d,* was found in Uganda at a frequency of 49% [14]. *Cyp6p4, Cyp6aa1* and *ZZB-TE* have been described as the triple mutant haplotype because of their a tight physical and statistical linkage. The frequency of this triple mutant haplotype was found at a frequency of approximately 80% in Uganda and Kenya and 90% in DRC [3].

In the studies that evaluated metabolic enzyme activity, elevated activity was reported in resistant *Anopheles* relative to susceptible mosquito strains (Tables 5b, 5c and 5d) [11, 12, 16, 19, 20, 23, 24, 27, 30, 32, 33]. For *An. gambiae* s.s., (Table 5b) activity of cyp450 monooxygenases including *Cyp6M2*, *Cyp6p3*, *Cyp6p4*, *Cyp9K1*, *Cyp6Z3*, and *Cyp6aa1* was significantly elevated in resistant mosquitoes exposed to permethrin, deltamethrin

and alphacypermethrin in Kenya [11, 16, 19], Tanzania [20] and DRC [27]. However, *Cyp9j5* was under-expressed in pyrethroid resistant *An. gambiae* s.s. from Tanzania [20]. Esterase enzymes were upregulated in resistant *An. gambiae* s.s. exposed to permethrin, deltamethrin and alphacypermethrin [16, 19, 30]. Glutathione-S-transferase enzymes were associated with resistance to both pyrethroids [19] and DDT.

In *An. arabiensis*, metabolic resistance to permethrin, deltamethrin and lambdacyhalothrin appeared to be mediated by a broader spectrum of genes (Table 5c). Including cuticle proteins, protein homologs, GABA receptor genes, nicotinic acetylcholine, ABC transporters and 'still life' genes [12, 33]. Cyp450 monooxygenases [19, 30, 32, 46] and GSTs [12, 19, 30] were upregulated in pyrethroid and DDT resistant *An. arabiensis.* Cuticle proteins and *Cyp4G16* associated with cuticular hydrocarbon synthesis were up-regulated in resistant *An. arabiensis* after exposure to deltamethrin [33], permethrin [12] and lambdacyhalothrin [32] resistant *An. arabiensis*. Two Cyp450 genes, including *Cyp6M2* and *Cyp6p3,* associated with pyrethroid resistance in *An. gambiae* s.s., were down-regulated and not significant in *An. arabiensis* [12].

For *An. funestus*, metabolic resistance in pyrethroids was largely mediated by Cyp450 monooxygenases (Table 5d) with only one report of upregulated esterase activity [23]. GSTs were associated with resistance to DDT in *An. funestus* [23, 24, 27]. Whilst studies in Uganda [23, 24], and DRC [27], reported increased expression of *Cyp6P9a* and

Cyp6P9b in the pyrethroid resistance phenotype, in Tanzania, *Cyp6N1, Cyp6M7, Cyp6Z1* and *Cyp6M1* were over-expressed [20]. *Cyp6M7* was also associated with pyrethroid resistance in DRC [27] but was not reported in Uganda [23, 24]. Cytochrome p450 duplicated genes *Cyp6P4a* and *Cyp6P4b* were only described in DRC with significant elevation in deltamethrin resistant *An. funestus* [27].

Table 2.5a: Metabolic Resistance to pyrethroid insecticides in *An. gambiae* (DNA based markers)

Country	Author, year of publication, (reference)	METABOLIC RESISTANCE-*Anopheles gambiae s.s.*			
		Metabolic resistance markers (DNA based)			
		Metabolic resistance marker	**Gene location**	**Marker frequency**	**Resistance association**
Uganda, Kenya, DRC	Weetman et al, 2018 [14]	*Cyp4j5*-L43F	Cytochrome p450	37%	Alphacypermethrin, deltamethrin
		Coeae1d	Carboxylesterase	49%	Alphacypermethrin, permethrin, deltamethrin
	Lynd et al, 2019 [15]	*Cyp4j5*-L43F	Cytochrome p450	60%	Pyrethroid
		Coeae1d	Carboxylesterase	54%	Pyrethroid
	Njoroge et al, 2022 [3]	*Cyp6p4*-1236M	Cytochrome p450	80%	Deltamethrin
		Cyp6aa1	Cytochrome p450	80%	Deltamethrin
		ZZB-TE	Cytochrome p450	80%	Deltamethrin

Table 2.5b Metabolic Resistance: Upregulated genes/elevated enzyme activity in pyrethroid exposed *An. gambiae*

Country	Author, year of publication, (reference)	METABOLIC RESISTANCE-*Anopheles gambiae*		
		Upregulated genes/enzyme activity		
		Up-regulated gene/Enzyme	**Enzyme activity**	**Resistance association**
Kenya	Chen et al, 2008 [11]	Cytochrome p450 Monooxygenases	From <30 pg/min/adult to >90 pg/min/ adult	Pyrethroid resistance
	Ochomo et al, 2013 [16]	*ß*-esterase enzyme	1.7-fold elevation vs susceptible strain	Permethrin
		Cytochrome p450 Monooxygenases	1.4-fold elevation vs susceptible strain	Permethrin
		Glutathione-S-transferase (GST)	No significant difference in GST activity	Not significant
	Owuor et al, 2021 [19]	β-esterases	1.2 fold elevation vs susceptible strain	Deltamethrin, permethrin, alphacypermethrin
		Cytochrome p450 Monooxygenases	1.3 fold elevation vs susceptible strain	Deltamethrin, permethrin, alphacypermethrin
		Glutathione-S-transferase (GST)	3.0 fold elevation vs susceptible strain	Deltamethrin, permethrin, alphacypermethrin
Tanzania	Matowo et al, 2022 [20]	*Cyp6M2, Cyp6P3, Cyp6P4, Cyp9K1, Cyp6Z3, & Cyp6aa1* (Cyp450)	4.0-11.0 fold higher vs susceptible strains	Permethrin
		GSTe2 (GST)	Under expressed	Not significant
		Cyp9j5 (Cyp450)	Under expressed	Not significant
DRC	Nardini et al, 2017 [27]	*GSTS1-2* (GST)	7.6 fold increase vs susceptible strain	DDT
		GSTe2 (GST)	Over-transcription in resistant insects	DDT
		Cyp6M2 (Cyp450)	Over-transcription in resistant insects	Deltamethrin
Tanzania	Kisinza et al, 2017 [30]	Glutathione-S-transferase (GST)	Upregulated in resistant insects	DDT
		Mixed function oxidases	Upregulated in resistant insects	Permethrin/ Deltamethrin
		Non-specific esterases (NSE)	Upregulated in resistant insects	Permethrin/ Deltamethrin

GST=Glutathione-s-transferase; Cyp=cytochrome; NSE=Non-specific esterase

Table 2.5c: Metabolic Resistance- Upregulated genes/elevated enzyme activity in pyrethroid exposed *An. arabiensis*

Country	Author, year of publication, (reference)	METABOLIC RESISTANCE-*Anopheles arabiensis*		
		Upregulated genes/enzyme activity		
		Up-regulated gene (s)	**Fold change/ Upregulation**	**Resistance association**
Kenya	Chen et al, 2008 [11]	Monooxygenases	From <30 pg/min/adult to >90 pg/min/ adult	Pyrethroid resistance
	Owuor et al, 2021 [19]	β-esterases	Not significant	Not done
		Monooxygenases /Oxidases	1.66-1.83 fold elevation vs susceptible strain	Permethrin, deltamethrin
		Glutathione-S-transferase (GST)	2.3 fold elevation vs susceptible strain	Permethrin, deltamethrin
Tanzania	Nkya et al, 2014 [33]	GABA receptor (AGAP006028)	Upregulated & Positively correlated	Deltamethrin
		nicotinic acetylcholine receptor (AGAP008588)	Upregulated & Positively correlated	Deltamethrin
		'still life' gene (AGAP006590)	Upregulated & Positively correlated	Deltamethrin
		cuticle proteins (AGAP006829 and AGAP008449).	Upregulated & Positively correlated	Deltamethrin
		P450 candidates (CYP6P3 & CYP9J5),	Up-regulated & Positively correlated	Deltamethrin
		Cuticle gene (AGAP000987)	Up-regulated & Positively correlated	Deltamethrin
		Gene AGAP002667 (protein homologous-tumour related protein	Up-regulated & Positively correlated	Deltamethrin
		Transcript AGAP004203 encoding for vitellogenin C	Negatively correlated	Deltamethrin
	Kisinza et al, 2017 [30]	Glutathione-S-transferase (GST)	Up-regulated	DDT
		Mixed function oxidases	Up-regulated	Permethrin/ Deltamethrin
		Non-specific Esterases (NSE)	Up-regulated	Permethrin/ Deltamethrin
	Matowo et al, 2014 [12]	*CYP4G16* (Cuticular hydrocarbon synthesis)	Up-regulated	Permethrin
		ABC transporters (ABC 2060)	Up-regulated	Not defined
		GSTe7 (GST)	Up-regulated	Not done
		CYP6M2 (Cyp450)	Down-regulated	Not significant
		CYP6P3 (Cyp450)	Down-regulated	Not significant
	Jones et al, 2013 [32]	*CYP4G16* (Cuticular hydrocarbon synthesis)	2.0-5.4 fold change vs susceptible strain	Lambdacyhalothrin
		CYP6Z2	1.6-2.6 fold change vs susceptible strain	Lambdacyhalothrin
		CYP6Z3	1.6-1.9 fold change vs susceptible strain	Lambdacyhalothrin

pg=pico grams; GST=Glutathione-s-transferase; Cyp=cytochrome; NSE=Non-specific esterase

Table 2.5d: Metabolic Resistance- Upregulated genes/ elevated enzyme activity in pyrethroid exposed *An. funestus*

Country	Author, year of publication, (reference)	METABOLIC RESISTANCE-*Anopheles funestus*		
		Upregulated genes/enzyme activity		
		Up-regulated gene	**Fold change / Up-regulation**	**Resistance association**
Uganda	Morgan et al, 2010 [31]	Esterase activity	Up regulated	Pyrethroids
		Glutathione-S-Transferase (GST)	3.47 fold increase vs susceptible strain	DDT
		Monooxygenases	2.35 fold increase vs susceptible strain	pyrethroids
		CYP6P9b (Cyp450)	12 fold increase	Permethrin/deltamethrin
		CYP6P9a (Cyp450)	Not found	Not found
	Mulamba et al, 2014 [24]	*CYP6P9a* (Cyp450)	2.5-3.8 fold change	Permethrin/Deltamethrin
		CYP6P9b (Cyp450)	5-3.8 fold change	Permethrin/Deltamethrin
		GSTe2 (GST)	3-1-4.4 fold change	DDT resistance
Tanzania	Matowo et al, 2022 [20]	*CYP6N1* (Cyp450)	17 fold higher than susceptible strain	Permethrin
		CYP6M7 (Cyp450)	21 fold higher than susceptible strain	Permethrin
		CYP6Z1 (Cyp450)	4-5 fold higher than susceptible strain	Permethrin
		CYP6M1 (Cyp450)	4-5 fold higher than susceptible strain	Permethrin
		Cyp6P9a	Not significantly expressed	Not significant
		Cyp6P9b	Not significantly expressed	Not significant
DRC	Nardini et al, 2017 [27]	*CYP6M7* (Cyp450)	7.7-fold increase vs susceptible strain	Deltamethrin
		CYP6P9b (Cyp450)	3.3-fold increase vs susceptible strain	Deltamethrin
		CYP6P9a (CypP450)	2.0-fold increase vs susceptible strain	Deltamethrin
		CYP6P4a (p450 duplicated gene)	2.0 fold increase vs susceptible strain	Deltamethrin
		CYP6P4b (p450 duplicated gene)	2.1 fold increase vs susceptible strain	Deltamethrin
		GSTe2 (GST)	2.0 fold increase vs susceptible strain	DDT
		GSTs1 (GST)	3.6 fold increase vs susceptible strain	un-annotated

GST=Glutathione-s-transferase; Cyp=cytochrome

2.5 Measures of association

Genotype and phenotype associations were assessed in 11 out of 40 studies (Table 2.6). Measures of association were based on target site mutations, including *Vgsc-L1014S, Vgsc-L1014F, G119S,* and DNA- based metabolic variants, including *Cyp4j5, Cyp4j10, Coeae1d*, triple mutant (*Cyp6p4, Cyp6aa1, ZZB-TE*) and the *L119F-GSTe2* mutation. There was considerable variability in the association between the frequency of the *Vgsc-L1014S* mutation with pyrethroid resistance. In Uganda, Ramphul et al, [34] using samples collected in 2008 showed that *An. gambiae* s.s with the *Vgsc-L1014S* mutation were 17.98 times more likely to survive exposure to permethrin compared to the wild type (OR 17.98, $p<0.001$) however, this association was not significant in exposure to deltamethrin [34]. Conversely, in another study from Uganda, Weetman et al [14] using samples collected in several study sites between 2008 and 2009 showed that *Vgsc-L1014S* was associated with *An. gambiae* s.s. survival to deltamethrin (model r^2 0.355, $p<0.005$). The stepwise regression analysis demonstrated that *Vgsc-L1014S* did not fully account for the observed resistance phenotype and further evidence showed that environmental determinants such as humidity and metabolic resistance variants such as *Cyp4j5* and *Coeae1d* affected the phenotype [14]. A significant relationship between *Vgsc-L1014S* and *An. gambiae* s.s. survival to lambdacyhalothrin (Fisher exact test; $p<0.001$) was reported in Tanzania by Kabula et al, [26] from samples collected in 2011. In *An. arabiensis* sampled from Kenya (2014), there was no significant relationship between *Vgsc-L1014S* and survival to pyrethroid exposure (OR=0.525, 95% CI 0.197-1.364, p=0.185) [36]. Notably, other studies also showed that *Vgsc-L1014S* was not associated with *An. gambiae* s.s [25, 35] or *An. arabiensis* [35] survival to pyrethroid exposure. The alternative *kdr* mutation, *Vgsc-L1014F* was associated with survival to

permethrin and deltamethrin in *An. gambiae* s.s. from DRC (Chi-square X^2=10.43; OR=3.7, p<0.01 in permethrin and Chi- square X^2=6.86; OR=3.8, p<0.05 in deltamethrin [22] and Burundi (Chi-square test, p<0.001 in permethrin and Chi-square test, p=0.019 in deltamethrin) [7]. In Burundi, *Vgsc- L1014F* was also associated with survival to DDT exposure in *An. gambiae* s.s. (Chi-squaretest, p<0.001) [7]. However, in Tanzania, *Vgsc-1014F* was not associated with and *An. gambiae* s.s. survival after exposure to lambdacyhalothrin (Chi-square X^2=0.68, p=0.409) [26]. Evaluation of the association between the target site mutation *G119S* (*Ace-1R*), showed found no association with survival of *An. gambiae* s.s. survival following exposure to the organophosphate pirimiphos methyl (Chi-square X^2=0.011, p=0.915) [29]

In *An. arabiensis,* one study from Kenya assessed the relationship between *Vgsc-1014F* and survival following exposure to pyrethroids and found a significant association between *Vgsc-1014F* and increased survival to deltamethrin in *An. arabiensis* (OR=3.495, 95% CI=1.809-7.102, p<0.001) [36]. Metabolic resistance variants, *Cyp4j5*, *Cyp4j10* and *Coeae1d* investigated by Weetman et al, [14] were significantly associated with *An. gambiae* s.s. survival to deltamethrin exposure (model r^2 0.284, p=0.0009; model r^2 0.393, p=0.021 and model r^2 0.363, p=0.026 respectively) [14]. *Cyp4j10* however, failed to attain statistical significance in subsequent resistance marker association tests [14]. The triple mutant haplotype (*Cyp6p4, Cyp6aa1, ZZB-TE)* investigated by Njoroge et al, [3] was found to be significantly associated with survival to deltamethrin in both WHO tube assays (OR=5.44 (1.41-31.28), p=5.71x10^{-3}) and deltamethrin impregnated long lasting insecticidal nets (LLINs) (OR= 2.56 (1.54-4.31), p=1.4x10^{-4}) [3]. The triple mutation however, was not significantly associated with survival to permethrin [3] (Table 6). Only one study evaluated genotype: phenotype associations in *An. funestus* showing a

significant relationship between the *L119F-GSTe2* mutation and survival to DDT exposure in this species (OR=12.9, $p<0.001$) [24].

Table 2.6: Genotype and Phenotype Measures of Association

Author, year of publication, (reference)	Genotype: Phenotype Measures of Association							
	Anopheles species	**Gene type (Metabolic/ Target site-Vgsc)**	**Predictor / Variable**	**Resistant allele frequency**	**Exposure Insecticide**	**Measure of association**	**Test statistic (Confidence intervals)**	**P-value**
Ramphul et al, 2009 [34]	*An. gambiae* s.s.	Target site	*Vgsc-L1014S*	54%-96%	DDT	*Vgsc-1014S* vs wild type (Vgsc-L1014L)	OR 61.99	P<0.001
					Permethrin	*Vgsc-1014S* vs wild type (Vgsc-L1014L)	OR 17.98	P<0.001
					Deltamethrin	*Vgsc-1014S* vs wild type (Vgsc-L1014L)	Not significant	N/A
Weetman et al, 2018 [14]	*An. gambiae* s.s.	Target site	*Vgsc-L1014S*	94%	Deltamethrin	Pyrethroid resistance vs candidate SNPs	Stepwise regression model r^2 0.335	0.005
		Metabolic (P450)	*Cyp4j5*	37%	Deltamethrin	Pyrethroid resistance vs candidate SNPs	Stepwise regression model r^2 0.284	0.00009
		Metabolic (P450)	*Cyp4j10*	45%	Deltamethrin	Pyrethroid resistance vs candidate SNPs	Stepwise regression model r^2 0.393	0.021
		Metabolic (COE)	*Coeae1d*	49%	Deltamethrin	Pyrethroid resistance vs candidate SNPs	Stepwise regression model r^2 0.363	0.026
Njoroge et al, 2022 [3]	*An. gambiae* s.s.	Metabolic	*Cyp6p4, Cyp6aa1*, ZZB-TE	90%	Deltamethrin (WHO Tube assay)	Triple mutant genotype vs survival to deltamethrin	OR 5.44 (1.41-31.28)	$5.71x10^{-3}$
			Cyp6p4, Cyp6aa1, ZZB-TE	90%	Deltamethrin (PermaNet 3.0)	Triple mutant genotype vs survival to deltamethrin	OR 2.56 (1.54-4.31)	$1.4x10^{-4}$
			Cyp6p4, Cyp6aa1, ZZB-TE	90%	Permethrin (WHO Tube assay)	Triple mutant genotype vs survival to permethrin	OR 1.23 (0.76-2.00)	0.42 (NS)
			Cyp6p4, Cyp6aa1, ZZB-TE	90%	Permethrin (Olyset Plus net)	Triple mutant genotype vs survival to permethrin	OR 0.93 (0.49-1.77)	0.88 (NS)
Mawejje et al, 2013 [35]	*An. gambiae* s.s.	Target site	*Vgsc-1014S*	95.04%	DDT	*Vgsc 1014S* genotype vs survival to DDT	Two-tailed exact tests	1.0 (NS)
					Permethrin	*Vgsc 1014S* genotype vs survival to permethrin	Two-tailed exact tests	0.051

Author, year of publication, (reference)	Genotype: Phenotype Measures of Association							
	Anophele sspecies	**Gene type (Metabolic/ Target site-Vgsc)**	**Predictor / Variable**	**Resistant allele frequency**	**Exposure Insecticide**	**Measure of association**	**Test statistic (Confidence intervals)**	**P-value**
					Deltamethrin	*Vgsc 1014S* genotype vs survival to Deltamethrin	Two-tailed exact tests	0.06 (NS)
Kitungulu et al, 2022 [29]	*An. gambiae* s.s.	Target site	*G119S* (Ace-1R)	0.0%-3.1%	Pirimiphos methyl	Ace-1R genotype vs survival to Pirimiphos-methyl	X^2 Chi-square test (X^2=0.011)	0.915 (NS)
Kabula et al, 2014 [26]	*An. gambiae* s.s.	Target site	*Vgsc-L1014S*	0%-33%	Lambdacyhalothrin	*Vgsc-L1014S* genotype vs survival to Lambda-cyhalothrin	Fisher exact test	P<0.001
			Vgsc-L1014F	7.1%	Lambdacyhalothrin	*Vgsc-L1014F* genotype vs survival to Lambda-cyhalothrin	X^2 Chi-square test (X^2=0.68)	0.409 (NS)
Protopopoff et al, 2013 [25]	*An. gambiae* s.s.	Target site	*Vgsc-L1014S*	98%	Lambdacyhalothrin, permethrin, deltamethrin	*Vgsc-L1014S* genotype vs survival to permethrin, Lambdacyhalothrin or deltamethrin	Chi-square test	0.59 (NS)
Lynd et al, 2018 [22]	*An. gambiae* s.s.	Target site	*Vgsc-L1014F*	93%-99%	Permethrin	*Vgsc-L1014F* genotype vs survival to permethrin (relative to *Vgsc-1014S*)	X^2 Chi-square test (X^2=10.43); OR=3.7	P<0.01
					Deltamethrin	*Vgsc-L1014F* genotype vs survival to deltamethrin (relative to *Vgsc-1014S*)	X^2 Chi-square test (X^2=6.86); OR=3.8	P<0.05
Protopopoff et al, 2008 [7]	*An. gambiae* s.s.	Target site	*Vgsc-L1014F*	22.6%-97.6%	DDT	*Vgsc-L1014F* genotype vs survival to DDT	Chi-square test	P<0.001
					Permethrin	*Vgsc-L1014F* genotype vs survival to permethrin	Chi-square test	P<0.001
					Deltamethrin	*Vgsc-L1014F* genotype vs survival to deltamethrin	Chi-square test	0.019
Mawejje et al, 2013 [35]	*An. arabiensis*	Target site	*Vgsc-1014S*	7%	DDT	*Vgsc 1014S* genotype vs survival to DDT	Two-tailed exact tests	1.0 (NS)
					Permethrin	*Vgsc 1014S* genotype vs survival to permethrin	Two-tailed exact tests	0.41 (NS)
					Deltamethrin	*Vgsc 1014S* genotype vs survival to Deltamethrin	Two-tailed exact tests	1.0 (NS)
Kitungulu et al, 2022 [29]	*An. arabiensis*	Target site	G119S (Ace-1R)	3.0%-8.9%	Pirimiphos methyl	Ace-1R genotype vs survival to pirimiphos methyl	X^2 Chi-square test (X^2=0.231)	0.631 (NS)

Author, publication year, (reference)	Genotype: Phenotype Measures of Association							
	Anophele sspecies	**Gene type (Metabolic/ Target site-Vgsc)**	**Predictor / Variable**	**Resistant allele frequency**	**Exposure Insecticide**	**Measure of association**	**Test statistic (Confidence intervals)**	**P-value**
Hemming-Schroeder et al, 2018 [36]	*An. arabiensis*	Target site	*Vgsc-L1014F*	1.7%-10.5%	Deltamethrin	*Vgsc 1014F* genotype vs survival to deltamethrin	Odds Ratio; OR=3.495, 95% CI=1.809-7.102; Fischer's exact	P<0.001
			Vgsc-L1014S	0.6%-54.7%	Deltamethrin	*Vgsc 1014S* genotype vs survival to deltamethrin	Odds Ratio; OR=0.525, 95% CI=0.197-1.364; Fischer's exact	0.0185
Mulamba et al, 2014 [24]	*An. funestus*	Metabolic	*L119F-GSTe2*	26.7%	DDT	*L119F-GSTe2* genotype vs survival to DDT	Odds Ratio; OR=12.9	P<0.001

NS= Not significant; N/A=Not applicable

2.6 Discussion

This systematic review summarizes evidence published over the last 30 years of insecticide resistance patterns in East Africa. Overall, the primary malaria vectors, *An. gambiae* s.s., *An. arabiensis* and *An. funestus* were present in varying proportions. This review did not explicitly quantify the proportions for each species, because different sources were used to obtain *Anopheles* mosquitoes for insecticide resistance testing. Including stored and desiccated samples, larval and adult resting collections and F1 generation adults. *An. gambiae* s.s. and *An. arabiensis* were sampled more commonly than *An. funestus,* with the latter reported in only 12 studies compared to 30 for *An. gambiae* s.s. and 31 for *An. arabiensis*. Although many studies used samples raised from larval collections, one study (in Uganda) suggested that this approach may underestimate resistance marker frequencies [21]. Work done by Abeku et al, [21] in Uganda, using samples collected in 2012, showed that mosquitoes collected as adults had significantly higher *Vgsc-1014S* frequency compared to those collected as larvae (91.6%; 95% CI: 85.3–95.4% vs 81.0%; 95% CI: 74.8–85.9%, p = 0.0109). This finding highlights the limitation of larval collections, where the likelihood of sampling individuals from the same parent is high introducing a sampling bias that is rarely taken into account.

Subtle differences in sampling of *Anopheles* vectors have been demonstrated to affect phenotypic resistance outcomes. For instance, in Tanzania, Matowo et al, [5] reports marked seasonal differences in phenotypic resistance as measured by WHO tube assays, in one study site (Minepa village), where *An. arabiensis* were fully susceptible to bendiocarb in the wet season (100% mortality) but strongly resistant in the dry season (24.6% mortality), surprisingly *An. arabiensi*s from neighboring villages were susceptible to bendiocarb in both seasons [5].

Most studies included in this review were of a cross-sectional study design, presenting results from a single time point. The dearth of longitudinal data limits the ability to monitor insecticide

resistance patterns and changes over time as well as our understanding of resistance development and the selection pressure driving resistance. In a longitudinal data set (from 1996 to 2010) in the Asembo area in Western Kenya [6], it was demonstrated that *kdr L1014S* homozygosity increased in *An. gambiae* s.s. from 0.0% in 1996 to 12.7% in 2004, to 35% in 2007, then nearly doubled to 66.1% in 2008, rising to 91.7% in 2009 and to 100% in 2010. This evolution of resistance was attributed to increased use of pyrethroid treated bed nets in the study area [6].

Similarly, longitudinal sampling before indoor residual spraying (IRS) with deltamethrin in Burundi [7], recorded *kdr L1014S* frequency of 1% (2002) in *An. gambiae* s.s. which increased significantly to 87% within 6 years of IRS implementation (by 2007) associated with increased implementation of pyrethroid based IRS [7]. This study underscores the utility of monitoring the impact of vector control interventions on patterns of resistance. Notably, however, the rapid increase in *kdr L1014S* frequency did not translate into a steep increase in the resistance phenotype to either permethrin or deltamethrin in WHO tube assays [7], highlighting the complexity of genotype-phenotype associations and gaps in our understanding of these relationships [47].

The geographic heterogeneity of insecticide resistance patterns limits generalizability of phenotypic results [5]. In this review, phenotypic resistance, particularly to DDT and pyrethroids varied widely with and between studies. Few patterns in the resistance phenotype were observed. Even within studies from the same country, areas of pyrethroid resistance were identified amongst areas of pyrethroid susceptibility [2, 7, 9]. The absence of longitudinal sampling limits interpretation of resistance patterns over time.

Overall, DDT and pyrethroid resistance appeared to be wide-spread with scattered reports of resistance to bendiocarb [5, 6, 16, 21, 25, 27, 33, 41]), pirimiphos methyl [29, 30, 41], fenitrothion [29, 38] and malathion [40] resistance. Most phenotypic assays (>70%) evaluated only permethrin and deltamethrin, with less attention given to other pyrethroids. Although the WHO [48]

recommends conducting insecticide resistance intensity assays, to establish the operational significance of phenotypic resistance, studies of this type were rare, only 4 studies out of 40 reviewed reported conducting pyrethroid intensity assays [17-20].

Synergist assays were largely (>80%) conducted with PBO, and showed increase in mortality to varying degrees in all *Anopheles* vectors tested; supporting the involvement of cytochrome p450 monooxygenase enzymes in mediating pyrethroid resistance. These results suggest that PBO based LLINs would be a useful pyrethroid resistance management tool in these *Anopheles* vector populations. Triphenyl phosphate (TPP) which inhibits esterase enzyme activity was only investigated by 2 studies [5, 27] and showed an increase mortality to bendiocarb but not to pyrethroids [5].

Target site mutations, largely *Vgsc-1014S* and *Vgsc-1014F* were assessed by nearly all studies (35 out of 40) for *An. gambiae* s.s. and *An. arabiensis.* The *Vgsc 1014S* mutation was widespread and had higher allele frequencies overall compared to *Vgsc-1014F* (Table 4). *Vgsc-N1575Y* which is found on the *L1014F* haplotype [45] was rarely investigated, with only one study reporting very low frequencies [22]. *Vgsc-1014S* and *Vgsc-1014F* were significantly associated with pyrethroid resistance in some studies [26, 36] but not in others [25, 35]. The acetylcholinesterase *Ace-1R (G119S)* mutation was found at low frequency in Kenya [19, 29] but was not significantly associated with observed resistance to bendiocarb [29].. Evidence of *Vgsc-1014S, Vgsc-1014F* and Vgsc-N1575Y mutations was also described in *An. coluzzii* [18, 22]. No target site mutations were described in *An. funestus* [24]. In examining a different approach to establishing insecticide resistance patterns, Owuor et al, [19] compared the resistance phenotype of indoor vs outdoor resting *An. arabiensis,* and found that indoor resting mosquitoes were more resistant to deltamethrin and permethrin than the outdoor resting however, the *Vgsc-1014S* and *Vgsc-1014F* frequencies were not significantly different either indoors or outdoors [19].

Metabolic resistance to pyrethroids was largely mediated by cytochrome p450 monooxygenases, particularly in *An. gambiae* s.s. and *An. funestus*. DNA based makers for metabolic resistance, [3, 14, 15] though not widely used are available in a form (DNA) that promotes wide scale surveillance for metabolic resistance variants [49]. Three classes of upregulated genes including monooxygenases, esterases and GSTs were the primary metabolic resistance systems investigated. Genes encoding cuticular proteins and hydrocarbon synthesis (*Cyp4G16*) were found to be associated with pyrethroid resistance in *An. arabiensis* [32, 33]. Measures of association to establish genotype and phenotype relationships were not the norm with only 11 out of 30 studies with both phenotypic and genotypic outcomes making these linkages. Several studies with both phenotype and genotype outcomes tended to present the two components as separate entities without examining causal relationships for *Anopheles* vector survival to insecticide exposure.

2.7 Gaps identified

The paucity of longitudinal studies in establishing spatial and temporal pattern of insecticide resistance was observed. Although most studies, reporting phenotypic resistance on the common pyrehthroids, intensity assays were rare. There is limited reporting on carbamate and organophosphate insecticides overall. The *Vgsc*-mutations *L1014S/F* were reasonably studied however, *Vgsc-N1575Y* was barely assessed even in areas where *L1014F* was found. Although target site resistance mechanisms were adequately reported, metabolic resistance was less addressed and the utilization of DNA based molecular markers for monitoring resistance variants was reported in less than 10% of the selected studies. A number of studies had both phenotype and genotype outcomes but did not evaluate the measures of association and linkages between the two data sets. Whereas PBO was the most common synergist used, none of the studies examined underlying genotypes for survivors of PBO and insecticide exposure. This thesis examined genotypic

and phenotypic measures of association, taking into account the underlying metabolic resistance mechanisms in *An. gambiae* s.s. that survived PBO plus insecticide exposure. DNA based markers of metabolic resistance were used to monitor resistance variants in 11 districts from Uganda. Phenotypic outcomes also took into account, the vector implementation status at the time of larval sampling to examine the potential impact of vector control interventions on the observed phenotypic and/or underlying genetic mechanisms of resistance.

2.8 Limitations

This systematic review had several limitations. First, heterogeneities in sampling of mosquitoes for phenotypic assays limited the ability to establish the species composition overall. Secondly, the cross-sectional nature of the study design in nearly all studies only provided a point in time assessment of insecticide resistance and curtailed the ability to establish outstanding patterns of resistance. Several studies (19) did not assess genotype and phenotype associations despite having measurements for both.

2.9 Conclusion.

This systematic review established that there was substantial heterogeneity in the insecticide resistance profiles of *Anopheles* vectors, for both phenotypic and genotypic outcomes. There were no clear insecticide resistance patterns for the phenotypic resistance due to the high level of variability, however for genotypic resistance, this review found that the kdr mutation Vgsc-L1014S is widely spread and approaching fixation in several *An. gambiae* s.s. populations. It was noted that the source of mosquitoes used in phenotypic assays as well as seasonal variations in sampling can affect estimations of resistance marker frequencies. Several resistance mechanisms define the resistance phenotype including target site, metabolic or both. Piperonyl butoxide (PBO) was found to increase mortality to pyrethroids across the studies that examined synergism albeit atvarying levels and confirmed the activity of cytochrome p450 monooxygenases in the resistance phenotype.

Overall, pyrethroid resistance was mediated by cytochrome p450 enzymes with recently isolated DNA markers whose potential as an insecticide resistance variant monitoring tool is yet to be realized. There were no reports on new generation insecticides such as clothianidin (neonicotinoid), chlorfenapyr (pyrrole) or pyriproxyfen (insect growth regulator).

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CHAPTER 3 METHODS

3.1 Overview

This thesis was embedded within the PRISM (Program for resistance, immunology, surveillance and modeling of malaria) project in Uganda, which is funded by the US National Institutes of Health under the East Africa International Centres of Excellence in Malaria Research (ICMER) program. The project started in 2010 and is currently in its second funding cycle. Samples for this thesis were collected from the PRISM 1 (2010-2017), PRISM 2-Insecticide Resistance project (2018-2020) and PRISM Border Cohort studies (2020-2021) as described below.

3.2 PRISM 1 Study

PRISM 1 study was conducted in 3 sub-counties, including Walukuba in Jinja, Kihiihi in Kanungu and Nagongera in Tororo, from August 2010 to July 2017. At the time of sampling (October 2011 to June 2016), the 3 districts were classified as low, moderate, and high malaria transmission areas respectively. Jinja and Tororo are in Eastern Uganda, whereas Kanungu is in southwestern Uganda (Figure 5.1).

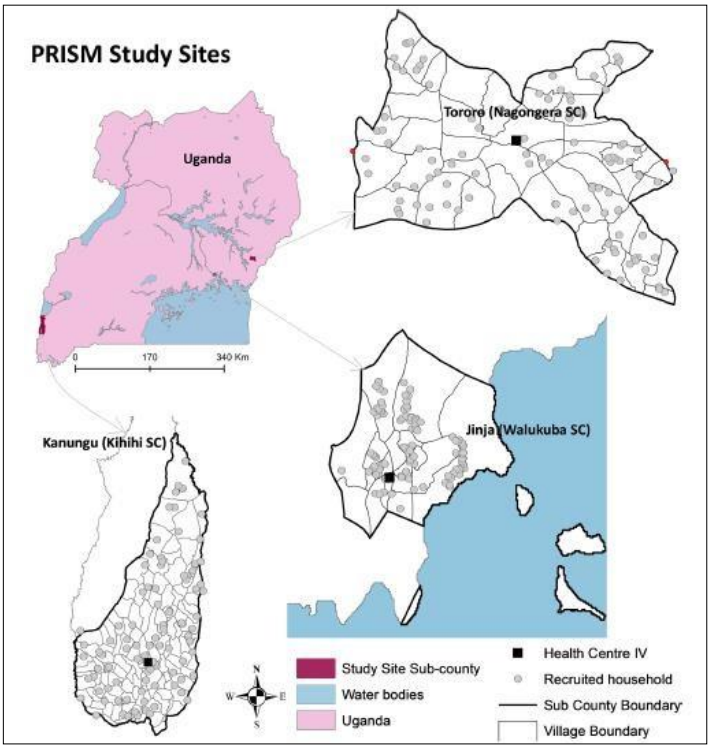

Figure 5.1: Map of Uganda showing study site location. Grey dots show location of households sampled for CDC light trap collections in the PRISM cohort (Programme for Resistance, Immunology, Surveillance and Modelling of Malaria). *Image from kigozi et al, [14]*

Enumeration surveys were conducted in the 3 sub-counties to generate a random list from which 100 households were enrolled per study site, making a total of 300 households enrolled in the entire cohort for both entomologic surveys and cohort studies [2]. The selection criteria used for

household enrollment included 1) having at least one resident between 6 months to 10 years of age and 2) having at least one resident adult to provide informed consent [2]. For the study clinical study, the following eligibility criteria was followed 1) documented age between 6 months and less than 10 years, 2) permanent resident of the household, 3) no intention to move out of the sub-county for the next 2 years, 4) agreement to come to a dedicated study clinic located within the sub-county for any febrile illness, 5) agreement to avoid antimalarial medications administered outside the study,and 6) provision of written informed consent from parent or guardian [2]. The PRISM 1 study was a dynamic cohort of children aged between 6 months and 10 years of age. All children that reached 6 months of age in the randomly selected households were added to the cohort upon consent of parents/caregivers and children beyond 10 years of age were automatically dropped out of the cohort [2]. All children within selected households meeting the selection criteria were enrolled into the PRISM 1 cohort.

3.2.1 Entomological study activities under PRISM 1

Entomological collections were conducted monthly to establish mosquito density, species composition and sporozoite infection rates using 4 mosquito collection methods from October 2011 to September 2012 per study site. The methods included monthly collections using CDC light traps conducted in all 100 randomly selected cohort households. Pyrethrum spraycollections (PSCs) and window exit traps were conducted monthly in 10 [3] randomly selected non-cohort households per study site and Human landing collections were conducted in an additional 8randomly selected non-cohort households per study site per month. After the first year of collections, all mosquito collection methods apart from CDC light traps were dropped due to resource limitations. CDC light trap collections were done from October 2011 to June 2016. Light traps were set per household, and positioned indoors, 1 meter above ground and at the foot end of a human occupied bed, with LLIN. Samples from the PRISM 1 study were used to address Objective 2 ‘to describe malaria vector species composition in areas with differing vector control interventions’ and methods are described in more detail in Chapter 5.

3.3 PRISM 2-Insecticide Resistance study

The PRISM 2 project was a successor project to the PRISM 1 cohort in the Nagongera-Tororo area, running from October 2017 to July 2020. As part of this project, insecticide resistance monitoring was conducted in the 11 sites using a cross-sectional study design. The PRISM 2-cohort was based at only one site (Nagongera sub county in Tororo) and entomological surveillance activities included bi-monthly collection of mosquitoes using CDC light traps. Eighty households were enrolled in the PRISM 2-Cohort.

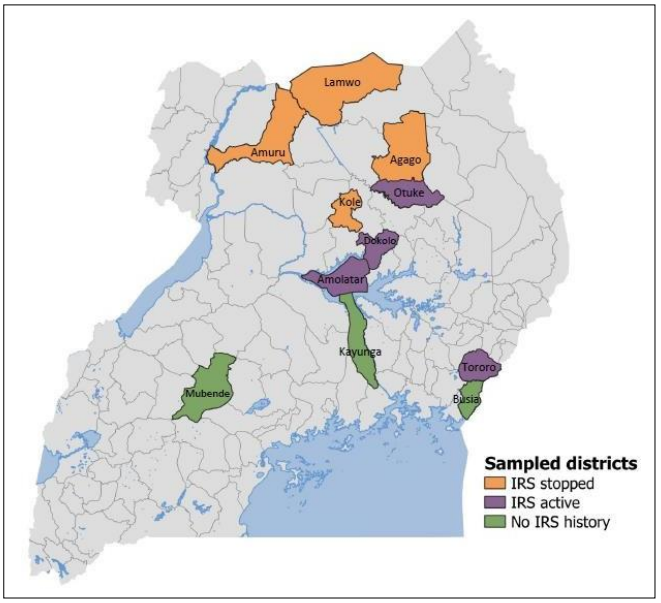

Figure 6.1: Map of study sites showing the location of sampled districts, and stratification by vector control measures. *Abbreviations*: IRS, indoor residual spraying; LLINs, long-lasting insecticidal nets. *Key*: green, No IRS (LLINs only); orange, IRS stopped (+ LLINs); purple, IRS active (+ LLINs).

The eligibility criteria included; 1) primary residence, 2) agreement to come tothe study clinic for any febrile illness, 3) agreement to avoid antimalarial medications outside the study, and 4) provision of written informed consent [4]. Mosquito larval collections were conducted from May 2018 to December 2020 in 11 districts geographically distributed in the northern, central and eastern parts of the country (Figure 6.1). The study sites were classified by vector control intervention including No IRS (Mubende, Kayunga and Busia), IRS stopped 2009 – 2014 with a single round of IRS in 2017 (Lamwo, Amuru, Kole and Agago) and IRS active (at time of larval sampling) 2014 – 2019 (Otuke, Dokolo, Amolatar and Tororo). All sites received two rounds of pyrethroid only LLINs in 2013–2014 and in 2017. Further details concerning the study site characteristics, IRS insecticide formulations and spray cycles are provided in Chapter 6.

Samples from the PRISM-Insecticide Resistance study were used to address Objective 3 'to determine insecticide susceptibility of malaria vectors in areas with differing vector control interventions' and Objective 4 'to evaluate the association between genetic polymorphisms (*Cyp6aa1, Cyp6p4, ZZB-TE, Cyp4j5, Coeae1d, Vgsc-L995S/L1014S* and *Vgsc-L995F/L1014F*) and mosquito survival in pyrethroid exposed *An. gambiae* s.l. adults' and are described in more detail in Chapter 6.

3.4 PRISM Border Cohort study

The PRISM Border Cohort study established between August 2020 and January 2021 in an area straddling the Busia and Tororo border in Eastern Uganda. Three parishes including Kayoro and Osukuru in Tororo and Buteba parsish in Busia were selected in a contiguous study area [1] to conduct the cohort study and entomological surveillance. Busia district borders Tororo district to the south and both districts border the Western Kenya region (Figure 4.1).

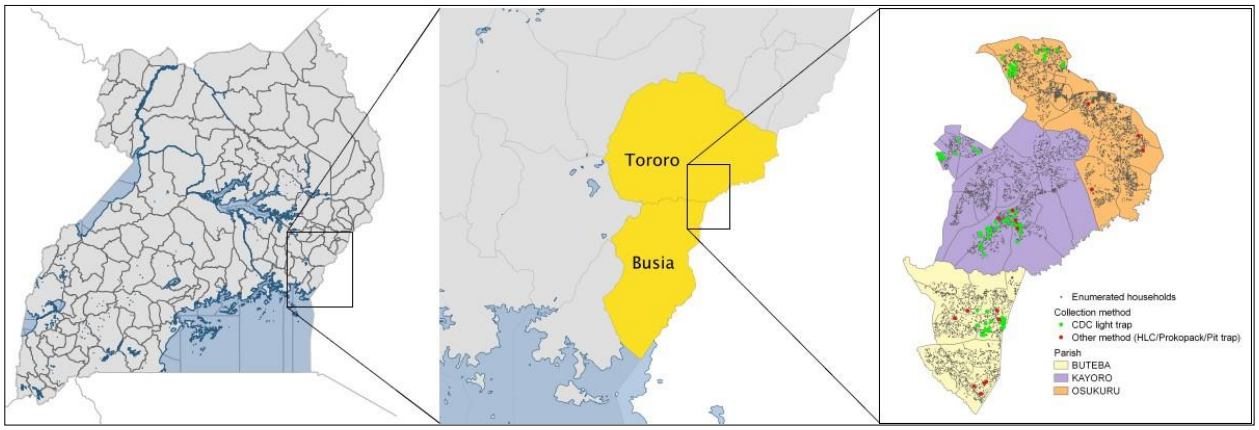

Figure 4.1: Map of study sites showing location of the 3 parishes including Buteba, Kayoro and Osukuru, in Busia and Tororo districts. The green dots highlight the positioning of the 80 border cohort households and the red dots show the position of the 16 households used for human landing catches, prokopack and pit trap collections. Image modified from Nankabirwa et al., [1]

Weekly aggregate data was collected from two health facilities, namely Osukuru Health Centre III (in Tororo near Busia border) and Nagongera Health Centre IV (in Tororo further away from the border) to generate temporal trends on malaria morbidity in comparison to Busia using the test

positivity rate statistic. All households in the 3 parishes were enumerated and mapped to generate a sampling frame for randomization and selection of households for the cohort study [1]

A cross-sectional survey of 300 randomly selected households was used to stratify the study area into high, medium and low transmission areas [1]. From the screened households, 80 were enrolled in the cohort including 20 households in Busia, 30 houses from Kayoro, Tororo near the Busia border, and 30 houses from Kayoro and Osukuru. Enrolled households met the following selection criteria 1) having at least two members aged 5 years or younger; 2) no more than 7 permanent residents currently residing; 3) no plans to move from the study catchment area in the next 2 years; and 4) willingness to participate in entomological surveillance studies [1].

Tororo has received IRS since December 2014 and within 5 years of sustained IRS between 2014 and 2019 malaria reached pre-elimination levels in Tororo with an EIR < 1% [1, 4]. However, in March 2020, the IRS insecticide was changed from the organophosphate pirimiphos methyl to the neonicotinoid-clothianidin (combined with deltamethrin in Fludora fusion) and this change in insecticide was coincident with the reported increase in the burden of malaria where a higher incidence of malaria was observed in Osukuru (Tororo district) compared to Buteba (Busia district) which had never received IRS [1]. At the time of sampling for Objective 1, there was no significant difference in *Plasmodium falciparum* parasite prevalence between the 3 parishes [1].

3.4.1 Entomological surveillance activities under the PRISM border cohort study

Entomological surveillance was conducted using CDC light traps and human landing collections. However, between June and November 2021, two additional collections including prokopack aspirators and pit traps were included for the purpose of comparing the 4 mosquito collection methods. Details on sampling and comparison procedures for each trap method are provided in Chapter 4. Briefly, CDC light trap collections were conducted in all the 80 cohort study households,

using miniature CDC light traps (Model 512; John W. Hock Company, Gainesville, Florida, USA) positioned at the foot end of a human occupied bed 1 meter above the ground). Light trap collections were done every 2 weeks. Human landing catches (HLCs) were conducted in an additional 16 randomly selected non-cohort households within the study area with 8 households in Busia and 8 households in Tororo. The location of the 16 households relative to the cohort households is illustrated in Figure 1. HLCs were conducted every 4 weeks. Indoor mosquito collections were conducted using prokopack aspirators every 4 weeks, scheduled a week before and after HLCs in the same non-cohort households. Outdoor mosquito collections were conductedusing pit traps 10 to 20 meters every 4 weeks outside the same non-cohort households used for HLCs. Samples from the PRISM Border Cohort study were used to address Objective 1 'to comparethe proportion of *An. gambiae* s.s. and *An. arabiensis* and *An. funestus* s.l. mosquitoes collected using different sampling methods' and are described in more detail in Chapter 4.

3.5 Mosquito Larval collections and insecticide resistance phenotypic assays

For Objectives 3 and 4, mosquito larvae were collected in 11 districts using the dipping method [5] between May 2018 and December 2020 as mentioned in Chapter 6. *Anopheles* larvae werecollected from a range of breeding sites including road side pools, rice fields, brick, sand and murrum pits. Larvae collected were reared using tetramin fish food to adults in a medical entomology insectary. Non-blood fed adult female mosquitoes were fed on 10% glucose solution and exposed at 3-5 days to diagnostic doses of permethrin (0.75%) and deltamethrin (0.05%) for 1 hour using World Health Organization (WHO) tube assays. Mortality was scored at 24 hours post insecticide exposure. Synergist assays were conducted using piperonyl butoxide (4.0%) prior to pyrethroid exposure to examine the involvement of cytochrome p450 monooxygenase activity. WHO tube assays were conducted in accordance to standard protocols [6]. A standard negative pyrethroid control with

silicone oil paper was run alongside each batch of tube tests. Temperature and humidity measurements were recorded for each bio-assay. Additional details regarding test procedures for insecticide resistance testing are included in Chapter 6.

3.6 Sample processing

All *Anopheles* mosquitoes collected independent of study objective were identified morphologically as belonging to the *An. gambiae* s.l. or *An. funestus* s.l. species complexes using morphological keys [7-9]. All mosquitoes were stored individually in 1.5 ml Eppendorf tubes and labelled with site location code, date and sample identification code to enable tracking and downstream molecular analysis.

3.7 Molecular analysis

3.7.1 Species identification by polymerase chain reaction (PCR)

Members of the *An. gambiae* s.l. species complex were isolated using the Scott et al, [10] standard PCR with species specific primers which identify *An. gambiae* s.s., *An. arabiensis*, *An. melas/An. merus* and *An. quadriannulatus.*

3.7.2 *Identifying genetic markers of insecticide resistance*

Target site resistance markers *Vgsc-L995S/L1014S* and *Vgsc-L995F/L1014F* were identified by a locked nucleic acid (LNA) assay recently described by Lynd et al, [11]. Briefly, the LNA assay simultaneously detected mutant and wild type Vgsc-alleles using LNA probes (LNAkdr-Ser: Cy5, LNAkdr-Phe: Fam, LNAkdr-Leu:Hex) and primers (VGSC-F, VGSC-R). The LNA assay was run on the AriaMX quantitative PCR platform at 95 °C for 3 minutes, 40 cycles of 95 °C for 5 seconds and 60 °C for 30 seconds (Lynd et al, 2018).

The triple mutation with *Cyp6aa1* duplication, *Cyp6p4-I236M* and *ZZB-TE* was assessed using three

independent and recently designed LNA assays by Njoroge et al.,[12] and run on AriaMx Real-Time PCR machine (Agilent, USA).

TaqMan assays described by Weetman et al, 2018 were used to genotype cytochrome p450 *Cyp4j5* and carboxylesterase *Coeae1d*. TaqMan assays used a primer/probe mix reaction conditions denaturing for 5 minutes at 95°C, then 40 cycles for 15 seconds at 92°C and annealing for 1 minute at 60°C. TaqMan assays were run were the Agilent MX3005P Real-Time PCR.

3.7.3 *Determining Plasmodium falciparum sporozoite infection*

Sporozoite infection rates were determined for samples collected using different mosquito collection methods (Objective 1) using the ELISA (Enzyme linked immunosorbent assay) method developed by Wirtz et al, [13]

3.8 Data management and statistical analysis

Mosquito collection data regardless of method of collection was recorded on standardized data forms which were double entered into a customized Microsoft access database and run for queries to check for discrepancies in the datasets. Original data forms were used to iron out any inconsistencies identified. All statistical analysis was done using STATA (version 14.2, Stata Corp, College Station, TX, USA). For each objective, statistical analysis was dependent on the independent variables and outcomes of interest. Detailed statistical analysis per objective is provided in Chapters 4, 5 and 6.

3.9 Ethical considerations

3.9.1 PRISM 1: Ethical approval and consent

In each study site, the head of household or adult representative was approached for consenting before household recruitment. A written informed consent was obtained as permission to conduct CDC light trap collections within the household. The study was approved by the Uganda National Council for Science and Technology (HS-119ES), Makerere University School of Medicine Research

and Ethics Committee (2017-099), the University of California, San Francisco Committee on Human Research (17-22544) and London School of Hygiene and Tropical Medicine Ethics Committee (14266-6). Used to consent households for objective 2 examining the impact of seasonality and malaria control interventions on *Anopheles* density and species composition

3.9.2 PRISM-Insecticide Resistance-Ethical approval

Mosquito collections for this study were approved by the Makerere University College of Health Sciences, School of Medicine research ethics committee (REF 2018-066), Uganda National Council of Science and Technology (REF: SS 4586), and London School of Hygiene and Tropical Medicine Ethics Committee (LSHTM Ethics Ref: 14584) under protocol study title "*Investigating spatial and localized interactions between insecticide resistance, insecticidal malaria vector control and malaria transmission in Anopheles mosquitoes from Uganda*" and by the School of Biomedical Sciences Research and Ethics Committee (REF: SBS-HDREC-669) and Uganda National Council of Science and Technology (REF: HS 2629) under study title "*Entomological surveillance of vector behaviour, vector density and insecticide resistance to inform malaria vector control in Uganda*." Used to access data collection sites in the target districts for to address Objectives 3 and 4. Consenting was not required to conduct larval collections.

3.9.3 PRISM Border Cohort: Ethical approval and consent.

For all study objectives ethical approval was obtained from institutional review boards (IRB) including the Uganda National Council for Science and Technology (UNCST), Makerere University School of Medicine Research and Ethics Committee (REC 2019-134), the University of California, San Francisco Committee on Human Research (19-28606) and London School of Hygiene and Tropical Medicine Ethics Committee (LSHTM Ethics Ref: 17777-09). Used to consent households for Objective 1 comparing mosquito collection methods.

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CHAPTER 4 IMPACT OF DIFFERENT MOSQUITO COLLECTION METHODS ON INDICATORS OF *ANOPHELES* MALARIA VECTORS

4.1 Chapter Introduction

This chapter addresses objective 1, to compare the proportion of *An. gambiae* s.s. and *An. arabiensis* and *An. funestus* s.l. mosquitoes collected using different sampling methods. This manuscript presents the results from measurements of mosquito density, species composition and sporozoite infection rates using 4 mosquito collection methods including human landing catches, CDC light traps, prokopack aspirators and pit traps. The manuscript was published in Malaria Journal. At the end of the manuscript are Tables for aggregate numbers of mosquitoes collected per method and measures of association. Figures showing the proportion of *Anopheles* vectors collected indoors and outdoors are also included.

4.2 Research paper

Below is the research paper cover sheet, followed by details of the manuscript, tables and figures.

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RESEARCH PAPER COVER SHEET

Please note that a cover sheet must be completed for each research paper included within a thesis.

SECTION A – Student Details

Student ID Number	1513692	**Title**	Mr
First Name(s)	Henry Ddumba		
Surname/Family Name	Mawejje		
Thesis Title	Anopheles species composition and insecticide resistance patterns in Uganda		
Primary Supervisor	Prof. Sarah Staedke		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?	Malaria Journal		
When was the work published?	December 2022		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	Not applicable		
Have you retained the copyright for the work?*	**Yes**	Was the work subject to academic peer review?	**Yes**

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Where is the work intended to be published?	
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Stage of publication	Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I participated and supervised data collection, conducted molecular analysis of the samples, provided the first draft of tables and figures with initial data analysis and wrote the first draft of the manuscript

SECTION E

Student Signature	Henry Ddumba Mawejje
Date	15th February 2023

Supervisor Signature	[redacted]
Date	23 Feb 2023

Mawejje *et al. Malaria Journal* (2022) 21:388
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Malaria Journal

RESEARCH Open Access

Impact of different mosquito collection methods on indicators of *Anopheles* malaria vectors in Uganda

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Abstract

Background

Methods used to sample mosquitoes are important to consider when estimating entomologic metrics. Human landing catches (HLCs) are considered the gold standard for collecting malaria vectors. However, HLCs are labour intensive, can expose collectors to transmission risk, and are difficult to implement at scale. This study compared alternative methods to HLCs for collecting *Anopheles* mosquitoes in eastern Uganda.

Methods

Between June and November 2021, mosquitoes were collected from randomly selected households in three parishes in Tororo and Busia districts. Mosquitoes were collected indoors and outdoors using HLCs in 16 households every 4 weeks. Additional collections were done indoors with prokopack aspirators, and outdoors with pit traps, in these 16 households every 2 weeks. CDC light trap collections were done indoors in 80 households every 4 weeks. Female *Anopheles* mosquitoes were identified morphologically and *Anopheles gambiae sensu lato* were speciated using PCR. *Plasmodium falciparum* sporozoite testing was done with ELISA.

Results

Overall, 4,891 female *Anopheles* were collected, including 3,318 indoors and 1,573 outdoors. Compared to indoor HLCs, vector density (mosquitoes per unit collection) was lower using CDC light traps (4.24 vs 2.96, density ratio [DR] 0.70, 95% CIs 0.63 – 0.77, p<0.001) and prokopacks (4.24 vs 1.82, DR 0.43, 95% CIs 0.37 – 0.49, p<0.001). Sporozoite rates were similar between indoor methods, although precision was limited. Compared to outdoor HLCs, vector density was higher using pit trap collections (3.53 vs 6.43, DR 1.82, 95% CIs 1.61 – 2.05, p<0.001), while the sporozoite rate was lower (0.018 vs 0.004, rate ratio [RR] 0.23, 95% CIs 0.07 – 0.75, p=0.008). Prokopacks collected a higher proportion of *Anopheles funestus* (75.0%) than indoor HLCs (25.8%), while pit traps collected a higher proportion of *Anopheles arabiensis* (84.3%) than outdoor HLCs (36.9%).

Conclusion

In this setting, the density and species of mosquitoes collected with alternative methods varied, reflecting the feeding and resting characteristics of the common vectors and the different collection approaches. These differences could impact on the accuracy of entomological indicators and estimates of malaria transmission, when using the alternative methods for sampling mosquitos, as compared to HLCs.

Keywords *Anopheles*, human landing catches, CDC light trap, prokopack aspirators, pit trap

Background

Malaria remains a major public health concern globally, and particularly in sub-Saharan Africa, despite considerable effort to control it [1]. Uganda is typical of high burden countries in Africa and ranked third in number of malaria cases worldwide in 2021, contributing 5.4% of the global burden [1, 2]. *Plasmodium falciparum* accounts for 97% of malaria cases in Uganda [3, 4]. In Uganda and elsewhere in sub-Saharan Africa, the primary malaria vectors are *Anopheles gambiae sensu stricto* (*s.s.*), *Anopheles arabiensis* and *Anopheles funestus sensu lato* (*s.l.*) [1, 3, 5]. Deployment of vector control tools, including long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) of insecticides, has been instrumental in reducing the burden of malaria, but the emergence and spread of insecticide resistance threatens the effectiveness of these measures [6]. Monitoring the impact of vector control tools through entomologic surveillance is essential to guide policy and programmes, but different sampling methods may influence entomologic outcome measures due to species-specific differences in the feeding and resting behaviours of *Anopheles* vectors. Moreover, the precision of the different collection methods varies, which may influence results [7-10].

Human landing catches (HLCs) are considered the 'gold standard' for monitoring human exposure to malaria mosquito vectors [11, 12]. HLCs involve overnight collection of mosquitoes from the exposed limbs of volunteers, using hand-held aspirators and torches; collections can be done both indoors and outdoors [12, 13]. HLCs provide a reliable estimate of key entomologic indicators including mosquito vector density, *Anopheles* species composition, sporozoite infection rate, and annual entomological inoculation rate (aEIR), defined as the number of infective bites per person per year [14, 15]. However, HLCs are expensive and labour intensive, and the positioning of collectors inside households overnight raises ethical issues, as does the intentional exposure of collectors to potentially infectious malaria vectors, even if prophylaxis is provided [7, 13]. These challenges have limited the widespread use of HLCs for entomological surveillance [16]. Alternative

sampling methods include Centers for Disease Control (CDC) light traps and prokopack aspirators for indoor collections and pit traps for use outdoors [17-20] CDC light traps are attractive alternatives to HLC for indoor mosquito collection [10, 18, 21-23]. These traps use a light source to attract free-flying mosquitoes and a rotating fan to create suction pressure to trap mosquitoes in a collection cup [21]. Compared to HLCs, CDC light traps provided equivalent estimates for human biting rates [9, 23], *Anopheles* age structure [21], and sporozoite rates [23, 24], while the density of mosquitoes captured in CDC light traps was higher in some environments [24]. However, measurements using CDC light traps can vary with trap position and presence of human hosts in the house during collections, and may underestimate *Anopheles* species abundance [10, 25] or overestimate human biting rates and aEIR [7]. Moreover, CDC light traps have limited application outdoors [23], and may require two visits to households per collection [18, 23, 26].

Prokopack aspirators are another alternative to HLCs which target indoor resting adult mosquitoes. Prokopacks utilize a battery-powered lightweight motor unit connected to a mosquito collection cup, with an extendable arm to reach mosquitoes resting on ceilings. Mosquitoes are captured by the suction pressure created by an inbuilt fan [19, 27, 28]. Prokopack aspirators are relatively inexpensive and easy to use, and require only a single visit to the households per collection, which is attractive for large-scale vector surveillance [19]. However, in some settings, the density of vectors collected with prokopacks was lower than with indoor HLCs and CDC light traps, which is a potential disadvantage [28, 29].

Pit traps were developed in the 1940s and are the oldest method for collecting outdoor resting mosquitoes [20, 30, 31]. Pit traps involve digging artificial pit shelters approximately 5-6 ft deep under a shaded area, with cavities carved into the vertical sides of the pit to capture mosquito vectors resting outside human dwellings [20, 30]. Pit traps have been used to examine the impact of vector control interventions on vector density, species composition, human blood index and

sporozoite infection rates [32]. Compared to HLCs, the density of mosquitoes captured in pit traps was higher [8, 33]. To further evaluate different mosquito collection methods both indoors and outdoors, this study compared four different methods to collect *Anopheles* vectors on key outcomes including vector density, species composition, sporozoite rate and aEIR. Indoors, prokopack aspirators and CDC light traps were compared to HLCs, and outdoors, pit traps were compared to HLCs.

Methods

Study sites

The study was conducted between June and November 2021 in Tororo and Busia districts. Both districts are in the Bukedi sub-region [34], in eastern Uganda bordering Kenya. The study area included Buteba parish in peri-urban Busia, and Kayoro and Osukuru parishes in rural Tororo (Fig. 1). These areas are characterized by low lying savannah plains, interspersed with bare rock and wetlands, and two annual rainfall peaks occurring between May-June and November-December [35]. Historically, Tororo district was a very high malaria transmission site with an aEIR measured at 562 infective bites per person per year in 2001 [36], and 125 in 2011-2012 [23]. Following implementation of regular rounds of IRS in 2014, combined with LLINs, which are delivered by the Ministry of Health every 3-4 years, malaria burden in Tororo reduced dramatically [37]. By 2019, the measured aEIR had dropped to 0.43 infective bites per person per year [37]. However, after five years of intensive vector control and sustained low-level transmission [38], a resurgence of malaria exceeding pre-IRS levels was documented in Tororo and other areas receiving IRS in 2020-2021 [39]. The etiology of the resurgence has not yet been established, but recent changes in the insecticide delivered by IRS is suspected [39]. In 2020-2021, coinciding with the mosquito sampling for this study, parasite prevalence in the study area was 19.5% by microscopy and 50.7% by qPCR, with no significant differences between Tororo and Busia [40]. In Tororo, the primary malaria vector species include *An. gambiae s.s*., *An. arabiensis* and *An. funestus s.l.* [23]. Following introduction of IRS, *An.*

arabiensis became the predominant species [41]. More recently, coincident with the change in IRS insecticide, increases in both *An. gambiae s.s*. and *An. funestus* mosquito density have been observed in Tororo district (unpublished data). Busia is also a site of very high malaria transmission [42, 43], but unlike Tororo, Busia has received LLINs only (without IRS) for vector control. Malaria transmission patterns in Busia are stable and characteristic of a high transmission area [38, 42]. The dominant malaria vectors in Busia are *An. gambiae s.s.* and *An. funestus,* and to a lesser extent *An. arabiensis* [44]. In 2020-2021, the annual EIR was higher in Busia (108.2 infective bites/person/year) than in Tororo (59.0 in Osukuru parish vs 27.4 in Kayoro parish) [40].

Households selected for entomological surveillance

Mosquito samples were collected under the PRISM (Program for Resistance, Immunology, Surveillance and Modeling of Malaria) Border Cohort study [40], initiated in August 2020 in three adjacent parishes (Fig. 1), including two parishes in Tororo district and one parish in Busia district. Prior to the study, all households in the study area were enumerated and mapped (n=10,474), to generate a sampling frame for the study. The study area was stratified into three transmission areas based on parasite prevalence data. In August 2020, randomly selected households from the three transmission areas were approached and screened for eligibility. Households were enrolled into the cohort study if they met the following selection criteria: (1) at least two members less than 5 years of age, (2) no more than 7 permanent members currently residing, (3) no plans to move from the study area in the next 2 years, and (4) willingness to take part in entomological surveillance activities [40]. A total of 80 randomly selected households were enrolled, including 20 households in Busia, 30 houses from Kayoro, Tororo near the Busia border, and 30 houses from Kayoro and Osukuru, Tororo away from the Busia border. In all 80 households participating in the cohort study, mosquitoes were collected using CDC light traps every 14 days. An additional 16 households (8 from Busia and 8 from Tororo) not taking part in the cohort study were randomly selected from the enumeration database to participate in indoor and outdoor HLCs, which were conducted every 4

weeks [40]. For the purposes of this study, prokopack aspirator collections and pit trap collections were also done in the same 16 non-cohort households one week prior and one week after the HLCs. Data collected between June and October 2021 were included in this analysis, covering 6 rounds of HLCs (every 4 weeks), 12 rounds of prokopack aspirator and pit trap collections (every 2 weeks, 1 week before and after HLCs), and 6 rounds of CDC light trap collections (every 4 weeks, closest date to when HLCs were done). All participating households provided written informed consent before study activities were conducted.

Mosquito collection methods

This study, aimed to evaluate different mosquito collection methods as compared to HLCs as the gold standard for both indoor and outdoor collections. Both HLCs and CDC light traps have been evaluated previously in this area [23], and prokopack aspirators were used in a large-scale trial conducted in 48 districts in Uganda [45]. Pit traps have not been evaluated in Uganda but provide an additional method for sampling outdoor resting mosquitoes [30].

Human-landing catches

HLC households were located > 300 meters from each other. To ensure comparability of results, four households were sampled per night for 4 consecutive nights in order to have the 16 households sampled within the same week for each 4-week interval. For the HLCs, four adult collectors were stationed at each house, with two indoors and two outdoors at a distance of at least 10 meters. Indoor and outdoor collections were conducted from 18:00 hours at dusk to 08:00 hours in the morning, with hourly recordings of mosquitoes caught. A 10-minute break was given for each hour of collection. Mosquito collectors used hand-held aspirators and torches to capture mosquitoes that landed on their exposed limbs. Collectors were rotated between sites and collection times to limit field collector bias. All mosquitoes collected were transferred to paper cups and transported for further processing.

CDC light trap collections

CDC light trap collections were conducted in all 80 households participating in the PRISM cohort study. Miniature CDC light traps (Model 512; John W. Hock Company, Gainesville, Florida, USA) were positioned 1 meter above the floor at the foot end of a human occupied bed covered by a standard pyrethroid-only LLIN. CDC light traps were set in all rooms where household members sleep. Traps were set at 19:00 hours and collected at 07:00 hours the following morning. All mosquitoes collected in the light traps were stored individually for further processing.

Prokopack aspirator collections

Prokopack collections were conducted using a battery powered mosquito aspirator (InsectaZooka) [27] with a lightweight motor and suction cups for mosquito collection. The prokopack was connected to a 12V battery, which was carried by the operator in a backpack to ease mobility. Prokopack collections were conducted a week before and the week following HLC sampling, 'sandwiching' HLCs to improve spatial comparison of mosquito density estimates. Prokopack collections were conducted on a single morning per household and scheduled not interfere with HLC collections. Resting mosquitoes were collected in the early morning hours (between 06.00 hours and 08.00 hours) while the temperature was cooler, to standardize collections and maximize yields. Two field workers spent at least 30 minutes inside each house, which was previously shown to be adequate in Uganda [45], and collected all mosquitoes resting on walls, on the ceiling, under tables and beds. Four houses were sampled each day, to ensure sampling of the 16 houses was done within the same week. All mosquitoes collected were transferred to paper cups and transported for further processing. Mosquitoes were transported using cool boxes to the study insectary, sorted and stored dry on desiccant (silica gel) for molecular analysis [23].

Pit trap collections

Mosquito pit traps were set up within 10-20m of each of the 16 households where HLCs and prokopack collections were done. Pit trap collections were conducted every two weeks with the same schedule as prokopack collections, 'sandwiching' HLCs, between 06:00 hours and 08:00 hours. Four pit shelters were assessed at a time, so that 16 pit shelters were covered within the same week, matching the prokopack collections. Artificial pit shelters were dug 5 to 6 ft deep, under natural shade so that their openings (4 to 5 x 3 to 4ft) were shaded from above [20]. A suitable cover using locally sourced timber and thatch was placed partially over the pit trap entrance for shielding. About 2ft from the bottom of the pit trap, small un-baited cavities, about 30cm deep were dug horizontally from each of the four sides of the pit. Mosquitoes were collected from these small cavities and from the wall of the pit itself. The pit traps were encircled with a thorn fence enclosure to prevent animals or children from falling into them or using them as toilets, as recommended by Muirhead-Thomson [20].

Species identification and *Plasmodium falciparum* sporozoite ELISA

All female *Anopheles* mosquitoes collected were identified morphologically using previously described keys [46] and stored dry, individually in 1.5 ml tubes for further molecular analysis. Morphologically identified species included 3 groups: *An. funestus*, *An. gambiae s.l.*, and other *Anopheles*, which were primarily *Anopheles chrysti* considered to be non-malaria vectors [47]. All female *An. gambiae s.l.* collected by HLC (both indoor and outdoor), prokopack and pit traps were differentiated as *An. gambiae s.s.* and *An. arabiensis* using PCR [48]. For CDC light trap collections, a random sample of 60 *An. gambiae s.l.* per month was speciated due to resource limitations. *Plasmodium falciparum* sporozoite ELISA was conducted on all female *An. gambiae s.l.* and *An. funestus s.l.* collected by HLC, prokopack, CDC light traps, and pit traps, using the protocol developed by Wirtz *et al* [49], which has previously been used in Uganda [23, 35, 40]. *Anopheles funestus s.l.* were only identified morphologically due to resource limitations.

Statistical analysis

Vector density was defined as the total number of female *Anopheles* mosquitoes collected divided by the total number of collections done per method and expressed as the average number of mosquitoes per day for each method. The sporozoite rate was defined as the number of female *Anopheles* mosquitoes testing positive using ELISA divided by the total number tested. The aEIR was expressed as a product of daily vector density and the sporozoite rate multiplied by 365 days per year [23, 50]. Analyses were done using Stata (version 14.2, Stata Corp, College Station, TX, USA). For all analyses, data were collapsed for each collection method across the entire collection period. For all measures of association, data were stratified by whether collections methods were indoor or outdoor, and HLCs were considered the reference group. Associations between collection methods and vector density or aEIR were made using a negative binomial regression model with the number of collections included as an offset and associations expressed as the density ratio (DR) or incident rate ratio (IRR), respectively. Associations between collection methods and sporozoite rates were made using the Chi-squared or Fisher's exact test. A two-sided p-value < 0.05 was considered statistically significant.

Ethical approval

For all methods, a written informed consent was obtained from household heads or their designate before mosquito collection could commence. HLCs included additional consenting of the mosquito collectors. Ethical approval was obtained from Makerere University School of Medicine Research and Ethics Committee (SOMREC), the Uganda National Council of Science and Technology (UNCST), the London School of Hygiene and Tropical Medicine Research and Ethics Committee and the University of California, San Francisco Committee on Human Research.

Results

Mosquito collection

A total of 4,891 female *Anopheles* were collected, including 3,318 indoors and 1,573 outdoors (Table 4.1). For indoor collections, most mosquitoes were collected using CDC light traps (2,562), while outdoors, the majority were collected using pit traps (1,234). Of the 3,313 mosquitoes captured indoors that were tested for sporozoites, 43 were positive, including 6 of 407 collected using HLCs (4 *An. gambiae s.s.*, 1 *An. arabiensis* and 1 *An. funestus*) and 6 of 349 collected with prokopack aspirators (1 *An. arabiensis* and 5 *An. funestus*). Of the 2,557 mosquitoes collected using CDC light traps that were tested for sporozoites, 31 were positive, however, due to the way these data were collected it was not possible to assign sporozoite positivity to the species level. Of the 1,573 mosquitoes captured outdoors, 11 were positive for sporozoites, including 6 of 339 collected using HLCs (1 *An. arabiensis* and 5 *An. funestus*) and 5 of 1,234 collected using pit traps (1 *An. gambiae s.s.*, 4 *An. arabiensis*).

Species composition

The dominant species of *Anopheles* captured varied by whether collections were done indoors or outdoors and the method of collection used. All three main vectors were collected using indoor HLCs (Fig. 4.2), with *An. arabiensis* dominating (49.9%). Using CDC light traps, all three main vectors were collected in fairly similar proportions (ranges 31.1% to 34.8%). In contrast, using prokopack aspirators, a higher proportion of *An*. *funestus* were collected (75.1%). Outdoors, HLCs captured all three main vectors (Fig. 3), with *An. arabiensis* (36.9%) and *An. funestus* (34.5%) dominating. However, pit traps captured a higher proportion of *An. arabiensis* (84.3%).

Measures of association between method of collection and key entomologic indicators

Compared to indoor HLCs, the density of mosquito vectors collected was lower using both CDC light traps (4.24 vs 2.96, DR 0.70, 95% CIs 0.63 – 0.77, p<0.001) and prokopack aspirators (4.24 vs 1.82,

density ratio [DR] 0.43, 95% CIs 0.37 – 0.49, p<0.001). *Plasmodium falciparum* sporozoite rates were similar between the three indoor collection methods, although precision was limited due to the low numbers of sporozoites that were detected, especially using prokopack and HLC. Overall, the aEIR using CDC light traps or prokopack aspirators was approximately half what was estimated using indoor HLCs, however these differences did not achieve statistical significance (Table 4.2). In contrast, compared to outdoor HLCs, vector density was higher using pit trap collections (3.53 vs 6.43, DR 1.82, 95% CIs 1.61 – 2.05, p<0.001), while the sporozoite rate was lower (0.018 vs 0.004, DR 0.23, 95% CIs 0.07 – 0.75, p=0.008). Overall, the aEIR using pit traps was less than half what was estimated using outdoor HLCs (22.81 vs. 9.51, IRR=0.42, 95% 0.13-1.37), although this difference did not reach statistical significance (Table 4.2).

Species-specific vector density and sporozoite rates, by method of collection

Compared to indoor HLCs, the density of *An. arabiensis* was significantly lower using CDC light traps (0.94 vs 2.11, DR 0.44, 95% CIs 0.38 – 0.52, p<0.001); but no significant differences in vector density of *An. gambiae s.s.* or *An. funestus* were observed when CDC light traps and indoor HLCs were compared (Table 4.3). The density of *An. gambiae s.s.* and *An. arabiensis* collected using prokopack aspirators were significantly lower than with indoor HLCs (0.31 vs 0.77, DR 0.40, 95% CIs 0.28 – 0.56, p<0.001; 0.14 vs 2.11, DR 0.06, 95% CIs 0.04 – 0.10, p<0.001); for *An. funestus*, vector density was higher using prokopack aspirators than HLCs, but this difference was not statistically significant (Table 4.3). No differences in sporozoite rates were observed for *An. gambiae s.s.*, *An. arabiensis* or *An. funestus* when mosquitoes collected indoors using prokopack aspirators were compared to indoor HLCs (Supplemental table 4.1).

The densities of *An. gambiae s.s.* and *An. funestus* collected using pit traps were lower than with outdoor HLCs (0.26 vs 0.65, DR 0.40, 95% CIs 0.27 – 0.57, p<0.001; 0.64 vs 1.22, DR 0.53, 95% CIs 0.41 – 0.68, p<0.001); for *An. funestus*, vector density was significantly higher using pit traps than

HLCs (5.42 vs 1.30, DR 4.16, 95% CIs 3.46 – 5.01, p<0.001; Table 4.3). No differences in sporozoite rates were observed for *An. gambiae s.s.* or *An. arabiensis* when mosquitoes collected outdoors using pit traps were compared to outdoor HLCs; however, for *An. funestus* the sporozoite rate in mosquitoes collected using pit traps was significantly lower than in those collected by outdoor HLCs (sporozoite rate 0.000 vs 0.043; 95% CIs 0.043 (0.0158-0.1018), fisher exact p=0.03) (Supplemental table 4.1).

Discussion

Human landing catches, considered the gold standard for collecting host-seeking *Anopheles* indoors and outdoors are challenging to use on a large scale [7, 9, 13]. In this study, CDC light traps and prokopack aspirators were compared to HLCs for indoor mosquito collection, and pit traps were compared to outdoor HLCs. The density of *Anopheles* vectors collected indoors was 30% lower with CDC light traps and 57% lower with prokopacks as compared to HLCs. Sporozoite rates and aEIRs were not significantly different between the 3 indoor collection methods but the precision of these comparisons was limited by the low sporozoite rate. The relative species composition was similar between indoor HLCs and CDC light traps, but prokopacks, which only collected mosquitoes resting in the morning indoors, captured a higher proportion of *An. funestus* compared to indoor HLCs. Given these findings, CDC light traps provided a reasonable alternative to indoor HLCs, but prokopacks may not provide an accurate sampling of mosquitoes responsible for malaria transmission. Outdoors, the density of *Anopheles* vectors collected via pit traps was significantly higher than HLCs, however, sporozoite rates were significantly lower and a higher proportion of *An. arabiensis* were collected. Pit traps could be a useful alternative to HLCs for simply sampling outdoor resting mosquitoes, but provided less accurate estimates of measures of transmission intensity [8, 33]. In this setting, the density and species of mosquitoes collected with alternative methods varied, reflecting the feeding and resting characteristics of the common vectors and the different collection approaches, which impacted on the entomological indicators and estimates of malaria transmission.

CDC light traps are the most common alternative to HLCs for collection of indoor resting *Anopheles* [10, 21, 23]. Overall, CDC light traps are mechanical, less intrusive, non-exposure and efficient tools that are relatively simple to use in field settings, permitting overnight collection of mosquitoes [23]. In this study, CDC light traps collected modestly fewer *An. arabiensis* compared to HLCs indoors, however there was no significant difference in vector density for both *An. gambiae s.s.* and *An. funestus* when compared to HLCs. Similar observations were reported by Briet et al, [10]; where the relative sampling efficiency of CDC light traps for *Anopheles* vectors was comparable to HLCs indoors. Notably, Briet *et al*, also observed that the relative sampling efficiency for CDC light traps was greater for *An. funestus s.l.* compared to *An. gambiae s.l.* [10]. In several observations from sub-Saharan Africa, CDC light traps collected equivalent or higher numbers of *Anopheles* compared to HLCs [10, 21, 23] and were used as reliable alternatives for estimating sporozoite infection rates and EIR [23]. However, early findings from Kenya by Mbogo *et al*, showed that CDC light traps underestimated the abundance of *An. gambiae s.l.* [25]. In examining mosquito sampling techniques and their reliability, including HLCs, CDC light traps and odour-baited traps, Mboera *et al*, reported an overestimation of EIR in CDC light traps arising from very high vector densities [7]. CDC light traps may not have universal appeal, as observed in Bioko Island, where this method did not reliably estimate mosquito biting rates [26]. Differences in vector density, species composition and sporozoite infection rates have been observed with CDC light traps in different settings, showing distinct geographical patterns but largely with a positive correlation in *Anopheles* vector density to indoor HLCs [10, 18, 23]. Differences in *Anopheles* vector density, species composition and sporozoite infection rates were observed in response to changes in CDC light source, trap position, collection time and presence or absence of a human bait [18, 25, 51, 52]. Limitations notwithstanding, CDC light traps collected similar vector densities to indoor HLCs for highly anthropophilic vectors; *An. gambiae s.s.* and *An. funestus.* In addition, CDC light traps have been shown to provide reliable estimates for mosquito vector density in comparison to HLCs with increase

in number of collection nights, making this tool suitable for longitudinal entomological surveillance [10, 23]. The recent deployment of solar-recharged CDC light traps in estimating *Anopheles* vector density, makes this tool an even more attractive alternative to HLCs in resource limited settings [53].

Prokopack aspirators are a relatively new tool for indoor mosquito collection [29]. Prokopacks are battery powered, light-weight motor units that collect indoor resting and free-flying mosquitoes using suction pressure [27]. Prokopack aspirators in this study collected significantly fewer mosquitoes indoors compared to HLCs, with significantly lower vector density for both *An. gambiae s.s.* and *An. arabiensis*. Comparison of prokopack aspirators with HLCs in coastal Kenya showed that prokopacks collected more *Culex quinquefasciatus* and other culicines than *Anopheles* vectors [29]. This finding, however, may have been influenced by the low density of *Anopheles* mosquitoes in the population sampled. Studies in Tanzania and Eritrea demonstrated the utility of prokopacks in estimating *Anopheles* vector density indoors, pre and post vector control interventions [28, 54, 55]. Prokopack aspirator collections provide an efficient mosquito collection technique operated by a single individual, requiring only 15 to 30 minutes in the household during a single visit, making prokopacks an attractive alternative to HLCs and a scalable tool for sampling indoor resisting mosquitoes [56]. In this study relatively more *An. funestus* were collected with prokopack aspirators compared to indoor HLCs. In contrast, prokopack collections across 48 districts in Uganda by Lynd *et al*, yielded significantly more *An. gambiae s.s.* than *An. funestus* [45]. Prokopack aspirators have been shown to be very effective in cross-sectional studies that require a snapshot assessment of *Anopheles* species composition, sporozoite infection rates and insecticide resistance variants [45].

Pit traps have been used for outdoor mosquito collections for over half a century [31]. Pit traps involve utilization of artificial pit shelters dug in the ground for collection of outdoor resting mosquitoes [20]. Comparison of pit traps with outdoor HLCs, showed significantly higher *Anopheles* vector density, albeit with significantly lower sporozoite infection rates. In addition, significantly

more *An. arabiensis* were collected with pit traps outdoors compared to HLCs. However, significantly fewer *An. gambiae s.s.* and *An. funestus* were collected in the pit traps compared to outdoor HLCs. Pit traps have been used for assessment of outdoor resting mosquitoes, estimates of mosquito gonotrophic cycles, sporozoite infection and EIR [30]. In this study, pit traps mainly caught *An. arabiensis* similar to observations made in Moshi, Tanzania [33] and Konso, southern Ethiopia [8]. Pit traps provide a stationary outdoor mosquito trap that can be used for prolonged periods with limited maintenance [20]. However, the stationary nature of pit traps is also a major limitation to the scale up of this tool [57], in addition to the fact that pit traps cannot be deployed in areas with a very low water table [32]. The comparison of pit traps to outdoor HLCs is indirect with regard mosquito behaviour, for instance, whilst HLCs target outdoor mosquito biting behaviour [13], pit traps target outdoor mosquito resting behaviour [20]. Pit traps are less likely to collect highly anthropophilic malaria vectors such as *An. funestus* that have been observed to bite outdoors in response to vector control [58-60]. This study shows that pit traps are a viable alternative to HLCs in sampling *Anopheles* vectors outdoors but did not provide accurate measures of transmission intensity. Pit traps, are relatively easy to set up, are very productive overall in terms of *Anopheles* vector density and assess a unique aspect of mosquito behaviour (outdoor resting) whose parameters are quite difficult to estimate [20, 31].

Whilst the choice for indoor/outdoor mosquito collection is most likely driven by entomologic measures of interest, HLCs provide measurements for both indoor and outdoor mosquito populations. Increased interest in mapping diurnal mosquito biting behaviour beyond night catches suggests that HLCs remain relevant [61]. Alternative indoor/outdoor collection methods including CDC light traps, prokopack aspirators and pit traps seem to be specialized mosquito collection methods targeting particular aspects of either indoor/outdoor HLCs. These aspects include, among others vector density, *Anopheles* species composition and sporozoite infection. As interest in alternative methods to HLCs gains momentum, some studies suggest using HLCs to calibrate

mosquito collection measurements for alternative collection methods which can then be scaled up [9, 10, 62, 63]. This would in part address the challenges of overestimation of mosquito biting rates and EIR associated with CDC light traps [7]. As scalable tools, CDC light traps and prokopack aspirators present viable alternatives to HLCs indoors, however for outdoor sampling on a large scale, other alternatives such as the human baited double net method may need to be considered [64].

Limitations

This study had several limitations. First, mosquito parameters such as parity, abdominal status and blood meal index, which may have provided additional granularity in the observed differences between trapping methods, were not measured. Second, not all indoor and outdoor alternatives were included. Alternative methods such as the human bait double net method and pyrethrum spray collections were not assessed due to resource limitations. Third, the study was limited to households located in 3 parishes within 2 districts in Eastern Uganda, and these findings may not be generalizable to other settings. Fourth, the houses used for CDC light trap collections were not the same as those used for other collection methods and variability in household characteristics was not accounted for. Finally, differences in the various methods, including the time period during which mosquitoes were collected and differences in targeting host-seeking vs resting mosquitoes, may have impacted on the results. Moreover, the data for this study were collected over only five months, not a complete calendar year, which may have affected aEIR estimates. Despite these limitations, the results of this study provide evidence on how alternative collection methods compare to HLCs to help guide future research studies and surveillance programmes.

Conclusion

The method used to collect mosquitoes is important to consider when measuring entomologic outcomes and estimating transmission intensity. In this study, the density and species of mosquitoes

collected with alternative methods varied, likely reflecting the feeding and resting characteristics of the common vectors and the different collection approaches. HLCs remain the gold standard for capturing host-seeking *Anopheles* mosquitoes indoors and outdoors during peak biting times, but the other methods evaluated have advantages. In this setting, CDC light traps provided a reasonable alternative to indoor HLCs, but prokopacks failed to collect a full representation of mosquitoes responsible for malaria transmission. Pit traps could be a useful alternative to HLCs for sampling outdoor resting mosquitoes, but mainly captured *An. arabiensis* and provided less accurate estimates of measures of transmission intensity. The potential impact of the method used to collect mosquitoes on the species composition of *Anopheles* collected and various entomologic endpoints should be carefully considered, particularly when assessing the effectiveness of vector control measures and estimating the impact on malaria transmission.

Figure legends

Figure 4.1: Map of study sites showing location of the 3 parishes including Buteba, Kayoro and Osukuru, in Busia and Tororo districts. The green dots highlight the positioning of the 80 border cohort households and the red dot show the position of the 16 households used for Human landing catches, prokopack and pit trap collections. Image modified from Nankabirwa et al., [40]

Figure 4.2: *Anopheles* vectors collected indoors using human landing catches (HLC), prokopack aspirators and CDC Light traps. The bars depict *Anopheles* mosquito species including *An. gambiae* s.s. (blue bar), *An. arabiensis* (red bar), *An. funestus* (grey bar) and *An. other* (orange bar).

Figure 4.3: *Anopheles* vectors collected outdoors using human landing catches (HLC), and Pit traps. The bars depict *Anopheles* mosquito species including *An. gambiae* s.s. (blue bar), *An. arabiensis* (red bar), *An. funestus* (grey bar) and *An. other* (orange bar).

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Table 4.1: Female Anopheles mosquito collections (*An. gambiae* s.l. and *An. funestus* s.l.) by different methods

Collection Method		Sampled HHs	Total Collections	Total *Anopheles* collected	Total number of *Anopheles* tested for sporozoites (number sporozoite positive)				
					All *Anopheles*	*An. gambiae* s.s.	*An. arabiensis*	*An. funestus*	Other *Anopheles*
Indoor	HLC	16	96	407	407 (6)	74 (4)	203 (1)	105 (1)	25 (0)
	CDC LT	80	867	2562	2557 (31)	798 (N/A)	813 (N/A)	891 (N/A)	60 (N/A)
	Prokopack	16	192	349	349 (6)	59 (0)	26 (1)	262 (5)	2 (0)
Outdoor	HLC	16	96	339	339 (6)	62 (0)	125 (1)	117 (5)	35 (0)
	Pit trap	16	192	1234	1234 (5)	49 (1)	1040 (4)	123 (0)	22 (0)

Table 4.2: Measures of association between method of collection and vector density, sporozoite rate and aEIR in female *Anopheles* mosquitoes

Collection method	Vector density	DR (95% CI)	P value	Sporozoite rate	RR (95% CI)	P value	aEIR	IRR (95% CI)	P value
Indoor									
HLC	4.24	reference	—	0.015	reference	—	22.81	reference	—
CDC LT	2.96	0.70 (0.63-0.77)	<0.001	0.012	0.82 (0.34-1.96)	0.66	13.08	0.57 (0.24-1.37)	0.21
Prokopack	1.82	0.43 (0.37-0.49)	<0.001	0.017	1.17 (0.38-3.58)	0.79	11.41	0.50 (0.16-1.55)	0.23
Outdoor									
HLC	3.53	reference	—	0.018	reference	—	22.81	reference	—
Pit trap	6.43	1.82 (1.61-2.05)	<0.001	0.004	0.23 (0.07-0.75)	0.008	9.51	0.42 (0.13-1.37)	0.15

Table 4.3: Measures of association between method of collection and vector density, stratified by species

	Anopheles gambiae s.s.			*Anopheles arabiensis*			*Anopheles funestus*		
Indoor	**Vector density**	**DR (95% CI)**	**P value**	**Vector density**	**DR (95% CI)**	**P value**	**Vector density**	**DR (95% CI)**	**P value**
HLC	0.77	reference	—	2.11	reference	—	1.09	reference	—
CDC LT	0.92	1.19 (0.94-1.52)	0.14	0.94	0.44 (0.38-0.52)	<0.001	1.03	0.94 (0.77-1.15)	0.55
Prokopack	0.31	0.40 (0.28-0.56)	<0.001	0.14	0.06 (0.04-0.10)	<0.001	1.36	1.25 (0.99-1.56)	0.06
Outdoor									
HLC	0.65	reference	—	1.30	reference	—	1.22	reference	—
Pit trap	0.26	0.40 (0.27-0.57)	<0.001	5.42	4.16 (3.46-5.01)	<0.001	0.64	0.53 (0.41-0.68)	<0.001

Supplemental Table 4.1: Measures of association between method of collection and sporozoite infection, stratified by species

	Anopheles gambiae s.s.					*Anopheles arabiensis*					*Anopheles funestus*				
Indoor	**N**	***Pf* positive**	***Pf* negative**	**SR (95% CI)**	**p-value**	**N**	***Pf* positive**	***Pf* negative**	**SR (95% CI)**	**p-value**	**N**	***Pf* positive**	***Pf* negative**	**SR (95% CI)**	**p-value**
HLC	74	4	70	0.054 (0.0175-0.1399)	—	203	1	202	0.005 (0.0003-0.0316)	—	105	1	104	0.010 (0.0005-0.0595)	—
Prokopack	59	0	59	0.000 (0.000-0.0762)	0.15	26	1	25	0.038 (0.0020-0.2159)	0.21	262	5	257	0.019 (0.0071-0.0465)	0.68
Outdoor															
HLC	62	0	62	0.000 (0.000-0.0727)	—	125	1	124	0.008 (0.0004-0.0508)	—	117	5	112	0.043 (0.0158-0.1018)	—
Pit trap	49	1	48	0.020 (0.0011-0.1247)	0.44	1036	4	1040	0.004 (0.0013-0.0106)	0.43	123	0	123	0.000 (0.000-0.0377)	0.03

Pf =*Plasmodium falciparum*; SR=Sporozoite rate

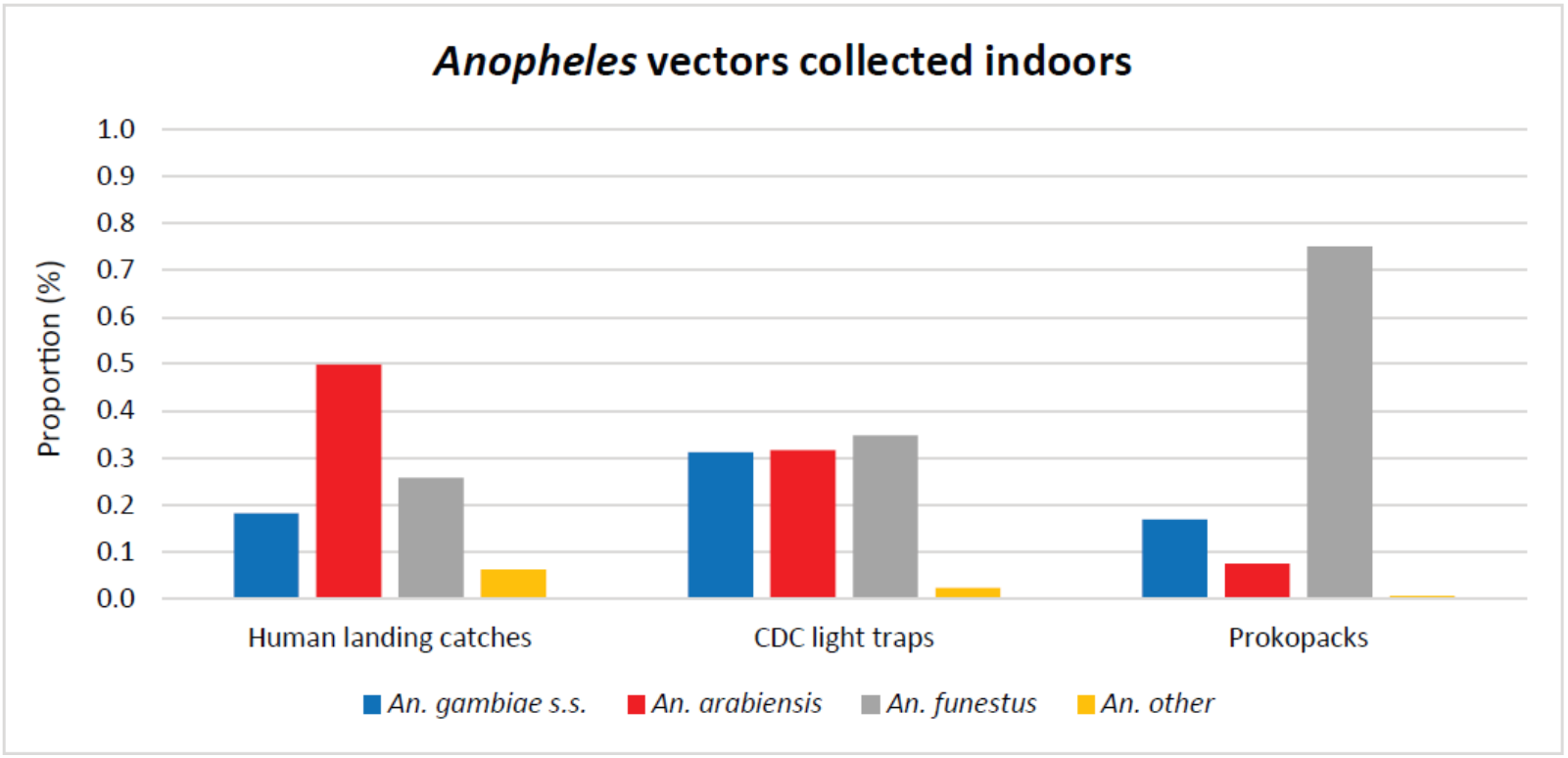

Figure 4.2: *Anopheles* vectors collected indoors using human landing catches (HLC), prokopack aspirators and CDC Light traps. The bars depict *Anopheles* mosquito species including *An. gambiae* s.s. (blue bar), *An. arabiensis* (red bar), *An. funestus* (grey bar) and *An. other* (orange bar).

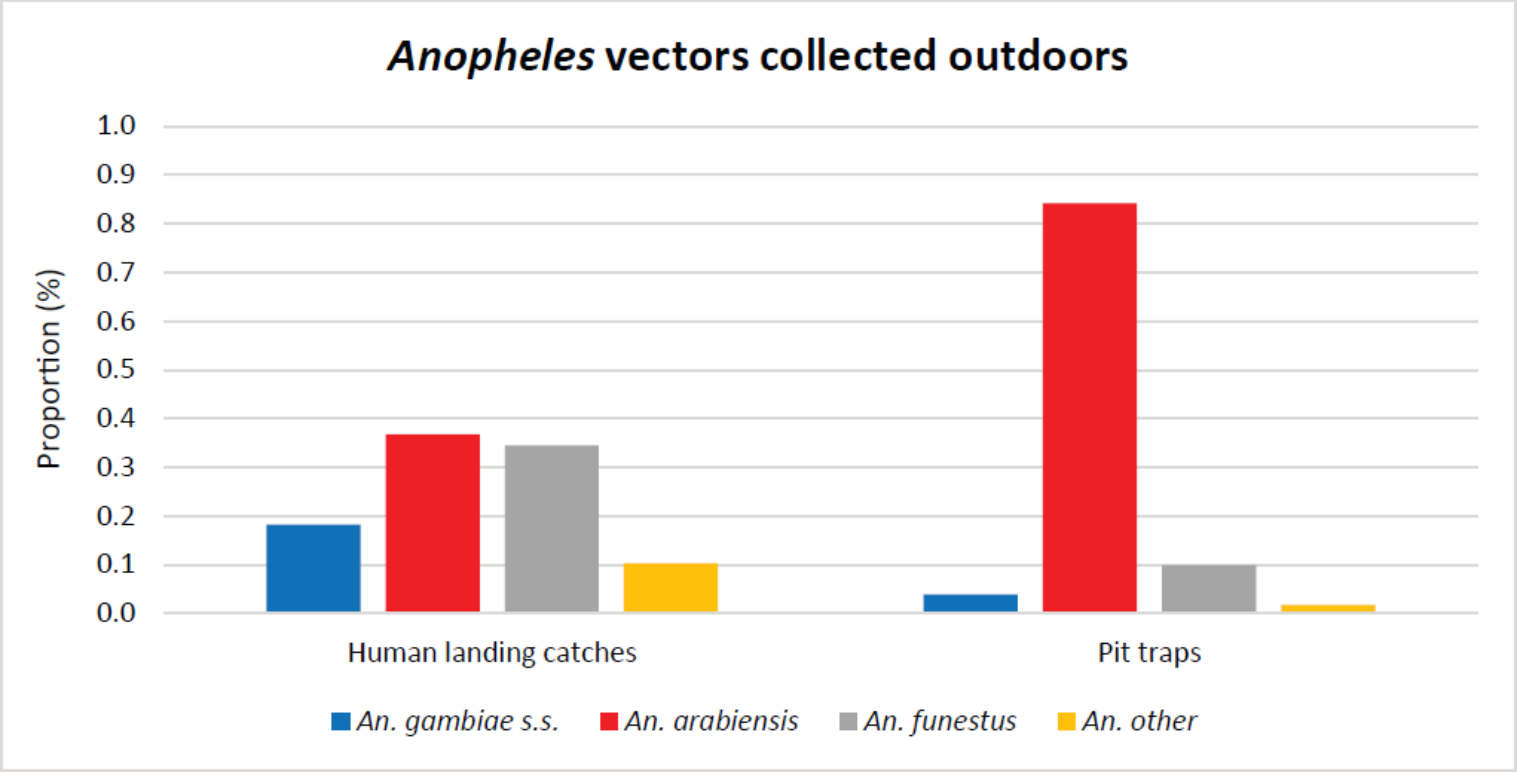

Figure 4.3: *Anopheles* vectors collected outdoors using human landing catches (HLC), and Pit traps. The bars depict Anopheles mosquito species including *An. gambiae* s.s. (blue bar), *An. arabiensis* (red bar), *An. funestus* (grey bar) and *An. other* (orange bar).

CHAPTER 5 IMPACT OF SEASONALITY AND MALARIA CONTROL INTERVENTIONS ON *ANOPHELES* DENSITY AND SPECIES COMPOSITION

5.1 Chapter Introduction

This chapter addresses objective 2, to describe malaria vector species composition in areas with differing vector control interventions. This manuscript presents findings from longitudinal data collected over 57 months to examine seasonal variations and the impact of long lasting insecticidal nets and indoor residual spraying on the species composition and density of sympatric *Anopheles* species in 3 study sites with differing malaria endemicity. This manuscript was published in Malaria Journal. At the end of the manuscriptare Tables showing characteristics of sites and collections, and stratified analysis of vector density. Figures showing the absolute numbers of *Anopheles* and the relative numbers collected over time are included.

5.2 Research paper

Below is the research paper cover sheet, followed by details of the manuscript, tables and figures.

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Student ID Number	1513692	**Title**	Mr
First Name(s)	Henry Ddumba		
Surname/Family Name	Mawejje		
Thesis Title	Anopheles species composition and insecticide resistance patterns in Uganda		
Primary Supervisor	Prof. Sarah Staedke		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

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SECTION E

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Impact of seasonality and malaria control interventions on *Anopheles* density and species composition from three areas of Uganda with differing malaria endemicity

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Abstract

Background

Long lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) are malaria control interventions primarily responsible for reductions in transmission intensity across sub-Saharan Africa. These interventions,however, may have differential impact on *Anopheles* species composition and density. Here, we studied thechanging pattern of *Anopheles* species in three areas of Uganda with markedly different transmission intensities and different levels of vector control.

Methods

From October 2011 to June 2016 mosquitoes were collected monthly using CDC light traps from 100 randomly selected households in three areas: Walukuba (low transmission), Kihihi (moderate transmission) and Nagongera (high transmission). LLINs were distributed in November 2013 in Walukuba and Nagongera and in June 2014 in Kihihi. IRS was implemented only in Nagongera, with three rounds of bendiocarb

delivered between December 2014 and June 2015. Mosquito species were identified morphologically and by PCR (Polymerase Chain Reaction).

Results

In Walukuba, LLIN distribution was associated with a decline in *Anopheles funestus* vector density (0.07 vs

0.02 mosquitoes per house per night, density ratio [DR] 0.34, 95% CI: 0.18-0.65, p=0.001), but not *An. gambiae* s.s. nor *An. arabiensis*. In Kihihi, over 98% of mosquitoes were *An. gambiae* s.s. and LLIN distributionwas associated with a decline in *An. gambiae* s.s. vector density (4.00 vs 2.46, DR 0.68, 95% CI: 0.49-0.94, p=0.02). In Nagongera, the combination of LLINs and multiple rounds of IRS was associated with significant reduction in *An. gambiae* s.s. (28.0 vs 0.17, DR 0.004, 95% CI: 0.002-0.009, p<0.001), and *An. funestus s.l*. (3.90 vs 0.006, DR 0.001, 95% CI: 0.0005-0.004, p<0.001), with a less pronounced decline in *An.arabiensis* (9.18 vs 2.00, DR 0.15 95% CI: 0.07-0.33, p<0.001).

Conclusions

LLIN distribution was associated with reductions in *An. funestus* s.l. in the lowest transmission site and *An. gambiae* s.s. in the moderate transmission site. In the highest transmission site, a combination of LLINs andmultiple rounds of IRS was associated with the near collapse of *An. gambiae* s.s. and *An. funestus* s.l. Following IRS, *An. arabiensis*, a behaviorally resilient vector, became the predominant species, which may have implications for malaria vector control activities.

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Background

Over the past two decades, improved funding and intensive malaria control efforts have increased coverageof vector control interventions worldwide, chiefly long lasting insecticidal nets (LLINs) and indoor residual spraying (IRS)[1-3]. Within this period, significant declines in the burden of malaria have been reported across sub-Saharan Africa with most of this reduction attributed to LLINs (68%), and to a lesser extent, use of IRS (13%) [1]. Global progress toward reducing the incidence of malaria and related deaths, however, hasstalled recently [3]. In response, the World Health Organization (WHO) has called for a locally tailored approach to malaria control rather than a 'one size fits all' policy [3].

In Uganda, focused efforts to ensure universal coverage of LLINs through mass distribution campaigns haveincreased household ownership of long lasting insecticidal nets, from 47% in 2009 to over 80% in 2015 and 2019 [4-6]. IRS has also been implemented, beginning with 10 districts from 2007 to 2014, and moving to 14new districts in 2014 [5, 7-9]. Concomitantly, malaria prevalence has declined in children under five years old, from 40% in 2009, to 19% in 2015 [5], and, further, to 9% in 2019 [6]. In Uganda [10], Kenya [11] and elsewhere [12], sustained vector control has not only resulted in reductions in transmission intensity, but also changes in *Anopheles* species composition, their behaviour [13, 14], and density [15].

Anopheles gambiae sensu lato and *Anopheles funestus* sensu lato are the primary malaria vector groups in Uganda [4, 16], and elsewhere in East Africa [17, 18]. Both groups are species complexes, comprising of genetically distinct but morphologically indistinguishable sibling species [19-23]. In the *An. gambiae* complex,*An. gambiae* sensu stricto and *An. arabiensis* differ in several aspects, including breeding environment, hostpreference, biting behaviours, malaria infection rates, and insecticide resistance patterns [14, 17, 24]. *Anopheles gambiae* s.s. prefer to feed on humans and rest indoors [17]. In contrast, *An. arabiensis* is less anthropophilic [25, 26]; feeding preferences vary with host availability

across the species range [27, 28], withexophilic tendencies [29, 30]. In some mosquito populations, *An. gambiae s.s.* has higher *Plasmodium falciparum* infection rates [31], and higher levels of pyrethroid resistance [32], than *An. arabiensis.* Hybrids between *An. gambiae* s.s and *An. arabiensis* have also been identified [33, 34], with evidence of gene flow between the two species [34]. The implication of hybrids for malaria control is still poorly understood, although in some populations adaptive introgression of insecticide resistance genes coincident with LLIN distribution has been observed [35]. In contrast, *An. funestus* s.l. breeds year-round in stable environments,such as marshland [20, 36], and may engage in early-morning biting [37]. *An. funestus* s.l remains an important vector in dry seasons as a result of its breeding habits [38, 39].

With the expansion of vector control, changes in *Anopheles* species composition and mosquito density havebeen observed in Uganda [10, 15], and elsewhere in sub-Saharan Africa [26, 40, 41]. Changes in malaria vector species composition in response to vector control interventions are not a new phenomenon and have been described previously [42]. Recent studies have demonstrated an increase in the relative abundance of*An. arabiensis* when compared to sympatric *An. gambiae* s.s. following deployment of LLINs and/or IRS [10, 11, 14]. Similarly, the apparent replacement of highly anthropophilic *An. funestus* s.s. by less anthropophilic(zoophilic) and more exophilic *An. rivulorum* in response to IRS in neighboring Tanzania, was observed in the*An. funestus* s.l. complex in the 1960s [42]. Due to their more zoophilic and exophilic behavior, vector controlinterventions have been less effective in controlling certain malaria vector species such as *An. arabiensis* [41,43], and *An. rivolurum* [42]. To further explore the species-specific impact of vector control interventions, we examined the impact of LLINs and IRS on sympatric *An. gambiae* s.s., *An. arabiensis* and *An. funestus* s.l.mosquito density in areas with differing malaria endemicity in Uganda.

Methods

Study sites

This study was conducted from October 2011 to June 2016 in three sites with differing malaria endemicity, within Walukuba, Kihihi and Nagongera sub-counties (Figure 5.1), as part of the PRISM1 (Program for Resistance, Immunology, Surveillance and Modelling of Malaria) project [10, 44], [45] [46]. Walukuba sub- county (00°26'33.2"N, 33°13'32.3"E), located on the fringes of Lake Victoria in Jinja District, eastern Uganda is a peri-urban area at an elevation of 1,215 m with low malaria transmission (baseline annual human bitingrate of 537 and *P. falciparum* entomological inoculation rate [EIR] of 3.2 infective bites per person per year)[46, 47]. *An. arabiensis* has been the predominant malaria vector species in this area [46, 48]. Kihihi sub- county (00°45'03.1"S, 29°42'03.6"E), located in Kanungu District, southwestern Uganda, is a rural highland area 1,310 m above sea level, with moderate malaria transmission (baseline annual human biting rate of 1,337 and *P. falciparum* EIR of 14.2 infective bites per person per year) [46]. *An. gambiae* s.s. has been the main malaria vector species in Kihihi [46, 48]. Nagongera sub-county (00°46'10.6" N, 34°01'34.1" E), locatedin Tororo District, eastern Uganda, is a rural area bordering Kenya with an elevation of 1,185 m with high malaria transmission (baseline annual human biting rate of 16,606 reported in 2014 and *P. falciparum* EIR of310 infective bites per person per year) [46]. *An. gambiae* s.s. has been described as the main malaria vectorin Tororo [48], however, in 2014 increasing proportions of *An. arabiensis* were documented [46]. Seasonality in Uganda is characterized by alternating rainy and dry seasons and a bimodal rainfall pattern. The longer rainy season occurs between July and November and the shorter rainy season between February and May [33]. During 2011-2016, the primary malaria control interventions deployed in Uganda included artemisinin-basedcombination therapy for treatment of uncomplicated malaria, distribution of LLINs through mass campaigns, and IRS in select districts [5]. LLINs were delivered to Walukuba and Nagongera in November 2013, and to Kihihi in June 2014. In Nagongera, three rounds of IRS with a

carbamate insecticide (bendiocarb) were implemented between December 2014 and June 2015 (1st: December 2014 to Feb 2015, 2nd: June-July 2015, and 3rd: November-December 2015).

Household selection

During the initial enrollment period in 2011, 100 households per site were randomly selected from a list of enumerated of households, as previously described [44]. In 2013, additional households were enrolled to replace households that had dropped out of the study to increase the number of enrolled households back to 100 per site (Figure 5.2).

Mosquito collections

Mosquitoes were collected monthly from cohort study households using miniature CDC light traps (Model 512; John W. Hock Company, Gainesville, FL, USA) set at 19:00 h and collected the following morning at 07:00

h. One trap was set per household each month from October 2011 to June 2016. Light traps were positioned indoors, 1 m above the ground at the foot end of the bed, next to a study participant, sleeping under a long-lasting insecticidal net (LLIN) [46]. Data were excluded from analysis if the target occupant did not sleep in the selected room or if the light trap was faulty.

Mosquito species identification

All anophelines collected were scored morphologically under dissecting microscopes at the study sites usingtaxonomic keys [21, 49]. A subset of 30-50 mosquitoes was randomly selected per month per site for the entire study period for purposes of identifying members of the *An. gambiae* s.l. species complex using PCR [50]. The *An. funestus* s.l. species complex was not processed beyond morphological identification due to resource limitations (henceforth referred to as *An. funestus*). Results from the species identification were extrapolated to the total dataset to establish the species composition of all

Anopheles collected at each siteevery month. Approximately, 10% of the *Anopheles* collected were non-malaria transmitting *An. christyi*, classified as 'other *Anopheles* species' and were not processed further.

Data management and analysis

Field entomologists recorded CDC light trap data on standardized forms. The data collection forms were double-entered into a Microsoft Access database and checked for discrepancies. Any subsequent inconsistencies were resolved using original data entry forms. Statistical analysis was done using Stata (version 14.2, Stata Corp, College Station, TX, USA).

The primary independent variables investigated were; seasonality (dry versus wet season) and the combinedvector control interventions (pre-intervention versus post-intervention). The outcomes of interest were vector density and species composition. Seasonality, denoted by rainy and dry seasons was generated for each site independently. For each site, the same consecutive months were divided into 2 rainy seasons and2 dry seasons over 1 calendar year. Months with rainfall above and below the median value for the entire observation period were classified as rainy or dry season, respectively, after including a 1-month lag period.Vector density was determined by the number of mosquitoes collected per household per month per site and stratified by seasonality and the period before intervention implementation versus the period after intervention implementation. Simple proportions were compared using a log-binomial regression model with generalized estimating equations to adjust for repeated measures from the same house.

Here, we expand on the PRISM1 results previously reported by Kilama et al. [46] from observations carried out over 12 months (October 2011 to September 2012), by describing species-specific changes in response to vector control interventions carried out over 57 months (October 2011 to June 2016). Musiime et al. alsoused PRISM1 data to examine the impact of vector control interventions on *Anopheles* mosquito composition in Nagongera only, as measured using indoor and outdoor human landing

catches [10]. This study analyzes mosquitoes collected indoors using CDC light traps using longitudinal sampling in the three study sites. The PRISM1 dataset can be accessed at https://clinepidb.org/ce/app/record/dataset/DS_0ad509829e.

Ethical Approval and Consent

In each study site, the head of household or adult representative was approached for consenting before household recruitment. A written informed consent was obtained as permission to conduct CDC light trap collections within the household. The study was approved by the Uganda National Council for Science and Technology (HS-119ES), Makerere University School of Medicine Research and Ethics Committee (2017-099),the University of California, San Francisco Committee on Human Research (17-22544) and London School ofHygiene and Tropical Medicine Ethics Comittee (14266-6).

Results

Total *Anopheles* mosquitoes collected

From October 2011 to June 2016, 16,002 light trap collections were performed monthly across the three study sites. Overall, 158,095 *Anopheles* mosquitoes were collected, including 4,640 (3%) from Walukuba, 18,474 (12%) from Kihihi, and 134,981 (85%) from Nagongera (Table 5.1, Figure 5.2). The number of *Anopheles* mosquitoes collected per household per night (vector density) varied across the sites from 0.89 in Walukuba to 25.11 in Nagongera (Table 5.1). Overall, *An. arabiensis* (n=2,391) was the predominant malaria vector species in Walukuba accounting for 52% of all collections. In Kihihi, nearly all *Anopheles* collected (98%) were *An. gambiae* s.s (n=18,135), while in Nagongera, 65% were *An. gambiae* s.s (n=87,936)(Table 5.1). Of the 1,413 'other' *Anopheles* species collected in the sites, 1,385 (98%) were identified morphologically as *An. christyi,* which is classified as a non-malaria vector [51]. There is historical evidence that *An. christyi* has the ability to transmit malaria parasites [52],

however, subsequent reports argue that this ability was either lost or suppressed independently [51] and is thus now considered to be a non-malariavector. As expected, more *Anopheles* mosquitoes were collected during rainy seasons, compared to the dryseasons (Table 5.2).

Trends in *Anopheles* mosquitoes in Walukuba

In Walukuba, the rainy season was associated with approximately a three-fold increase in vector density forall three main vectors, including *An. gambiae* s.s. (density ratio [DR] 3.21, 95% confidence interval [CI]: 2.15-4.79), *An. arabiensis* (DR 2.84, 95% CI: 1.87-4.32) and *An. funestus* (DR 2.57, 95% CI: 1.36-4.88; Table 5.2).

Following LLIN distribution, approximately a 3-fold decline in *An. funestus* vector density (DR 0.34, 95% CI: 0.18-0.65; Table 5.2) was observed in Walukuba. The density of *An. gambiae* s.s or *An. arabiensis* following distribution of LLINs was similar to levels before deployment (Table 5.2). This corresponded with the pattern of distribution observed in the graphical plots examining the absolute numbers of *Anopheles* collected in Walukuba (Figure 5.3a) and the relative proportions (Figure 5.4a) of mosquito species.

Trends in *Anopheles* mosquitoes in Kihihi

In Kihihi, the rainy season was associated with over a five-fold increase in *An. gambiae* s.s density (DR 5.56, 95% CI: 3.90-7.92) compared to the dry season. Insufficient numbers of both *An. arabiensis* and *An. funestus*were collected however, precluding further analysis. LLIN distribution in this area was associated with a decrease in *A. gambiae* s.s. vector density (DR 0.68, 95% CI: 0.49-0.94). This observation is supported by thelongitudinal patterns for absolute numbers of *Anopheles* mosquitoes collected per household (Figure 5.3b). When focusing only on trends in relative proportions of *Anopheles* over time, however, this finding is not obvious (Figure 5.4b).

Trends in Anopheles mosquitoes in Nagongera

In Nagongera, there were substantially more *An. gambiae* s.s. (DR 12.2, 95% CI: 7.05-21.3) and *An. arabiensis* (DR 7.75, 95% CI 4.21-14.3) during the rainy season, but no significant difference was observed for *An. funestus* (DR 1.61, 95% CI: 0.97-2.66). LLINs were associated with a significant decrease in vector density for *An. gambiae* s.s. (DR 0.40, 95% CI: 0.21-0.73) and *An. arabiensis* (DR 0.36, 95% CI 0.18-0.72), but not *An. funestus* (DR 0.61, 95% CI: 0.36-1.04). In Nagongera, three rounds of IRS with bendiocarb were delivered following LLIN distribution. The first round of IRS was associated with a 20-fold decline in *An. gambiae* s.s. vector density compared to the pre-LLIN period (DR 0.05, 95% CI: 0.02-0.16), while the impact on *An. funestus* was close to elimination (DR 0.02, 95% CI: 0.008-0.06). There was no difference in *An. arabiensis* densities before and after the first round of IRS (DR 0.33, 95% CI: 0.10-1.09). The 2nd and 3rd rounds of IRS (combined) were associated with further declines in vector density for both *An. gambiae* s.s. (DR 0.004, 95%CI: 0.002-0.009), and *An. funestus* (DR 0.001, 95% CI: 0.0005-0.004), but a less pronounced decline was observed in *An. arabiensis* vector density (DR 0.15, 95% CI: 0.07-0.33). In contrast to Walukuba and Kihihi, substantial reductions in the absolute numbers of *An. gambiae* s.s. and *An. funestus* s.l. were observed following the addition of IRS to LLINs (Figure 5.3c). The absolute number of *An. arabiensis* changed less after the introduction of the mass vector control measures, and, as a result, the relative proportion of *An. arabiensis* increased markedly as the populations of *An. gambiae* s.s. and *An. funestus* collapsed, with *An. arabiensis* left as the predominant species after IRS (Figure 5.4c).

Discussion

Over the past 13 years (2007-2020), vector control interventions have been scaled-up substantially across Uganda. Whilst the impact of LLINs and IRS on epidemiological outcomes has been assessed routinely [4, 5, 7, 32, 53, 54], the effect of these interventions on malaria vector species is less commonly

investigated. Here,we characterized vector species composition and density in three epidemiologically diverse settings from 2011 to 2016 while vector control interventions were implemented across the country by the Uganda Ministry of Health (National Malaria Control Division).

As expected, we found that *Anopheles* densities were higher during the rainy season in all study sites, consistent with other studies [48, 55]. Prior to the widespread implementation of vector control interventions, *Anopheles* species were sympatric but composition varied between the sites, with *An. arabiensis* predominant in Walukuba (the lowest transmission site) and *An. gambiae* s.s. predominant in both Kihihi and Nagongera (the moderate and high transmission sites respectively). Delivery of LLINs was associated with significant declines in vector density for *An. funestus* in Walukuba, *An. gambiae* s.s. in Kihihiand in both *An. gambiae* s.s. and *An. arabiensis* in Nagongera. Addition of IRS to LLINs in Nagongera was associated with a decline in all vector species, albeit with a greater impact on *An. gambiae* s.s. and *An. funestus,* as reported elsewhere [56, 57]. Consequently, An. *arabiensis* became the predominant species in this area. Understanding the impact of vector control interventions on local malaria vector species is paramount for assessing gaps in current vector control tools.

Malaria vector control interventions, mainly LLINs and IRS have been associated with changes in sympatric *Anopheles species* composition in Uganda [10], and elsewhere in East Africa [11, 39, 43]. However, a shift invector species composition and a decline in vector numbers has also been reported in absence of systematic vector control in north-east Tanzania [58, 59], which underscores the possibility of other causes for these changes such as epidemics of mosquito pathogens, improvements in housing, and changes in climate and land use. Inherent differences in malaria vector ecological characteristics [25], host preference [17], and exophagic and exophilic behavior [29, 60, 61], could be a threat to vector control especially for *An. arabiensis*[41]. *An. arabiensis* is considered to have a lower vectorial capacity than *An. gambiae* s.s. and *An. funestus* in parts of East Africa [38]. In other settings, however, where *An. arabiensis* is the principal vector, evidenceof strong anthropophagic behavior and

outdoor malaria transmission have been described [60]. The opportunistic feeding behavior of *An. arabiensis*, enables this species to avoid contact with LLINs and walls sprayed with insecticides which are applied indoors [27, 60, 62, 63]. Empirical evidence shows that highly anthropophilic malaria vectors such as *An. gambiae* s.s. and *An. funestus* s.s. are more responsive to vector control, particularly IRS programs [10, 39, 42]. A shift in biting patterns of *An. funestus*, however, including early morning biting [37, 64], and broad daytime biting [65], following introduction of LLINs has been documented.

Current vector control tools target highly anthropophagic and endophilic behavior [63]. However, there is growing evidence of outdoor biting especially in *An. arabiensis* [62, 66], which poses a threat to vector control. A similar study, within the study area in Nagongera found a high proportion of *An. arabiensis* bitingoutdoors [10]. In this study, the combination of LLIN and IRS had a lower impact on *An. arabiensis* vector density compared to *An. gambiae* s.s. and *An. funestus*, making it the predominant malaria vector post-

intervention. The impact of this apparent increase in *An. arabiensis* vector density on malaria transmission remains unclear, however. A similar study in Nagongera showed limited malaria transmission despite relatively abundant *An. arabiensis* [10]. In Kenya, there was a decline in malaria transmission following increased LLIN coverage, coincident with the replacement of primary malaria vectors, *An. gambiae s.s* and *An. funestus* by *An. arabiensis* [39]. It is plausible that *An. arabiensis* may maintain residual transmission until the primary malaria vectors *An. gambiae* s.s. or *An. funestus* 'bounce back'. This occurred in western Kenya, where previously dominant *An. funestus* was suppressed following long term use of LLINs, but then recovered, becoming the predominant vector again within a period of almost 20 years, possibly due to highlevels of pyrethroid resistance in this species [67]. In a key example of vector control failure in Kwazulu Natal,previously 'eliminated' *An. funestus* was replaced by less endophilic *An. arabiensis*, but returned after almost40 years, highly resistant to pyrethroids, and associated with a malaria resurgence in this area [68].

Outdoor biting behavior of *An. arabiensis* poses a challenge to malaria vector control. Larval source management with microbial larvicides combined with LLINs has been shown to be protective against malariainfections in rural Kenya [69], and there are several measures including treating cattle with insecticide [60],use of odor-baited traps dispensing spatial repellents [70], and transfluthrin-treated chairs and ribbons [71], which could be deployed as control interventions in the future. In Uganda, there is still an information gap regarding the zoophilic behavior of *An. arabiensis* and host choice in the presence of animals and humans. There is need for further research to assess the efficacy of interventions for controlling *An. arabiensis*.

This study had several limitations. First, we present findings from three sub-counties from only three districts. Thus, our study has limited geographical scope and the results may not be generalizable to other

settings. We did, however, select sites representing markedly different transmission settings, and all mosquito collections were made from randomly selected households after enumeration. Second, only indoor mosquito collections were done using light traps. Therefore, these results are subject to inherent biases presented by the mosquito trapping method used. Third, species-specific sporozoite data were not collected, therefore implications to malaria control regarding residual transmission are implied.

Anopheles species composition may change from highly anthropophagic to less anthropophagic malaria vectors in response to vector control. However, the implications of these shifts in species composition on malaria transmission and control programmes are not well understood and require an in-depth examinationof *Anopheles* species specific contribution to local malaria transmission. We found that LLINs and IRS effected vector densities and species composition differently in different settings. Measuring absolute numbers of mosquitoes to quantify the impact of interventions instead of relying on relative

proportions is important inorder to understand the full picture.

In areas of low- and moderate- malaria transmission large-scale deployment of LLINs resulted in substantial reductions in *An. gambiae* s.s. and *An. funestus* s.l. In the area of intense malaria transmission, the introduction of LLINs and IRS, resulted in the near collapse of these main vectors, with *An. arabiensis* becoming the principal vector, but at lower densities than prior to wide-scale vector control. Current vector control interventions are effective against malaria, but will not lead to elimination of the disease unless additional tools are included as supplementary interventions. Larval source management using chemical or microbial larvicides, combined with environmental management, could be used to improve control, especially in areas of high transmission. Development of interventions targeted at outdoor biting remains apriority.

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Figure legends

Figure 5.1: Map of Uganda showing study site location. Grey dots show location of households sampled for CDC light trap collections in the PRISM cohort (Programme for Resistance, Immunology, Surveillance and Modelling of Malaria). *Image from kigozi et al, [45]*

Figure 5.2: Study profile of Walukuba, Kihihi and Nagongera sub counties.

Figure 5.3: Absolute numbers of *An. gambiae* s.s (blue line), *An. arabiensis* (red line) and *An. funestus* s.l. (yellow line), collected per month in the three study sites. The grey line shows the rainfall pattern, the grey bar depicts Long Lasting Insecticidal Net (LLIN) distribution and the green bars depict Indoor Residual Spraying (IRS) deployment.

Figure 5.4: Relative numbers / proportion of *An. gambiae* s.s (blue), *An. arabiensis* (red) and *An. funestus* s.l. (yellow), collected per month in the three study sites

Table 5.1: Characteristics of sites and collections

	Walukuba	Kihihi	Nagongera
District	Jinja	Kanungu	Tororo
Entomological Inoculation Rate (EIR) [1]	3.2	14.2	310.0
Transmission intensity at baseline	Low	Medium	High
Households sampled (N)	5,212	5,414	5,376
Total Anopheles collected (n)	4.640	18,474	134,981
Vector density	0.89	3.41	25.11
Mosquito collections			
***An. gambiae* s.s. (n, %)**	1736 (37%)	18,135 (98%)	87,936 (65%)
***An. arabiensis* (n, %)**	2391 (52%)	117 (0.6%)	32,485 (24.2%)
***An. funestus* s.l. (n, %)**	234 (5%)	115 (0.6%)	13,533 (10%)
Other Anopheles (n, %)	279 (6%)	107 (0.6%)	1,027 (0.8%

[1] *Infectious bites per person per year*

Table 5.2: Stratified analysis of vector density (density ratio) by seasonality and intervention period

Study site	Variable	Categories	*An. gambiae s.s.*			*An. arabiensis*			*An. funestus*		
			Vector density	DR (95% CI)	p-value	Vector density	DR (95% CI)	p-value	Vector density	DR (95% CI)	p-value
Walukuba[1]	Seasonality	Dry seasons[a]	0.16	reference		0.22	reference		0.02	reference	
		Rainy seasons[b]	0.49	3.21 (2.15-4.79)	<0.001	0.67	2.84 (1.87-4.32)	<0.001	0.06	2.57 (1.36-4.88)	0.004
	Intervention Period	Before LLINs[c]	0.34	reference		0.58	reference		0.07	reference	
		After LLINs[d]	0.33	1.29 (0.86-1.93)	0.21	0.35	0.85 (0.56-1.30)	0.45	0.02	0.34 (0.18-0.65)	0.001
Kihiihi[2]	Seasonality	Dry seasons[e]	0.78	reference		Insufficient number of *An. arabiensis* collected			Insufficient number of *An. funestus s.l* collected		
		Rainy seasons[f]	4.50	5.56 (3.90-7.92)	<0.001						
	Intervention Period	Before LLINs[g]	4.00	reference		Insufficient number of *An. arabiensis* collected			Insufficient number of *An. funestus s.l* collected		
		After LLINs[h]	2.46	0.68 (0.49-0.94)	0.02						
Nagongera[3]	Seasonality	Dry seasons[i]	2.92	reference		1.54	reference		2.24	reference	
		Rainy seasons[j]	25.4	12.2 (7.05-21.3)	<0.001	9.06	7.75 (4.21-14.3)	<0.001	2.70	1.61 (0.97-2.66)	0.06
	Intervention Period	Before LLINs[k]	28.0	reference		9.18	reference		3.90	reference	
		After LLINs[l]	11.2	0.40 (0.21-0.73)	<0.003	3.65	0.36 (0.18-0.72)	0.004	2.56	0.61 (0.36-1.04)	0.07
		After 1st Round of IRS[m]	2.71	0.05 (0.02-0.16)	<0.001	5.44	0.33 (0.10-1.09)	0.07	0.10	0.02 (0.008-0.06)	<0.001
		After 2nd Round of IRS[n]	0.17	0.004 (0.002-0.009)	<0.001	2.00	0.15 (0.07-0.33)	<0.001	0.006	0.001 (0.0005-0.004)	<0.001

[1]Low malaria transmission, [2]Moderate malaria transmission, [3]High malaria transmission. [a] February-April, July-September; [b] May-June, October-January; [c] Oct 2011-Nov 2013; [d] Dec 2013-June 2016; [e] January-February, July-August; [f] March-June, September-December; [g] Oct 2011-June 2014; [h] July 2014-June 2016; [i] January-March, August-September; [j] April-July, October-December; [k] Oct 2011-Nov 2013; [i] Dec 2013-Feb 2015; [m] March 2015-June 2015; [n] July 2015-June 2016

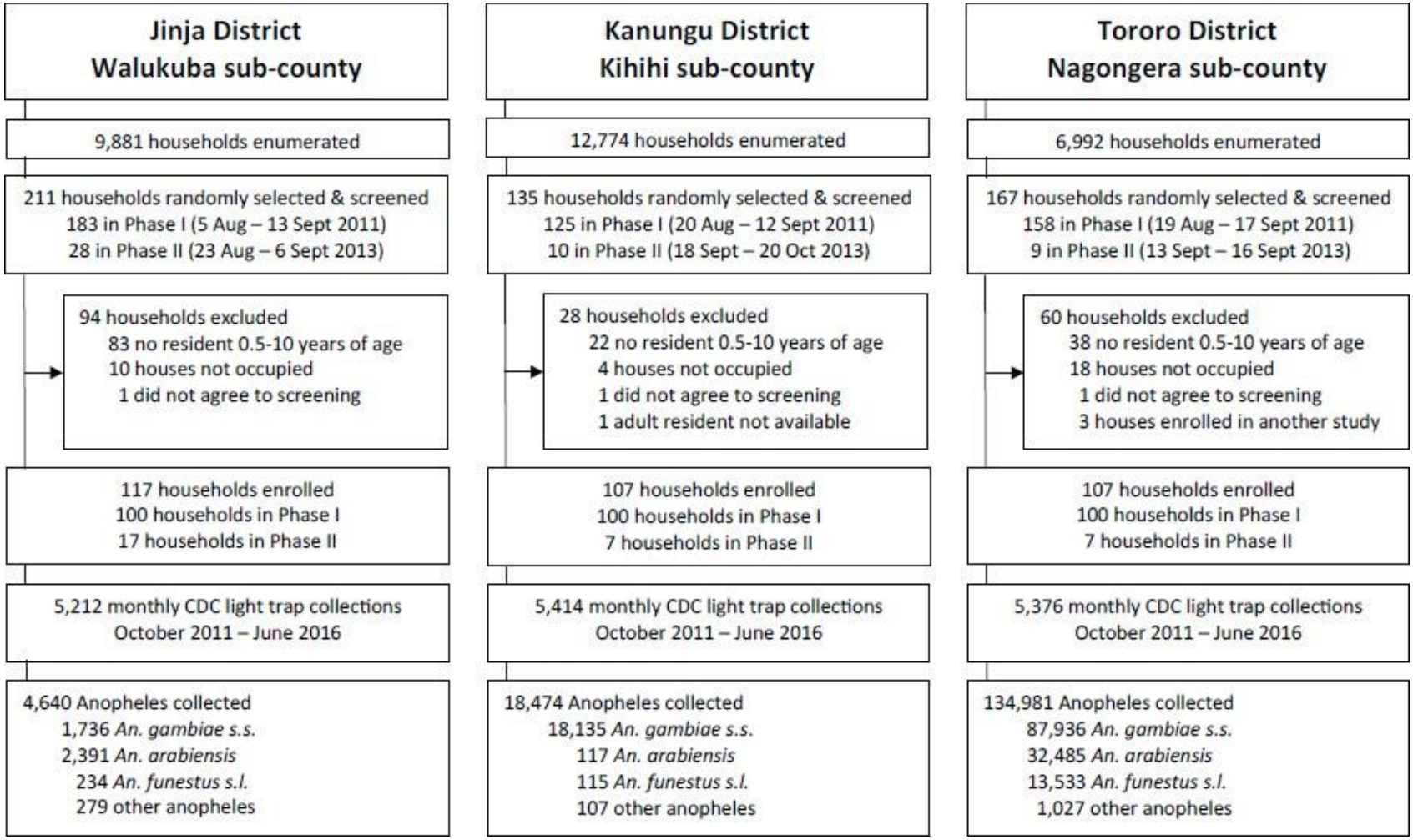

Figure 5.2: Study profile of Walukuba, Kihihi and Nagongera sub counties

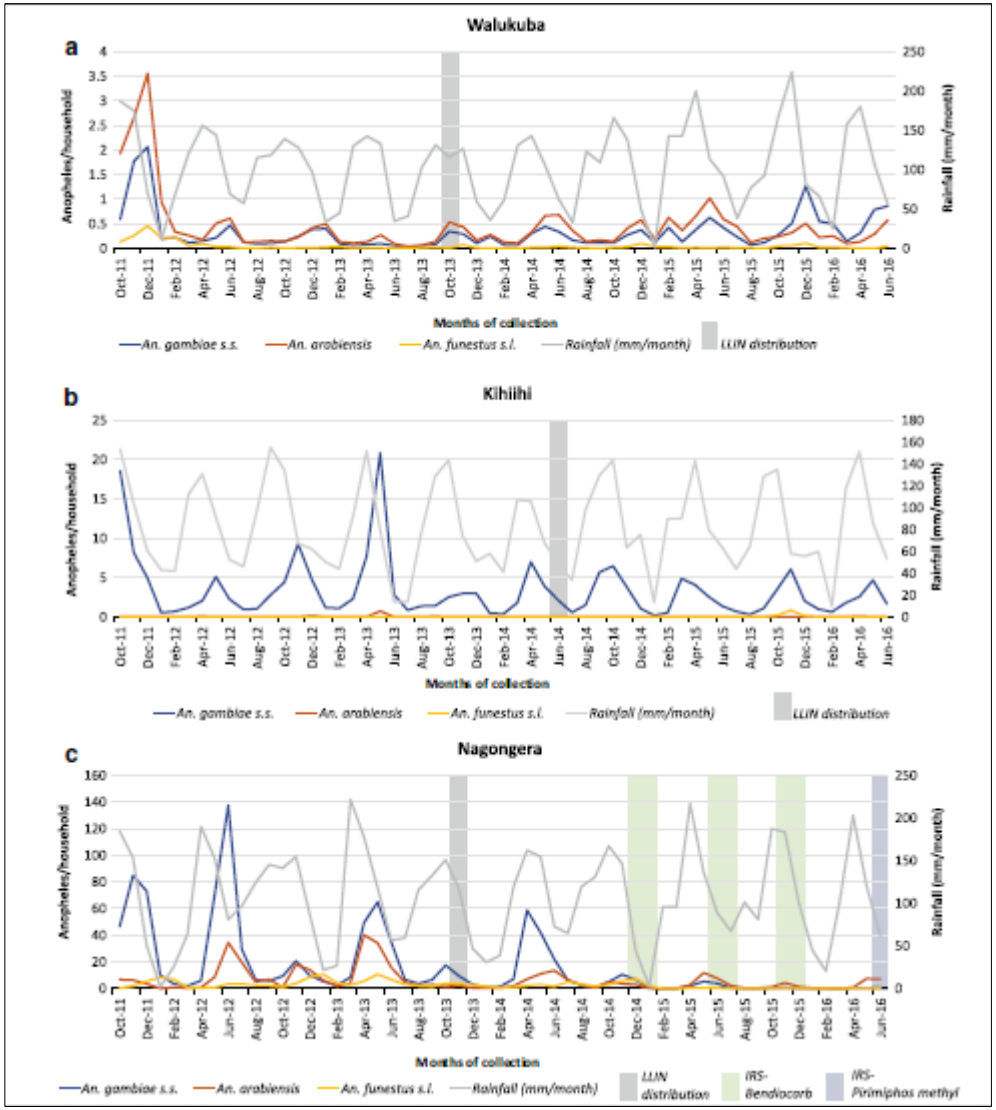

Figure 5.3: Absolute numbers of *An. gambiae* s.s (blue line), *An. arabiensis* (red line) and *An. funestus* s.l. (yellow line); collected per month in the three study sites. The grey line shows the rainfall pattern, the gray bar depicts Long Lasting Insecticidal Net (LLIN) distribution and the green bars depict Indoor Residual Spraying (IRS) deployment

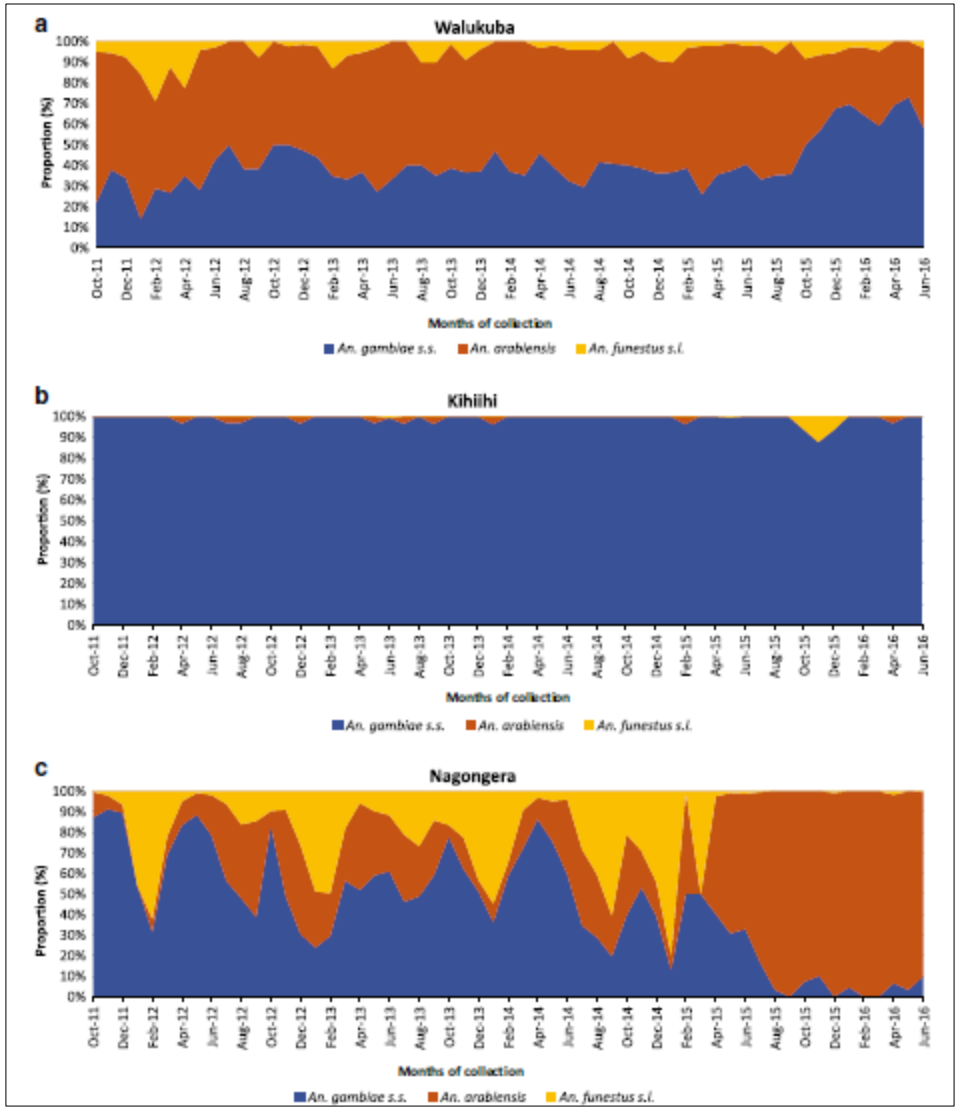

Figure 5.4: Relative numbers / proportion of *An. gambiae* s.s (blue line), *An. arabiensis* (red line) and *An. funestus* s.l. (yellow line); collected per month in the three study sites.

CHAPTER 6 CHARACTERIZING PYRETHROID RESISTANCE AND MECHANISMS IN *ANOPHELES GAMBIAE* S.S. AND *ANOPHELES ARABIENSIS*

6.1 Chapter Introduction

This chapter addresses objectives 3 and 4, to determine insecticide susceptibility of malaria vectors in areas with differing vector control interventions and to evaluate the association between genetic polymorphismsand mosquito survival in pyrethroid exposed *Anopheles gambiae* s.l. adults. Objective 3 examines phenotypicresistance, whereas objective 4 examines underlying mechanisms of resistance. In order to present the resistance data comprehensively, the outputs from objective 3 and 4 were combined in the manuscript.

This manuscript therefore presents outcomes of phenotypic assays to permethrin and deltamethrin with orwithout the synergist piperonyl butoxide and examines the underlying target site and metabolic resistance polymorphism. This manuscript was published in the Current Research in Parasitology and Vector-BorneDiseases journal. At the end of the manuscript are Tables showing characteristics of sites and collections, and stratified analysis of vector density. Figures showing the outcomes of phenotypic and genotypic assays.

6.2 Research paper

Below is the research paper cover sheet, followed by details of the manuscript, tables and figures.

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SECTION A – Student Details

Student ID Number	1513692	**Title**	Mr
First Name(s)	Henry Ddumba		
Surname/Family Name	Mawejje		
Thesis Title	Anopheles species composition and insecticide resistance patterns in Uganda		
Primary Supervisor	Prof. Sarah Staedke		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?	Current Research in Parasitology and Vector-Borne Diseases		
When was the work published?	December 2022		
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SECTION E

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Date	15th February 2023

Supervisor Signature	[redacted]
Date	16 Feb 2023

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Characterizing pyrethroid resistance and mechanisms in *Anopheles gambiae* (*s.s.*) and *Anopheles arabiensis* from 11 districts in Uganda

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Abstract

Background: Insecticide resistance threatens recent progress on malaria control in Africa. To characterize pyrethroid resistance in Uganda, *Anopheles gambiae* (*s.s.*) and *Anopheles arabiensis* were analyzed from 11 sites with varied vector control strategies.

Methods: Mosquito larvae were collected between May 2018 and December 2020. Sites were categorized as receiving no indoor-residual spraying ('no IRS', *n* = 3); where IRS was delivered from 2009–2014 and in 2017 and then discontinued ('IRS stopped', *n* = 4); and where IRS had been sustained since 2014 ('IRS active', *n* = 4). IRS included bendiocarb, pirimiphos methyl and clothianidin. All sites received long-lasting insecticidal nets (LLINs) in 2017. Adult mosquitoes were exposed to pyrethroids; with or without piperonyl butoxide (PBO). *Anopheles gambiae* (*s.s.*) and *An. arabiensis* were identified using PCR. *Anopheles gambiae* (*s.s.*) were genotyped for *Vgsc-995S/F*, *Cyp6aa1*, *Cyp6p4- I236M*, *ZZB-TE*, *Cyp4j5-L43F* and *Coeae1d,* while *An. arabiensis* were examined for *Vgsc-1014S/F*.

Results: Overall, 2753 *An. gambiae* (*s.l.*), including 1105 *An. gambiae* (*s.s.*) and 1648 *An. arabiensis* were

evaluated. Species composition varied by site; only nine *An. gambiae* (*s.s*.) were collected from 'IRS active' sites, precluding species-specific comparisons. Overall, mortality following exposure to permethrin and deltamethrin was 18.8% (148/788) in *An. gambiae* (*s.s*.) and 74.6% (912/1222) in *An. arabiensis*. Mortality was significantly lower in *An. gambiae* (*s.s*.) than *An. arabiensis* in 'no IRS' sites (permethrin: 16.1 *vs* 67.7%, $P < 0.001$; deltamethrin: 24.6 *vs* 83.7%, $P < 0.001$) and in 'IRS stopped' sites (permethrin: 11.3 *vs* 63.6%, $P < 0.001$; deltamethrin: 25.6 *vs* 88.9%, $P < 0.001$). When PBO was added, mortality increased for *An. gambiae* (*s.s*.), and *An. arabiensis*. Most *An. gambiae* (*s.s*.) had the *Vgsc-995S/F* mutation (95% frequency) and the *Cyp6p4-I236M* resistance allele (87%), while the frequency of *Cyp4j5* and *Coeae1d* were lower (52% and 55%, respectively).

Conclusions: Resistance to pyrethroids was widespread and higher in *An. gambiae* (*s.s*.). Where IRS was active, *An. arabiensis* dominated. Addition of PBO to pyrethroids increased mortality, supporting deployment of PBO LLINs. Further surveillance of insecticide resistance and assessment of associations between genotypic markers and phenotypic outcomes are needed to better understand mechanisms of pyrethroid resistance and to guide vector control.

Keywords: *Anopheles gambiae*; *Anopheles arabiensis*; Pyrethroid resistance; Triple mutation; Piperonyl butoxide (PBO)

1. Introduction

Remarkable progress in malaria control has been achieved over the past two decades following the scale-up of vector control interventions including long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) (Bhatt et al., 2015; Cibulskis et al., 2016; WHO, 2021). Nearly 70% of clinical malaria cases averted between 2000 and 2015 were attributed to use of LLINs (Bhatt et al., 2015). LLINs have been shown to reduce parasite prevalence, malaria morbidity, and malaria mortality in children (Kleinschmidt et al., 2018; Pryce et al., 2018); more recently, use of LLINs in early childhood has been associated with better survival outcomes through adulthood (Fink et al., 2022). In Uganda, LLINs serve as the backbone of malaria control, and mass campaigns are conducted every 3–4 years to distribute LLINs nationwide, supplemented by a targeted IRS program (Uganda National Malaria Control Division, 2019). IRS conducted in high-transmission areas has also been very effective (Katureebe et al., 2016; Nankabirwa et al., 2020; Namuganga et al., 2021). Various non-pyrethroid insecticides have been deployed, including bendiocarb (a carbamate), pirimiphos-methyl (an organophosphate) and clothianidin (a neonicotinoid), all with differing modes of action found to be suitable alternatives to pyrethroids (Akogbéto et al., 2010; Agossa et al., 2014; Fongnikin et al., 2020). However, the substantial benefits of LLINs and IRS are threatened by widespread insecticide resistance in Uganda (Mawejje et al., 2013; Mulamba et al., 2014; Okia et al., 2018; Tchouakui et al., 2021), and elsewhere (Ochomo et al., 2013, 2014; Yipmo et al., 2022). The long-term application of insecticides for public health (WHO, 2021) and control of agricultural pests (Nkya et al., 2014) has increased selection pressure on malaria vectors (Lines, 1988; Nauen, 2007; Ranson & Lissenden, 2016; Mathias et al., 2011), driving the development and spread of insecticide resistance (Mathias et al., 2011; Ranson et al., 2011; Ranson & Lissenden, 2016; Hancock et al., 2020; Wat'senga et al., 2020). Conventional LLINs prequalified by the World Health Organization (WHO) rely on pyrethroid insecticides, including permethrin and deltamethrin, which are favored because of low mammalian toxicity (WHO, 1999), excito-repellency, (Elliott et al., 1978; WHO, 2011) and relatively low cost

compared to alternative insecticides (Hancock et al., 2020). Mosquitoes with relevant resistance mutations are more likely to survive if exposed to insecticides, thus extending their lifespan and the likelihood of transmitting malaria parasites (Verhaeghen et al., 2010; Kabula et al., 2016). Pyrethroid resistance has been shown to compromise vector control (Kigozi et al., 2012; Toé et al., 2014; Hargreaves et al., 2000), although the impact of insecticide resistance on malaria metrics is less conclusive (Kleinschmidt et al., 2018). Widespread resistance to pyrethroids has been reported across sub-Saharan Africa (Hancock et al., 2020; Lissenden et al., 2021), including in Uganda (Verhaeghen et al., 2006, 2010; Ramphul et al., 2009; Mawejje et al., 2013; Okia et al., 2013, 2018; Katureebe et al., 2016). To combat the spread of pyrethroid resistance, newer generation LLINs have been developed, which incorporate additional chemicals into the nets, such as piperonyl butoxide (PBO), a synergist (WHO, 2017; Protopopoff et al., 2018; Staedke et al., 2020; Gleave et al., 2021), pyriproxyfen, an insect growth regulator (Tiono et al., 2018; Ngufor et al., 2020), and chlorfenapyr, a pyrrole insecticide (Mosha et al., 2022). Initial studies of these dual active-ingredient nets are promising (Mosha et al., 2022). Current WHO guidelines on malaria control (WHO, 2022) recommend deployment of PBO-LLINs in areas with pyrethroid resistance and strategic co-deployment of LLINs and non-pyrethroid IRS, as a strategy to limit insecticide resistance (WHO, 2014, 2015, 2022). Further evidence of the impact of combining LLINs with IRS using non-pyrethroid insecticides on malaria burden and the selection for pyrethroid resistance is needed.

Resistance to pyrethroids is primarily mediated by changes in the voltage-gated sodium channel (*Vgsc*) (Ranson & Lissenden, 2016), which serves as the target site for these insecticides, and through metabolic mechanisms (Donnelly et al., 2009). Non-synonymous point mutations in *Vgsc*, commonly referred to as knockdown resistance (*kdr*) (Martinez-Torres et al., 1998), most commonly involve either an *L995S* (Ranson et al., 2000) or *L995F* (Martinez-Torres et al., 1998) mutation (numbering for *An. gambiae* (*s.s.*); the orthologous codon in *An. arabiensis* is *1014*). Both mutations have been described previously in Uganda, with the *L995S* mutation at greater frequency (Verhaeghen et al., 2006; Mawejje et al., 2013;

Okia et al., 2013, 2018; Lynd et al., 2019). Metabolic resistance in *An. gambiae* (*s.s*.) is often associated with changes in cytochrome p450 enzymes that potentially increase insecticide detoxification; in Uganda these include *Cyp4j5* (Weetman et al., 2018), *Cyp6p4* and an associated 'Zanzibar-like' transposable element (*ZZB-TE*) (Njoroge et al., 2021), and the *Cyp6aa1*/*Cyp6aap* duplication (Lucas et al., 2019; Njoroge et al., 2021). A carboxylesterase gene (*Coeae1d*) (Weetman et al., 2018) has also been associated with pyrethroid resistance in Uganda and Kenya. Previous analysis of *An. gambiae* (*s.s*.) mosquitoes collected from Uganda and Kenya (Weetman et al., 2018) showed that marker polymorphisms in *Cyp4j5* and *Coeae1d* were found at relatively high frequency (0.61 and 0.53, respectively) and were associated with pyrethroid resistance. In Uganda and parts of the Democratic Republic of the Congo, the *Cyp6aa1* duplication, *Cyp6p4* point mutation and *ZZB-TE* insertion are found at high frequency as a triple-mutant (Njoroge et al., 2021), with the two p450 genes shown to be capable of metabolizing pyrethroids *in vitro* in *An. gambiae* (*s.s*.) None of these mechanisms are known to be associated with resistance to the insecticides (bendiocarb, pirimiphos-methyl and clothianidin) used for recent IRS in Uganda. To further characterize pyrethroid resistance in Uganda and explore patterns associated with non-pyrethroid IRS, we collected *An. gambiae* (*s.s*.) and *An. arabiensis* from 11 districts around Uganda under conditions of varying malaria control, including sites with and without IRS programmes, and analysed them using both phenotypic and genotypic assays.

2.0 Materials and methods

2.1 Study site characteristics

This study was conducted in 11 districts across Uganda (Fig. 6.1). Mubende and Kayunga districts are in the central region (North Buganda sub-region), characterised by forest-savannah mosaic vegetation (Roberts & Ocaya, 2009); prevalence of malaria parasitemia in children aged 0–59 months, as measured by microscopy, was 9% in the 2019 Malaria Indicator Survey (MIS) (Uganda National Malaria Control

Division, 2019). Kole, Otuke, Dokolo and Amolatar districts are found in the Lango sub-region of northern Uganda, which is characterised by short grassland vegetation (Roberts & Ocaya, 2009), and a regional parasite prevalence of 13% in 2019 (Uganda National Malaria Control Division, 2019). Amuru, Lamwo and Agago districts are located Acholi sub-region, also in northern Uganda, bordering South Sudan, with a parasite prevalence of 12% in 2019. Busia and Tororo districts are in Bukedi sub-region in eastern Uganda, bordering western Kenya. This area is characterized by moist savannah vegetation (Roberts & Ocaya, 2009), and parasite prevalence of 3% in 2019 (Uganda National Malaria Control Division, 2019). Previous meteorological data demonstrated that districts in the central and eastern regions experience bimodal rainfall with two peaks, one in March-May and the second in September-December (MOH, 2014), whilst the northern region receives less rainfall, with only one rainy season between March and October (MOH, 2014).

Study sites were stratified by vector control status. In all 11 districts, two mass campaigns were conducted to deliver conventional (pyrethroid only) LLINs in 2013–2014 and in 2017 (Fig. 6.2). 'No IRS' sites (Busia, Mubende and Kayunga) received LLINs only; the Ministry of Health did not implement IRS in these areas. 'IRS stopped' sites (Kole, Amuru, Lamwo, Agago) received LLINs plus annual rounds of IRS from 2009 to 2014, followed by a single round of IRS in 2017. 'IRS active' sites (Otuke, Tororo, Dokolo and Amolatar) received LLINs plus routine IRS from 2014 to 2019 (active at the time of larval sampling). Details of insecticides used are provided in Fig. 6.2 and have also been described elsewhere (Namuganga et al., 2021). Briefly, both 'IRS stopped' and 'IRS active' districts received IRS with two insecticide compounds, namely bendiocarb followed by pirimiphos methyl. Dokolo received IRS with clothianidin, rather than pirimiphos methyl, in 2019 (illustrated in Fig. 6.2).

2.2 Mosquito collections and identification

Mosquito larvae were collected between May 2018 and December 2020 (Fig. 6.2) using the dipping

method (Service, 1993) from a range of breeding sites including man-made pits to excavate sand, brick, or murram, cow watering holes, tyre tracks, stagnant roadside pools, rice fields, and harvested gardens. Larvae were transported to the medical entomology insectary at the Central Public Health Laboratories in Kampala and were raised to adults using finely ground Tetramin fish food. Subsequent adult mosquitoes were identified morphologically using keys (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987) and classified as members of the *Anopheles gambiae* (*sensu lato*) species complex. Subsequent identification of sibling species was done using standard polymerase chain reaction (PCR) protocols (Scott et al., 1993).

2.3 Insecticide susceptibility tests

Assessment of insecticide susceptibility was performed using standard WHO tube bioassays (WHO, 1998, 2016). Adult non-blood-fed female *An. gambiae* (*s.l.*), aged 3–5 days-old were exposed to permethrin or deltamethrin at WHO diagnostic concentrations of 0.75% and 0.05%, respectively. Four replicates of 20–25 mosquitoes were exposed per insecticide for 1 h under temperatures ranging from 23.3 °C to 26.7 °C and relative humidity between 80% and 95%. Mortality was scored 24-h post-insecticide exposure. Mosquito samples were stored individually and preserved using desiccant silica gel for subsequent molecular analysis. For quality control, each assay was run with a control tube of 20–25 mosquitoes containing (standard pyrethroid control) silicone oil papers. Phenotypic data from larvae collected at different sampling points (Fig. 6.2) were pooled within each study site to improve test power.

2.4 Synergist bioassays

To further investigate underlying mechanisms of pyrethroid resistance *via* the synergist PBO, which acts primarily to block detoxification by cytochrome P450 monooxygenases, adult female *An. gambiae* (*s.l.*) were exposed to WHO papers treated with PBO (4%) for 1 h followed by permethrin or deltamethrin exposure for an additional diagnostic period of 1 h. Mortality was scored after 24 h. In control samples,

PBO control papers were used prior to pyrethroid control paper exposure. Mosquito samples were stored singly over silica gel for further molecular analysis.

2.5 Molecular analysis

Genomic DNA was extracted from whole mosquitoes using the DNeasy kit (Qiagen, Hilden, Germany) and used as a template for molecular analyses. The *Vgsc* genotype at codon *1014* (*995* using *An. gambiae* (*s.s.*) numbering) (The *Anopheles gambiae* 1000 Genomes Consortium, 2017) were determined using a locked nucleic allele (LNA) assay, which detects wild type and *kdr* mutants serine or phenylalanine (Lynd et al., 2018). The triple mutation with *Cyp6aa1* duplication, *Cyp6p4-I236M* and *ZZB-TE* (cytochrome p450-linked 'Zanzibar-like' transposable element) was assessed using three independent LNA assays (Njoroge et al., 2021). All assays were run on AriaMx Real-Time PCR machine (Agilent, USA). TaqMan assays were used to genotype *Cyp4j5* and *Coeae1d* (Weetman et al., 2018). TaqMan assays used a primer/probe mix in addition to 1× sensimix (Bioline) and DNA template (1 µl) in a 10 µl volume reaction with denaturing for 5 min at 95 °C, followed by 40 cycles of denaturing for 15 s at 92 °C and annealing for 1 min at 60 °C. The TaqMan assays were performed on an Agilent MX3005P Real-Time PCR machine.

2.6 Statistical analysis

Statistical analysis using Stata (version 14.2, Stata Corp, College Station, TX, USA) generated measures of association (odds ratios) using mixed effects logistic regression, adjusting for repeated observations from the same study site. Key exposure variables were insecticide exposure, IRS status and species status. The primary outcome was mosquito phenotype, assessing whether changes in the exposure resulted in mortality or survival. To examine associations between genotypic markers of resistance and phenotypic outcomes, a logistic regression model was used. The nonsynonymous point mutation *Cyp6p4* was

selected as the marker of reference in the triple mutant haplotype due to the high level of correlation. Data were pooled by site and categorized by IRS status to improve the statistical power of the model. Pyrethroid resistance markers included in the model were *Vgsc-L995S, Vgsc-L995F, Cyp6p4-I236M, Cyp4j5-L43F* and *Coeae1d*.

2.7 Ethical approval

Mosquito collections for this study were approved by the Makerere University College of Health Sciences, School of Medicine research ethics committee (REF: 2018-066), Uganda National Council of Science and Technology (REF: SS 4586), and London School of Hygiene and Tropical Medicine Ethics Committee (LSHTM Ethics Ref: 14584) under protocol study title "*Investigating spatial and localized interactions between insecticide resistance, insecticidal malaria vector control and malaria transmission in Anopheles mosquitoes from Uganda*" and by the School of Biomedical Sciences Research and Ethics Committee (REF: SBS-HDREC-669) and Uganda National Council of Science and Technology (REF: HS 2629) under study title "*Entomological surveillance of vector behaviour, vector density and insecticide resistance to inform malaria vector control in Uganda*."

3.0 Results

3.1 Species composition

Overall, 2753 *An. gambiae* (s.l.) adults were raised from larvae collected in 11 sites were phenotyped for pyrethroid resistance and speciated, including 1105 *An. gambiae* (s.s.) and 1648 *An. arabiensis* (Table 6.1). In the 'no IRS' sites, where vector control was limited to LLINs, the proportion of mosquitoes identified as *An. gambiae* (s.s.) ranged between 33.6–83.8%, while *An. arabiensis* ranged between 16.2–66.4%. In the sites where IRS was stopped 1.8–3.8 years prior to completing larval collections, most mosquitoes were identified as *An. gambiae* (s.s.) at 3 sites (76.5–99.4%), but at one site (Agago) 100%

of mosquitoes were *An. arabiensis*. In the four IRS-active sites, in which IRS had been sustained for at least 3.5 years prior to larval collection, nearly all mosquitoes were identified as *An. arabiensis* (98–100%); only nine *An. gambiae* (s.s.) were collected from sites with active IRS, and these were excluded from subsequent analyses due to the small sample size.

3.2 Phenotypic bioassay results stratified by IRS categories, mosquito species, and insecticides

Anopheles gambiae (*s.s.*) and *An. arabiensis* were exposed to diagnostic concentrations of permethrin and deltamethrin, and mortality was measured (Supplementary Table S1). Overall, mortality of *An. gambiae* (*s.s.*) following exposure to pyrethroids was low, indicating high prevalence of resistance: 12.9% (53/411) for permethrin and 25.2% (95/377) for deltamethrin. Mortality of *An. arabiensis* was high, indicating greater susceptibility to pyrethroids at 65.5% (402/614) for permethrin and 82.4% (510/619) for deltamethrin. Phenotypic assay results were pooled and compared between IRS category, species, and insecticide (Table 6.2). When different IRS category sites were compared, no significant difference in mortality was observed after exposure to either permethrin and deltamethrin, for either *An. gambiae* (*s.s.*) or *An. arabiensis*. When mosquito species were compared, mortality after exposure to both permethrin and deltamethrin was significantly lower for *An. gambiae* (*s.s.*) than *An. arabiensis* in both 'no IRS' (16.1 *vs* 67.7%, $P < 0.001$ for permethrin; 24.6 *vs* 83.7%, $P < 0.001$ for deltamethrin) and 'IRS stopped' sites (11.3 *vs* 63.6%, $P < 0.001$ for permethrin; 25.6 *vs* 88.9%, $P < 0.001$ for deltamethrin). In the 'IRS active' sites, the limited number of *An. gambiae* (*s.s.*) precluded species-specific comparisons. When the two pyrethroids were compared, *An. gambiae* (*s.s.*) mortality was significantly lower following exposure to permethrin than to deltamethrin in 'IRS stopped' sites (11.3 *vs* 25.6%, $P = 0.001$), but not in 'no IRS' sites (16.1 *vs* 24.6%, $P = 0.10$). For *An. arabiensis*, mortality was significantly lower following exposure to permethrin than to deltamethrin in 'no IRS' sites (67.7 *vs* 83.7%, $P = 0.002$), 'IRS stopped' sites (63.6 *vs* 88.9%, $P < 0.001$), and 'IRS active' sites (65.4 *vs* 79.2%, $P < 0.001$).

3.3 Synergist bioassays with piperonyl butoxide

Overall, when *An. gambiae* (*s.s.*) were exposed to the synergist PBO, mortality to both pyrethroids increased (Supplementary Table S1); for permethrin from 12.9% (53/411) to 56.5% (96/170), and for deltamethrin from 25.2% (95/377) to 68.7% (101/147). In *An. arabiensis*, mortality following PBO exposure also increased, from 65.5% (402/614) to 93.3% (195/209) for permethrin, and from 82.4% (510/619) to 89.8% (185/206) for deltamethrin. In the 'no IRS' sites, mortality of *An. gambiae* (*s.s.*) was significantly higher when PBO was added compared to that with the pyrethroid alone (permethrin: 54.5 *vs* 16.1%, $P < 0.001$; deltamethrin: 55.6 *vs* 24.6%, $P < 0.001$), indicating at least partial restoration of susceptibility to both permethrin and deltamethrin by PBO (Table 6.3). Similar results were observed in the 'IRS stopped' sites (permethrin: 57.0 *vs* 11.3%, $P < 0.001$; deltamethrin: 78.6 *vs* 25.6%, $P < 0.001$). When *An. arabiensis* from the 'no IRS' sites were exposed to PBO, mortality increased slightly, but not significantly, with permethrin (82.0 *vs* 67.7%, $P = 0.36$). Unexpectedly, mortality following exposure to PBO and deltamethrin was significantly lower compared to that with deltamethrin alone (66.0 *vs* 83.7%, $P = 0.01$). When *An. arabiensis* from the 'IRS stopped' sites were exposed to PBO, mortality increased to 100% for both permethrin and deltamethrin, but statistical significance could not be determined because all *An. arabiensis* died and comparisons could not be made. In the 'IRS active' sites, mortality of *An. arabiensis* increased significantly when PBO was added to both permethrin (96.5 *vs* 65.4%, $P < 0.001$) and deltamethrin (96.0 *vs* 79.2%, $P < 0.001$).

3.4 Molecular markers of insecticide resistance in *An. gambiae* s.s.

A subset of *An. gambiae* (*s.s.*) (Supplementary Table S2) were genotyped for molecular markers associated with pyrethroid resistance, including the *kdr* target site mutations *Vgsc-L995S* and *Vgsc-L995F*, and *Cyp6aa1*, *Cyp6p4*, *ZZB-TE*, *Cyp4j5* and *Coeae1d*, associated with metabolic resistance. The frequency of the *Vgsc-L995S* resistance allele was high in the 'no IRS' sites, ranging from 83% in Kayunga

to 96% in Busia, but was low in the 'IRS stopped' sites, ranging from 62% in Lamwo to 74% in Kole (Fig. 6.3, Supplementary Table S3). The frequency of the *Vgsc-L995F* resistance allele was low in *An. gambiae* (*s.s.*), but was highest in the northern 'IRS stopped' sites, ranging from 15% in Kole to 37% in Lamwo (Fig. 6.3, Supplementary Table S3). (a summary of *Vgsc* genotypes in *An. gambiae* (s.s.) is shown in supplementary table S7). Comparison of resistance allele frequencies showed significantly higher *Vgsc-L995F* frequency in the 'IRS stopped' compared to 'no IRS' sites (28.40 *vs* 3.43, Fisher's exact test, *P* = 0.02). There was no significant difference in *Vgsc-L995S* resistance allele frequencies between the 'IRS stopped' and 'no IRS' sites (Supplementary Table S4). A high level of agreement was found between the metabolic resistance markers *Cyp6aa1, Cyp6p4* and *ZZB-TE* (Spearman's rank correlation = 0.72 for *Cyp6p4* and 0.74 for *ZZB-TE* relative to *Cyp6aa1*). Thus, analyses were restricted to *Cyp6p4.* The frequency of the *Cyp6p4-I236M* resistance allele was very high in *An. gambiae* (*s.s.*) from all sites regardless of IRS status, ranging from 80% in Kayunga to 93% in Mubende, while the frequency of *Cyp4j5* and *Coeae1d* ranged from 42% in Amuru to 65% in Kole and from 44% in Mubende to 62% in Amuru respectively (Fig. 6.3, Supplementary Table S3).

3.5 Molecular markers of insecticide resistance in *An. arabiensis*

For *An. arabiensis*, only target-site resistance mutations (*Vgsc-1014S* and *Vgsc-1014F*) were genotyped (Supplementary Tables S5 and S6). *Anopheles arabiensis* were predominantly wild type (*Vgsc-1014L)* for *kdr* (Fig. 6.4); *Vgsc-1014S* was found only in Kayunga (3%) and in Kole (11%), while *Vgsc-1014F* was found in Agago (1%), Lamwo (2%) and Kole (9%). *Vgsc-1014S* was not detected in *An. arabiensis* from the 'IRS active' sites. However, *Vgsc-1014F* was found in a single *An. arabiensis* mosquito in Tororo and in one other *An. arabiensis* mosquito from Amolatar (Supplementary Table S5). (a summary of *Vgsc* genotypes in *An. arabiensis* is represented in supplementary table S8)

3.6 Association between genotypic resistance markers and phenotypic resistance in *An. gambiae* s.s.

Analysis of the associations between genotypic resistance markers and phenotypic resistance in *An. gambiae* (*s.s.*) from 'no IRS' sites (Table 6.4) revealed significant associations between the target site mutations, *Vgsc-L995S*/*F*, and survival when exposed to deltamethrin (odds ratio, OR: 3.44; 95% CI: 1.02–11.57; *P* = 0.046) and between *Cyp4j5* and survival when exposed to deltamethrin + PBO (OR: 2.27; 95% CI: 1.08–4.80; *P* = 0.031). In 'IRS stopped' sites (Table 6.5), significant associations were found between *Cyp6p4* and survival when exposed to permethrin + PBO (OR: 3.19; 95% CI: 1.16–8.80; *P* = 0.025) and when exposed to deltamethrin (OR: 2.27; 95% CI: 1.02–5.05, *P* = 0.045). All other measures of association were found to be non-significant in the 'no IRS' sites (Table 6.4) and 'IRS stopped' sites (Table 6.5).

4.0 Discussion

Resistance to pyrethroid insecticides threatens the effectiveness of malaria vector control. To further characterize pyrethroid resistance in Uganda, we collected *An. gambiae* (*s.l.*) from 11 districts implementing different IRS-based vector control strategies. We found high levels of pyrethroid resistance, particularly in *An. gambiae* (*s.s.*), but in settings where IRS was active, *An. arabiensis* dominated and almost no *An. gambiae* were identified. Combining PBO with a pyrethroid increased mortality for *An. gambiae* (*s.s.*), as well as *An. arabiensis* in some settings, indicating partial restoration of pyrethroid susceptibility and supporting the use of PBO LLINs in Uganda. The underlying genotypes only partially explained the resistance phenotype in *An. gambiae* (*s.s.*), while *An. arabiensis* were predominantly wild type for the target site resistance mutation.

In this study, resistance to permethrin and deltamethrin was widespread. Mortality in phenotypic assays was significantly lower in *An. gambiae* (*s.s.*) than *An. arabiensis* in sites without ongoing IRS. Mortality following exposure to permethrin was significantly lower than to deltamethrin for *An. gambiae* (*s.s.*) in sites where IRS had been stopped (but not in 'no IRS' sites), and for *An. arabiensis* in all sites, suggesting

greater resistance to permethrin (a type I pyrethroid) than to deltamethrin (type II). Most *An. gambiae* (*s.s.*) had *Vgsc-995* target site mutations, while these mutations were uncommon in *An. arabiensis*. The *Cyp6p4-I236M* resistance allele, a marker of metabolic resistance, was also common in *An. gambiae* (*s.s.*), while *Cyp4j5* and *Coeae1d* were less common, present in just over half of *An. gambiae* (*s.s.*) tested. Some associations between genotypic markers of resistance and phenotypic outcomes were observed in *An. gambiae* (*s.s.*), although results were inconsistent, suggesting mechanisms of pyrethroid resistance are complex and insufficiently explained by currently recognized resistance markers.

The target site resistance mutation *Vgsc-995S* was found at very high frequency in *An. gambiae* (*s.s.*), consistent with prior observations in Uganda and Kenya (Okia et al., 2018; Lynd et al., 2019). The presence of the *Vgsc-995F* mutation, which has been associated with a strong resistance phenotype (Reimer et al., 2008), suggests pyrethroid selection pressure, in the study sites. The *Vgsc-995F* mutation has also been noted to confer greater resistance to type I (permethrin) than type II (deltamethrin) pyrethroids (Reimer et al., 2008), which may partially account for the significantly lower *An. gambiae* (*s.s.*) mortality to permethrin compared to deltamethrin observed in the 'IRS stopped' but not in the 'no IRS' sites. However, the very low frequency of this mutation (*Vgsc-L1014F* alternative) in *An. arabiensis*, suggests that the observed difference in insecticide specific mortality may be driven by other resistance mechanisms. The prevalence of the *Vgsc-995F* mutation seems to be increasing in Uganda, since the first report of this mutation at very low frequency in *An. gambiae* (*s.s.*) approximately 15 years ago (Verhaeghen et al., 2006). We found *kdr* mutations (*Vgsc-995S* and *Vgsc-995F*) within the same sample, particularly in *An. gambiae* (*s.s.*) The presence of both mutations (F/S heterozygotes) within the same mosquito is associated with a strong pyrethroid resistance phenotype, similar to that of F/F homozygotes. In *An. arabiensis*, both *kdr* mutations (*L1014S* and *L1014F*) were at relatively low frequency, with most individuals found to be wild type homozygotes, akin to findings elsewhere in Uganda (Mawejje et al., 2013; Lynd et al., 2019). Nevertheless, *kdr* mutations (*Vgsc-L1014S*) in *An.*

arabiensis have been found at frequencies as high as 63% in mosquitoes from Western Kenya (Hemming-Schroeder et al., 2018), neighboring Tororo (IRS active) and Busia (No IRS) districts, and as high as 89.5% in *An. arabiensis* from Dakar, Senegal (Dia et al., 2018).

The recently described mutants *Cyp6aa1*, *Cyp6p4* and *ZZB-TE* (Njoroge et al., 2021) were found to be strongly correlated in *An. gambiae* (*s.s.*), indicating strong, though imperfect linkage disequilibrium and a high frequency of the triple mutant haplotype. The triple-mutant (represented by *Cyp6p4*) suggested strong positive selection in geographically distinct *An. gambiae* (*s.s.*) and was found at a frequency ranging from 80 to 93% in the target sites. This is consistent with observations of *An. gambiae* (*s.s.*) collected in Busia, Uganda and in Kenya (Njoroge et al., 2021). The *Cyp6p4* mutation was associated with resistance to deltamethrin, similar to findings from western Kenya described by Njoroge et al. (2021). However, the association between the triple-mutant and mosquito survival following exposure to permethrin and PBO observed in this study has not previously been described and is unexpected given the expected blocking effects of PBO on P450 enzyme activity (Farnham, 1999). However, Njoroge et al. (2021) found that PBO LLINs were effective against a pyrethroid-resistant colony (from Busia, Uganda) with a triple-mutant frequency of 29.7%. The association between the *Cyp4j5* P450 marker, and mosquito survival following exposure to deltamethrin plus PBO is another novel finding and similarly unexpected, although previous reports have found significant association between *Cyp4j5* and deltamethrin (as well as permethrin) resistance (Weetman et al., 2018) and to our knowledge the marker association's relationship with PBO has not previously been assessed.

Cluster-randomized trials in Uganda (Staedke et al., 2020) and Tanzania (Protopopoff et al., 2018) demonstrated significant declines in mosquito density and parasite prevalence associated with PBO LLINs, supported by the recently revised Cochrane review on PBO LLINs (Gleave et al., 2021). The WHO's Vector Control Advisory Group concluded that PBO LLINs are more effective than pyrethroid-only LLINs in settings of high-level pyrethroid resistance, and the WHO now recommends PBO LLINs for the

prevention and control of malaria in areas where malaria vectors demonstrate substantial pyrethroid resistance (WHO, 2022). As PBO LLINs are scaled-up, surveillance of markers of metabolic resistance will be essential.

We observed differences in the distribution of *An. gambiae* (*s.s*.) and *An. arabiensis* relative to IRS status. In sites with 'no IRS', *An. gambiae* (*s.s*.) and *An. arabiensis* were fairly evenly distributed, in contrast with the predominance of *An. gambiae* (*s.s*.) in 'IRS stopped' sites (apart from Agago) and *An. arabiensis* in 'IRS active' sites. Observed differences in species composition suggested an impact of IRS on malaria vectors, similar to other reports from this region (Musiime et al., 2019). Sustained vector control has previously been associated with changes in *Anopheles* mosquito species composition whereby highly anthropophagic *An. gambiae* (*s.s*.) is replaced by the less anthropophagic *An. arabiensis* (Bayoh et al., 2010; Mwangangi et al., 2013; Mawejje et al., 2021) potentially arising from the tendency of *An. arabiensis* to rest outdoors (Mahande et al., 2007), and behavioral patterns limiting contact with indoor based vector control interventions (Yohannes & Boelee, 2012). Similarly, a study in Tororo (one of the 'IRS active' sites) showed predominant *An. gambiae* (*s.s*.) (up to 77% abundance) prior to IRS, being replaced by *An. arabiensis* after IRS (Musiime et al., 2019). Stopping vector control has been associated with a rebound of primary vector species in some settings (Hargreaves et al., 2000; McCann et al., 2014). Pyrethroid-resistant primary vectors (such as *An. gambiae* (*s.s*.) and *An. funestus*) may have a selective advantage enabling them to overcome pyrethroid-based vector control or less effective non-pyrethroid IRS, resulting in a resurgence of malaria morbidity (Hargreaves et al., 2000). In the 'IRS stopped' district of Agago, in which we recorded predominantly *An. arabiensis*, it is plausible that there were spillover effects from sustained IRS (Namuganga et al., 2021) in the neighboring district of Otuke (Fig. 6.1), with the 'invasion' of *An. gambiae* (*s.s*.) in this district limited by IRS activity in Otuke. The absence of historical data on species composition pre-vector control implementation in the 'IRS stopped' area, however, limits interpretation of the impact of IRS on malaria vector-species composition. This noted, the

consequences of stopping IRS in this region on malaria epidemiology have been associated with a rapid resurgence of the disease to pre-IRS levels (Raouf et al., 2017; Namuganga et al., 2021).

Highly anthropophilic and endophilic mosquitoes (*An. gambiae* (*s.s*.) and *An. funestus*) (Mwangangi et al., 2003) are more likely than zoophilic species (White et al., 1972; Molineaux et al., 1980) to be exposed to LLINs and IRS (Russell et al., 2010). Sympatric populations of *An. gambiae* (*s.s*.) and *An. arabiensis* or *An. funestus*, and zoophilic *An. rivulorum* (Kawada et al., 2012) have often revealed differential levels of mortality to insecticides in *An. gambiae* (*s.s*.) or *An. funestus* compared to *An. arabiensis* (Ochomo et al., 2014) or *An. rivulorum* (Kawada et al., 2012), respectively. In addition, the mechanisms mediating resistance in *An. gambiae* (*s.s*.) and *An. funestus* are more widespread and established (Ranson et al., 2011; Kawada et al., 2011; Mulamba et al., 2014; Ranson & Lissenden, 2016). Here, *An. gambiae* (*s.s*.) was significantly more resistant to pyrethroids than *An. arabiensis,* similar to reports from elsewhere (Ochomo et al., 2013). The significantly higher levels of pyrethroid resistance observed in *An. gambiae* (*s.s*.) in the 'IRS stopped' sites suggest that halting IRS interventions which have a different target site may open a population to selection by insecticides used for public health and/or agricultural purposes.

4.1 Limitations

This study had several limitations. First, the findings are limited by the cross-sectional sampling done in only 11 districts. This may have introduced bias; however, sampling from several districts provided a snapshot of pyrethroid resistance in geographically distinct areas. Second, the definitions of insecticide resistance are based on WHO cut-offs using diagnostic concentrations of permethrin (0.75%) and deltamethrin (0.05%). Pyrethroid intensity assays to determine the operational significance of insecticide resistance were not conducted due to sample size limitations. Third, sample size limitations limited the statistical power available to adequately test genotype- phenotype associations, nonetheless there are significant associations between target site/metabolic resistance markers with pyrethroid resistance.

Fourth, the concentration of PBO used was 4.0% which may not be directly comparable to the concentration of PBO on LLINs. In a study of PBO LLINs distributed by the Ugandan Ministry of Health in 2017–2018, the concentration of PBO at baseline was 26.81 g/kg in PermaNet 3.0, and 8.17 g/kg in Olyset Plus (Mechan et al., 2022) which may not be equivalent to the concentration included in the WHO tube assay. Finally, the absence of historical data before LLIN and/or IRS implementation limited the inferences that could be made on the development and spread of pyrethroid resistance mutations. Moreover, metabolic resistance mechanisms were not explored in *An. arabiensis* due to resource limitations.

5.0 Conclusion

Resistance to pyrethroids was widespread across Uganda, underscoring the importance of insecticide resistance management strategies targeting both *An. gambiae* (*s.s*.) and *An. arabiensis*. Adding PBO to pyrethroids improved mosquito mortality in both species, supporting the WHO's new recommendation to deploy PBO LLINs for vector control in settings of pyrethroid resistance. Whilst target site resistance marker *Vgsc 995S* seems to be approaching fixation in *An. gambiae* (*s.s*.), the moderate frequency of *Vgsc 995F* in the 'IRS stopped' sites suggests intense insecticide selection pressure in northern Uganda. Our results also suggest an association between metabolic resistance variants (the triple-mutant-*Cyp6p4* and *Cyp4j5*) and *An. gambiae* (*s.s*.) survival following exposure to PBO and pyrethroids underscoring the need for further research on the relationship between markers of metabolic resistance and PBO. Further surveillance of insecticide resistance and assessment of correlations between genotypic markers and phenotypic outcomes are needed to better understand mechanisms of pyrethroid resistance as PBO LLINs are scaled-up and to guide vector control measures.

Figure legends

Fig. 6.1 Map of study sites showing the location of sampled districts, and stratification by vector control measures. *Abbreviations*: IRS, indoor residual spraying; LLINs, long-lasting insecticidal nets. *Key*: green, No IRS (LLINs only); orange, IRS stopped (+ LLINs); purple, IRS active (+ LLINs).

Fig. 6.2 Timeline of vector control measures and mosquito larval collections in study sites, stratified by IRS status. *Abbreviations*: IRS, indoor residual spraying; LLINs, long-lasting insecticidal nets. *Key*: purple, IRS with bendiocarb; green, IRS with pirimiphos methyl (Actellic); gold, IRS with Sumishield 50W (clothianidin); blue circle, LLINs distributed nationwide by Uganda's Ministry of Health through the 2017–2018 universal coverage campaign; red-inverted triangles, mosquito larval collection.

Fig. 6.3 Heat maps showing the frequencies of target site mutations *Vgsc-995S* and *Vgsc-995F*, the triple mutant (represented by *Cyp6P4*), a cytochrome p450 *Cyp4j5-L43F* and carboxylesterase *Coeae1d*, associated with resistance to pyrethroids in *An. gambiae* (*s.s.*). The color scale ranges from white (0%) to dark orange (100%); the darker the shade, the higher the resistant allele frequency.

Fig. 6.4 Heat maps showing the frequency of target site mutation *Vgsc-L1014S* and *Vgsc-L1014F* in *An. arabiensis*. The color scale ranges from white (0%) to dark purple (12%); the darker the shade, the higher the resistant allele frequency.

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Table 6.1. Mosquitoes tested using phenotypic assays stratified by species, insecticide exposure, study site and vector control measures

Species	Insecticide exposure	No IRS			IRS stopped				IRS active			
		Busia	Mubende	Kayunga	Kole	Amuru	Lamwo	Agago	Otuke	Tororo	Dokolo	Amolatar
An. gambiae (*s.s.*)	Total	58	171	135	241	169	322	0	0	0	6	3
	Permethrin	32	66	26	113	89	82	0	0	0	2	1
	Permethrin + PBO	0	16	39	0	39	75	0	0	0	1	0
	Deltamethrin	26	74	22	128	41	81	0	0	0	3	2
	Deltamethrin + PBO	0	15	48	0	0	84	0	0	0	0	0
An. arabiensis	Total	71	33	267	74	1	81	158	112	365	293	193
	Permethrin	34	15	78	41	0	16	72	57	119	86	96
	Permethrin + PBO	0	1	49	0	1	15	0	0	70	73	0
	Deltamethrin	37	17	93	33	0	16	86	55	111	74	97
	Deltamethrin + PBO	0	0	47	0	0	34	0	0	65	60	0

Table 6.2. Mosquito mortality after exposure to pyrethroid insecticides using phenotypic assays, stratified by species, IRS category andinsecticide

Comparison between IRS category, stratified by species							
Species	IRS category	Permethrin			Deltamethrin		
		Mortality (%)	Odds ratio (95% CI)	*P*-value	Mortality (%)	Odds ratio (95% CI)	*P*-value
An. gambiae (*s.s.*)	No IRS	20/124 (16.1)	Reference		30/122 (24.6)	Reference	
	IRS stopped	32/284 (11.3)	0.64 (0.15–2.71)	0.55	64/250 (25.6)	0.66 (0.19–2.33)	0.52
An. arabiensis	No IRS	86/127 (67.7)	Reference		123/147 (83.7)	Reference	
	IRS stopped	82/129 (63.6)	0.90 (0.28–2.94)	0.86	120/135 (88.9)	1.37 (0.51–3.64)	0.53
	IRS active	234/358 (65.4)	1.39 (0.47–4.10)	0.55	267/337 (79.2)	0.83 (0.37–1.87)	0.66
Comparison between mosquito species, stratified by IRS category							
IRS category	Species	Permethrin			Deltamethrin		
		Mortality (%)	Odds ratio (95% CI)	*P*-value	Mortality (%)	Odds ratio (95% CI)	*P*-value
No IRS	*An. arabiensis*	86/127 (67.7)	Reference		123/147 (83.7)	Reference	
	An. gambiae (*s.s.*)	20/124 (16.1)	0.10 (0.05–0.19)	< 0.001	30/122 (24.6)	0.06 (0.03–0.12)	<0.001
IRS stopped	*An. arabiensis*	82/129 (63.6)	Reference		120/135 (88.9)	Reference	
	An. gambiae (*s.s.*)	32/284 (11.3)	0.20 (0.10–0.38)	< 0.001	64/250 (25.6)	0.08 (0.03–0.18)	<0.001
Comparison between insecticides, stratified by IRS category							
IRS category	Insecticide	*An. gambiae* (*s.s.*)			*An. arabiensis*		
		Mortality (%)	Odds ratio (95% CI)	*P*-value	Mortality (%)	Odds ratio (95% CI)	*P*-value
No IRS	Deltamethrin	30/122 (24.6)	Reference		123/147 (83.7)	Reference	
	Permethrin	20/124 (16.1)	0.59 (0.31–1.11)	0.10	86/127 (67.7)	0.40 (0.22–0.72)	0.002
IRS stopped	Deltamethrin	64/250 (25.6)	Reference		120/135 (88.9)	Reference	
	Permethrin	32/284 (11.3)	0.44 (0.27–0.71)	0.001	82/129 (63.6)	0.21 (0.11–0.41)	< 0.001
IRS active	Deltamethrin				267/337 (79.2)	Reference	
	Permethrin	Insufficient *An. gambiae* (*s.s.*) collected			234/358 (65.4)	0.48 (0.34–0.68)	< 0.001

Table 6.3. Mosquito mortality after exposure to pyrethroid insecticides with and without piperonyl butoxide, by species and IRS category

IRS category	Insecticide	*An. gambiae* (*s.s.*)			*An. arabiensis*		
		Mortality (%)	Odds ratio (95% CI)	*P*-value	Mortality (%)	Odds ratio (95% CI)	*P*-value
No IRS	Permethrin	20/124 (16.1)	Reference		86/127 (67.7)	Reference	
	Permethrin + PBO	30/55 (54.5)	6.81 (3.08–15.1)	< 0.001	41/50 (82.0)	1.52 (0.62–3.70)	0.36
	Deltamethrin	30/122 (24.6)	Reference		123/147 (83.7)	Reference	
	Deltamethrin + PBO	35/63 (55.6)	3.83 (2.01–7.31)	< 0.001	31/47 (66.0)	0.38 (0.18–0.80)	0.01
IRS stopped	Permethrin	32/284 (11.3)	Reference		82/129 (63.6)	Reference	
	Permethrin + PBO	65/114 (57.0)	15.0 (7.23–31.2)	< 0.001	16/16 (100)	Omitted because of collinearity	
	Deltamethrin	64/250 (25.6)	Reference		120/135 (88.9)	Reference	
	Deltamethrin + PBO	66/84 (78.6)	18.1 (8.36–39.3)	< 0.001	34/34 (100)	Omitted because of collinearity	
IRS Active	Permethrin	Insufficient *An. gambiae* (*s.s.*) collected			234/358 (65.4)	Reference	
	Permethrin + PBO				138/143 (96.5)	16.1 (6.31–41.2)	< 0.001
	Deltamethrin				267/337 (79.2)	Reference	
	Deltamethrin + PBO				120/125 (96.0)	7.37 (2.82–19.3)	< 0.001

Table 6.4. Associations between resistant alleles and mosquito survival in *An. gambiae* s.s. mosquitoes following exposure to pyrethroid insecticides with and without piperonyl butoxide in sites with no IRS

Resistant alleles	Resistant allele frequency *n*/*N* (%)	Wild type alleles-survived *n*/*N* (%)	Resistant alleles-survived *n*/*N* (%)	Odds ratio (95% CI)	*P*-value	Resistant allele frequency *n*/*N* (%)	Wild type alleles-survived *n*/*N* (%)	Resistant alleles-survived *n*/*N* (%)	Odds ratio (95% CI)	*P*-value
	Permethrin					Permethrin + PBO				
Vgsc-L995S/F	214/218 (98.2)	4/4 (100)	180/214 (84.1)	0.62 (0.17–2.26)	0.46	94/106 (88.7)	2/12 (16.7)	44/94 (46.8)	1.17 (0.23–5.94)	0.85
Cyp6P4 (triple mutant)	204/226 (90.3)	19/22 (86.4)	173/204 (84.8)	0.93 (0.23–3.67)	0.91	87/106 (82.1)	6/19 (31.6)	40/87 (46.0)	1.22 (0.27–5.58)	0.80
Cyp4j5	113/216 (52.3)	83/103 (80.6)	99/113 (87.6)	1.85 (0.80–4.29)	0.15	60/106 (56.6)	22/46 (47.8)	24/60 (40.0)	0.84 (0.33–2.16)	0.72
Coeae1d	107/218 (49.1)	90/111 (81.1)	94/107 (87.9)	1.97 (0.82–4.77)	0.13	51/106 (48.1)	25/55 (45.5)	21/51 (41.2)	0.85 (0.32–2.25)	0.74
	Deltamethrin					Deltamethrin + PBO				
Vgsc-L995S/F	271/278 (97.5)	2/7 (28.6)	206/271 (76.0)	3.44 (1.02–11.6)	0.046	102/125 (81.6)	14/23 (60.9)	42/102 (41.2)	1.41 (0.40–4.97)	0.59
Cyp6P4 (triple mutant)	248/278 (89.2)	20/30 (66.7)	188/248 (75.8)	0.82 (0.31–2.21)	0.70	95/126 (75.4)	15/31 (48.4)	41/95 (43.2)	0.94 (0.32–2.80)	0.91
Cyp4j5	147/278 (52.9)	102/131 (77.9)	106/147 (72.1)	0.82 (0.46–1.47)	0.51	75/134 (56.0)	15/59 (25.4)	41/75 (54.7)	2.27 (1.08–4.80)	0.031
Coeae1d	141/278 (50.7)	103/137 (75.2)	105/141 (74.5)	0.98 (0.50–1.89)	0.94	63/124 (50.8)	30/61 (49.2)	26/63 (41.3)	0.65 (0.27–1.55)	0.33

Table 6.5. Associations between resistant alleles and mosquito survival in *An. gambiae* s.s. mosquitoes following exposure to pyrethroid insecticides with and without piperonyl butoxide in sites where IRS was stopped

Resistant alleles	Resistant allele frequency *n*/*N* (%)	Wild type alleles-survived *n*/*N* (%)	Resistant alleles-survived *n*/*N* (%)	Odds ratio (95% CI)	*P*-value	Resistant allele frequency *n*/*N* (%)	Wild type alleles-survived *n*/*N* (%)	Resistant alleles-survived *n*/*N* (%)	Odds ratio (95% CI)	*P*-value
	Permethrin					Permethrin + PBO				
Vgsc-L995S/F	424/432 (98.2)	6/8 (75)	370/424 (87.2)	1.40 (0.85–2.30)	0.18	199/202 (98.5)	0/3 (0)	100/199 (50.3)	1.25 (0.77–2.03)	0.37
Cyp6P4 (triple mutant)	400/440 (90.9)	25/40 (62.5)	351/400 (87.8)	1.87 (0.86–4.09)	0.12	175/198 (88.4)	6/23 (26.1)	92/175 (52.6)	3.19 (1.16–8.80)	0.025
Cyp4j5	214/430 (49.8)	189/216 (87.5)	185/214 (86.4)	0.75 (0.22–2.57)	0.64	95/200 (47.5)	49/105 (46.7)	51/95 (53.7)	1.35 (0.80–2.28)	0.27
Coeae1d	254/432 (58.8)	157/178 (88.2)	219/254 (86.2)	0.78 (0.42–1.48)	0.45	119/202 (58.9)	45/83 (54.2)	55/119 (46.2)	0.70 (0.37–1.32)	0.27
	Deltamethrin					Deltamethrin + PBO				
Vgsc-L995S/F	270/306 (88.2)	6/36 (16.7)	190/270 (70.4)	1.64 (0.99–2.71)	0.056	72/72 (100)	0/0 (0)	36/72 (50)	1.60 (0.67–3.83)	0.30
Cyp6P4 (triple mutant)	256/306 (83.7)	13/50 (26.0)	183/256 (71.5)	2.27 (1.02–5.05)	0.045	67/70 (95.7)	0/3 (0)	36/67 (53.7)	–	–
Cyp4j5	204/306 (66.7)	71/102 (69.6)	125/204 (61.3)	1.0 (0.52–1.91)	0.99	7/70 (10.0)	30/63 (47.6)	4/7 (57.1)	1.19 (0.21–6.74)	0.85
Coeae1d	164/306 (53.6)	84/142 (59.2)	112/164 (68.3)	1.55 (0.83–2.88)	0.17	42/72 (58.3)	14/30 (46.7)	22/42 (52.4)	2.07 (0.64–6.66)	0.22

Supplementary Table 6.1: Phenotypic Bioassay outcomes by study site using standard WHO diagnostic concentrations for both permethrin and deltamethrin

Site	*An. gambiae* (*s.s.*)				*An. arabiensis*			
	Total	Alive	Dead	Mortality (%)	Total	Alive	Dead	Mortality (%)
No IRS								
Busia	N=58				N=71			
-Permethrin	32	30	2	6.3	34	11	23	67.7
-Deltamethrin	26	19	7	26.9	37	6	31	83.8
Mubende	N=171				N=33			
-Permethrin	66	51	15	22.7	15	12	3	20.0
-PBO + Permethrin	16	6	10	62.5	1	1	0	0
-Deltamethrin	74	56	18	24.3	17	3	14	82.4
-PBO + Deltamethrin	15	6	9	60.0				
Kayunga	N=135				N=267			
-Permethrin	26	23	3	11.5	78	18	60	76.9
-PBO + Permethrin	39	19	20	51.3	49	8	41	83.7
-Deltamethrin	22	17	5	22.7	93	15	78	83.9
-PBO + Deltamethrin	48	22	26	54.2	47	16	31	66.0
IRS stopped								
Kole	N=241				N=74			
-Permethrin	113	92	21	18.6	41	19	22	53.7
-Deltamethrin	128	79	49	38.3	33	4	29	87.9
Amuru	N=170				N=1			
-Permethrin	89	88	1	1.1	–	–	–	–
-PBO + Permethrin	40	13	27	66.7	1	0	1	–
-Deltamethrin	41	40	1	2.4	–	–	–	–
Lamwo	N=322				N=81			
-Permethrin	82	72	10	12.2	16	11	5	31.3
-PBO + Permethrin	75	36	39	52.0	15	0	15	100
-Deltamethrin	81	67	14	17.3	16	5	11	68.8
-PBO + Deltamethrin	84	18	66	78.6	34	0	34	100
Agago	N=0				N=158			
-Permethrin	–	–	–	–	72	17	55	76.4
-Deltamethrin	–	–	–	–	86	6	80	93.0

Site	*An. gambiae* (*s.s.*)				*An. arabiensis*			
	Total	**Alive**	**Dead**	**Mortality (%)**	**Total**	**Alive**	**Dead**	**Mortality (%)**
IRS active								
Otuke	N=0				N=112			
-Permethrin	–	–	–	–	57	19	38	66.7
-Deltamethrin	–	–	–	–	55	8	47	85.5
Tororo	N=0				N=365			
-Permethrin	–	–	–	–	119	58	61	51.3
-PBO + Permethrin	–	–	–	–	70	3	67	95.7
-Deltamethrin	–	–	–	–	111	37	74	66.7
-PBO + Deltamethrin	–	–	–	–	65	5	60	92.7
Dokolo	N=6				N=293			
-Permethrin	2	1	1	–	86	19	67	77.9
-PBO + Permethrin	1	0	1	–	73	2	71	97.3
-Deltamethrin	3	2	1	–	74	8	66	89.2
-PBO + Deltamethrin	–	–	–	–	60	0	60	100
Amolatar	N=3				N=193			
-Permethrin	1	1	0	–	96	28	68	70.8
-Deltamethrin	2	2	0	–	97	17	80	82.5

Supplementary Table 6.2: *Anopheles gambiae* s.s. genotyped for molecular markers associated with pyrethroid resistance

***An. gambiae* (*s.s.*)**	**Permethrin**			**Deltamethrin**			**PBO + Permethrin**			**PBO + Deltamethrin**		
IRS status	Alive	Dead	Sum	Alive	Dead	Sum	Alive	Dead	Sum	Alive	Dead	Sum
No IRS	93	17	110	104	35	139	23	30	53	28	35	63
IRS stopped	188	32	220	112	67	179	36	39	75	18	18	36

Supplementary Table 6.3: Resistance marker allele frequencies by study site in *An. gambiae* s.s.

Insecticide Resistance-*An. gambiae* (*s.s.*)																					
Resistance marker			*Vgsc-995*				*Cyp6aa1*			*Cyp6p4*			*ZZB*			*Cyp4j5*			*Coeae1d*		
Allele			***L***	***S***	***F***	**Total**	***N***	***D***	**Total**	***I***	***M***	**Total**	***L***	***Z***	**Total**	***L***	***F***	**Total**	***S***	***R***	**Total**
Busia	Phenotype	Alive	4	94	0	98	15	83	98	16	82	98	15	87	102	52	44	96	40	58	98
		Dead	1	17	0	18	3	15	18	5	13	18	4	14	18	8	10	18	8	10	18
	Sum		5	111	0	116	18	98	116	21	95	116	19	101	120	60	54	114	48	68	116
	Allele frequency		0.04	0.96	0.00	1.00	0.16	0.84	1.00	0.18	0.82	1.00	0.16	0.84	1.00	0.53	0.47	1.00	0.41	0.59	1.00
Mubende	Phenotype	Alive	0	214	24	238	15	223	238	18	220	238	18	220	238	115	123	238	131	107	238
		Dead	0	97	7	104	11	91	102	7	97	104	7	97	104	55	49	104	58	44	102
	Sum		0	311	31	342	26	314	340	25	317	342	25	317	342	170	172	342	189	151	340
	Allele frequency		0.00	0.91	0.09	1.00	0.08	0.92	1.00	0.07	0.93	1.00	0.07	0.93	1.00	0.50	0.50	1.00	0.56	0.44	1.00
Kayunga	Phenotype	Alive	18	136	4	158	25	137	162	26	140	166	25	135	160	55	103	158	77	81	158
		Dead	23	87	1	111	29	85	114	30	82	112	27	85	112	54	66	120	50	62	112
	Sum		41	223	5	269	54	222	276	56	222	278	52	220	272	109	169	278	127	143	270
	Allele frequency		0.15	0.83	0.02	1.00	0.20	0.80	1.00	0.20	0.80	1.00	0.19	0.81	1.00	0.39	0.61	1.00	0.47	0.53	1.00
Kole	Phenotype	Alive	9	178	43	230	41	225	266	20	210	230	20	210	230	87	143	230	99	131	230
		Dead	30	78	10	118	43	75	118	43	79	122	42	80	122	36	82	118	61	57	118
	Sum		39	256	53	348	84	300	384	63	289	352	62	290	352	123	225	348	160	188	348
	Allele frequency		0.11	0.74	0.15	1.00	0.22	0.78	1.00	0.18	0.82	1.00	0.18	0.82	1.00	0.35	0.65	1.00	0.46	0.54	1.00
Amuru	Phenotype	Alive	1	109	56	166	12	154	166	9	157	166	9	157	166	89	77	166	62	104	166
		Dead	1	16	7	24	8	20	28	7	21	28	7	21	28	21	3	24	11	13	24
	Sum		2	125	63	190	20	174	194	16	178	194	16	178	194	110	80	190	73	117	190
	Allele frequency		0.01	0.66	0.33	1.00	0.10	0.90	1.00	0.08	0.92	1.00	0.08	0.92	1.00	0.58	0.42	1.00	0.38	0.62	1.00
Lamwo	Phenotype	Alive	2	188	122	312	15	297	312	15	295	310	14	298	312	163	145	308	139	173	312
		Dead	4	106	52	162	22	140	162	22	136	158	22	140	162	90	70	160	61	101	162
	Sum		6	294	174	474	37	437	474	37	431	468	36	438	474	253	215	468	200	274	474
	Allele frequency		0.01	0.62	0.37	1.00	0.08	0.92	1.00	0.08	0.92	1.00	0.08	0.92	1.00	0.54	0.46	1.00	0.42	0.58	1.00

Supplementary Table 6.4*:* Differences in *Vgsc-995* allele frequencies by IRS category in *An. gambiae* s.s. exposed to both permethrin and deltamethrin

Vgsc-995L vs Vgsc-995S				
IRS category	**Proportion resistant (%)**	***Vgsc-995L frequency*** *(Wild type)*	***Vgsc-995S frequency*** *(Mutant)*	**Fisher Exact (p-value)**
No IRS	197/240 (82.1%)	3.44	93.13	0.46
IRS stopped	300/399 (75.2%)	4.51	67.09	
Vgsc-995L vs Vgsc 995F				
IRS category	**Proportion resistant (%)**	***Vgsc-995L frequency*** *(Wild type)*	***Vgsc-995F frequency*** *(Mutant)*	**Fisher Exact (p-value)**
No IRS	197/240 (82.1%)	3.44	3.43	0.02
IRS stopped	300/399 (75.2%)	4.51	28.40	
Vgsc-995S vs Vgsc 995F				
IRS category	**Proportion resistant (%)**	***Vgsc-995S frequency*** *(Mutant)*	***Vgsc-995F frequency*** *(Mutant)*	**Fisher Exact (p-value)**
No IRS	197/240 (82.1%)	93.13	3.43	<.0001
IRS stopped	300/399 (75.2%)	67.09	28.40	

Supplementary Table 6.5: Resistant marker allele frequencies by study site in *An. arabiensis*

Insecticide resistance-*An. arabiensis*						
Resistance marker			*Vgsc-1014*			
Allele			***L***	***S***	***F***	**Total**
Busia	Phenotype	Alive	42	0	0	42
		Dead	114	0	0	114
	Sum		156	0	0	156
	Allele frequency		1.00	0.00	0.00	1.00
Mubende	Phenotype	Alive	30	0	0	30
		Dead	32	0	0	32
	Sum		62	0	0	62
	Allele frequency		1.00	0.00	0.00	1.00
Kayunga	Phenotype	Alive	60	4	0	64
		Dead	108	2	0	110
	Sum		168	6	0	174
	Allele frequency		0.97	0.03	0.00	1.00
Kole	Phenotype	Alive	32	6	6	44
		Dead	52	5	3	60
	Sum		84	11	9	104
	Allele frequency		0.81	0.11	0.09	1.00
Lamwo	Phenotype	Alive	31	0	1	32
		Dead	32	0	0	32
	Sum		63	0	1	64
	Allele frequency		0.98	0.00	0.02	1.00
Agago	Phenotype	Alive	45	0	1	46
		Dead	128	0	1	129
	Sum		173	0	2	175
	Allele frequency		0.99	0.00	0.01	1.00
Otuke	Phenotype	Alive	52	0	0	52
		Dead	122	0	0	122
	Sum		174	0	0	174
	Allele frequency		1.00	0.00	0.00	1.00
Tororo	Phenotype	Alive	159	0	1	160
		Dead	192	0	0	192
	Sum		351	0	1	352
	Allele frequency		0.997	0.00	0.003	1.00
Dokolo	Phenotype	Alive	56	0	0	56
		Dead	120	0	0	120
	Sum		176	0	0	176
	Allele frequency		1.00	0.00	0.00	1.00
Amolatar	Phenotype	Alive	91	0	1	92

		Dead	84	0	0	84
	Sum		175	0	1	176
	Allele frequency		0.994	0.00	0.006	1.00

L: *Vgsc 1014* Wild type (leucine) allele; S: *Vgsc 1014* (*kdr*-east) serine allele; F: *Vgsc 1014* (*kdr*-west) phenylalanine allele

Supplementary Table 6.6: *Anopheles arabiensis* genotyped for molecular markers associated with pyrethroid resistance

An. arabiensis	**Permethrin**			**Deltamethrin**		
IRS status	**Alive**	**Dead**	**Sum**	**Alive**	**Dead**	**Sum**
No IRS	40	52	92	23	60	83
IRS stopped	47	69	116	14	45	59
IRS active	109	147	256	71	112	183

Supplementary Table 6.7: Knock down resistance (*kdr*) genotype frequencies by study site in *An. gambiae* s.s.

***Anopheles gambiae* s.s. *kdr* genotype frequencies**									
Resistance marker			***Vgsc-995***						
Genotype			***LL***	***LS***	***LF***	**FS**	**FF**	**SS**	**Total**
Busia	Phenotype	Alive	2	0	0	0	0	47	49
		Dead	0	1	0	0	0	8	9
	Sum		2	1	0	0	0	55	58
	Genotype frequency		0.03	0.02	0.00	0.00	0.00	0.95	1.00
Mubende	Phenotype	Alive	0	0	0	22	1	96	119
		Dead	0	0	0	7	0	45	52
	Sum		0	0	0	29	1	141	171
	Genotype frequency		0.00	0.00	0.00	0.17	0.006	0.82	1.00
Kayunga	Phenotype	Alive	9	0	0	4	0	66	79
		Dead	11	1	0	1	0	43	56
	Sum		20	1	0	5	0	109	135
	Genotype frequency		0.15	0.007	0.00	0.04	0.00	0.81	1.00
Kole	Phenotype	Alive	2	0	1	34	4	72	113
		Dead	9	0	0	8	0	34	51
	Sum		11	0	1	42	4	106	164
	Genotype frequency		0.067	0.00	0.006	0.26	0.02	0.65	1.00
Amuru	Phenotype	Alive	0	0	1	37	9	36	83
		Dead	0	1	0	5	1	5	12

	Sum		0	1	1	42	10	41	95
	Genotype frequency		0.00	0.01	0.01	0.44	0.11	0.43	1.00
Lamwo	Phenotype	Alive	1	0	0	76	23	56	156
		Dead	2	0	0	38	7	34	81
	Sum		3	0	0	114	30	90	237
	Genotype frequency		0.01	0.00	0.00	0.48	0.13	0.38	1.00

Supplementary Table 6.8: Knock down resistance (*kdr*) genotype frequencies by study site in *An. arabiensis*

***Anopheles arabiensis kdr* genotype frequencies**									
Resistance marker			***Vgsc-1014***						
Genotype			***LL***	***LS***	***LF***	***FS***	***FF***	***SS***	**Totals**
Busia	Phenotype	Alive	16	0	0	0	0	0	16
		Dead	40	0	0	0	0	0	40
	Sum		56	0	0	0	0	0	56
	Genotype frequency		1.00	0.00	0.00	0.00	0.00	0.00	1.00
Mubende	Phenotype	Alive	15	0	0	0	0	0	15
		Dead	16	0	0	0	0	1	17
	Sum		31	0	0	0	0	1	32
	Genotype frequency		0.97	0.00	0.00	0.00	0.00	0.03	1.00
Kayunga	Phenotype	Alive	30	0	0	0	0	2	32
		Dead	54	0	0	0	0	1	55
	Sum		84	0	0	0	0	3	87
	Genotype frequency		0.97	0.00	0.00	0.00	0.00	0.03	1.00
Kole	Phenotype	Alive	16	0	0	0	3	3	22
		Dead	26	0	0	1	1	2	30
	Sum		42	0	0	1	4	5	52
	Genotype frequency		0.8	0.00	0.00	0.02	0.08	0.1	1.00
Lamwo	Phenotype	Alive	15	0	1	0	0	0	16
		Dead	16	0	0	0	0	0	16
	Sum		31	0	1	0	0	0	32
	Genotype frequency		0.97	0.00	0.03	0.00	0.00	0.00	1.00
Agago	Phenotype	Alive	22	0	1	0	0	0	23
		Dead	63	0	2	0	0	0	65
	Sum		85	0	3	0	0	0	88
	Genotype frequency		0.97	0.00	0.03	0.00	0.00	0.00	1.00
Otuke	Phenotype	Alive	25	0	2	0	0	0	27
		Dead	61	0	0	0	0	0	61
	Sum		86	0	2	0	0	0	88
	Genotype frequency		0.98	0.00	0.02	0.00	0.00	0.00	1.00
Tororo	Phenotype	Alive	79	0	1	0	0	0	80
		Dead	96	0	0	0	0	0	96
	Sum		175	0	1	0	0	0	176
	Genotype frequency		0.99	0.00	0.01	0.00	0.00	0.00	1.00

Dokolo	Phenotype	Alive	27	0	0	0	0	2	29
		Dead	60	0	0	0	0	1	61
	Sum		87	0	0	0	0	3	90
	Genotype frequency		0.97	0.00	0.00	0.00	0.00	0.03	1.00
Amolatar	Phenotype	Alive	45	0	1	0	0	0	46
		Dead	42	0	0	0	0	0	42
	Sum		87	0	1	0	0	0	88
	Genotype frequency		0.99	0.00	0.01	0.00	0.00	0.00	1.00

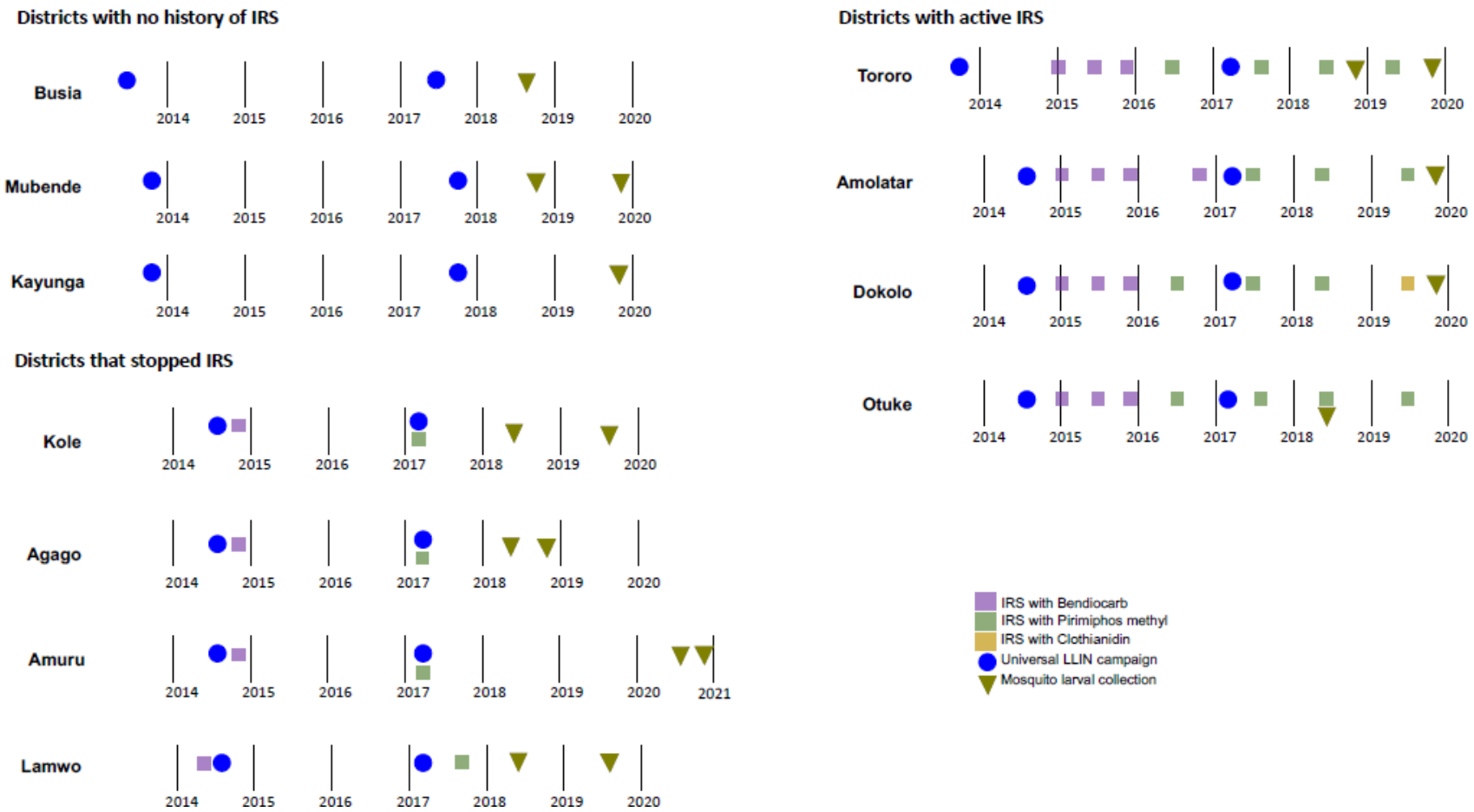

Fig. 6.2 Timeline of vector control measures and mosquito larval collections in study sites, stratified by IRS status. *Abbreviations*: IRS, indoor residual spraying; LLINs, long-lasting insecticidal nets. *Key*: purple, IRS with bendiocarb; green, IRS with pirimiphos methyl (Actellic); gold, IRS with Sumishield 50W (clothianidin); blue circle, LLINs distributed nationwide by Uganda's Ministry of Health through the 2017–2018 universal coverage campaign; red-inverted triangles, mosquito larval collection.

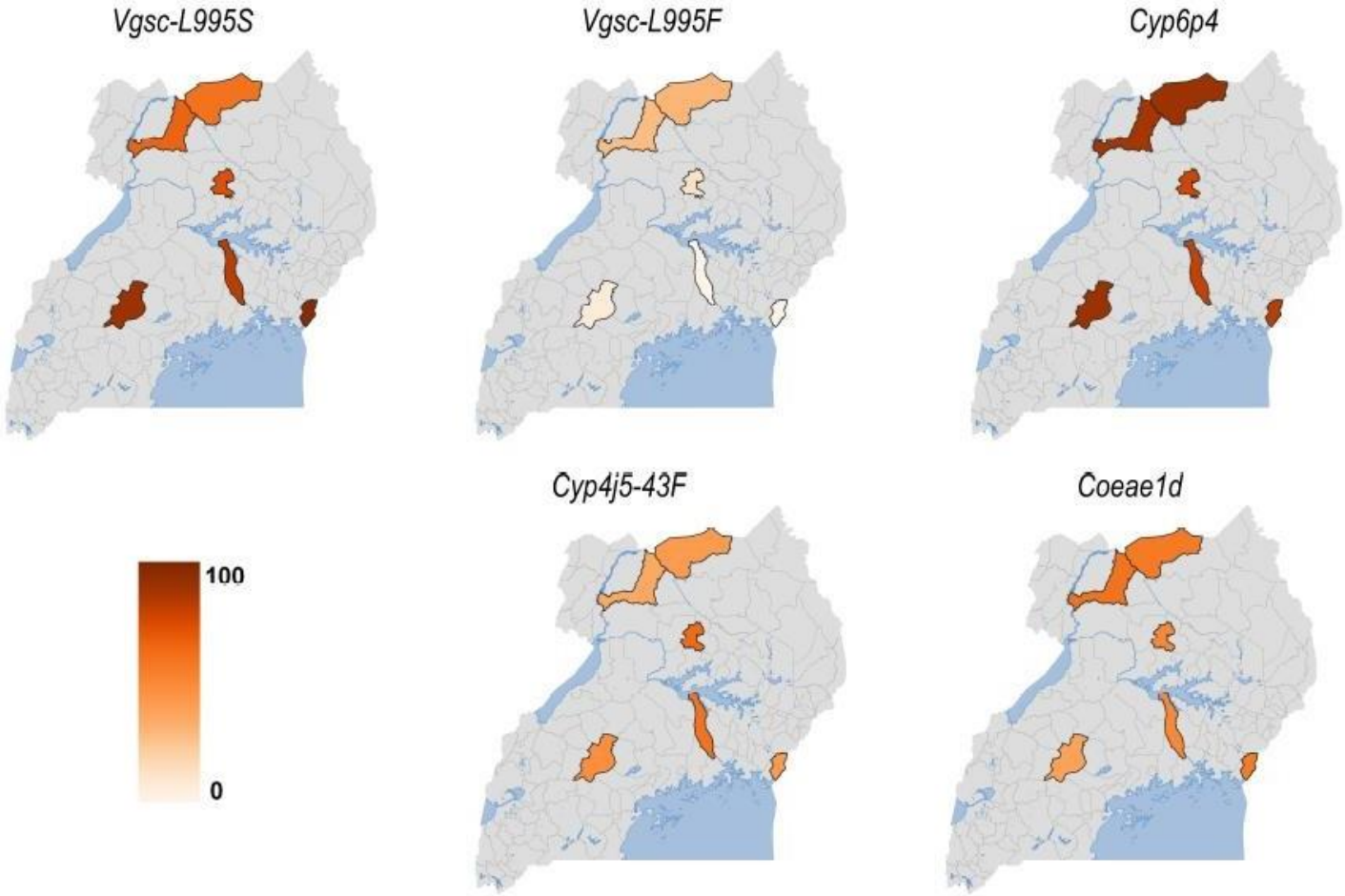

Fig. 6.3 Heat maps showing the frequencies of target site mutations *Vgsc-995S* and *Vgsc-995F*, the triple mutant (represented by *Cyp6P4*), a cytochrome p450 *Cyp4j5-L43F* and carboxylesterase *Coeae1d*, associated with resistance to pyrethroids in *An. gambiae* (*s.s.*). The color scale ranges from white (0%) to dark orange (100%); the darker the shade, the higher the resistant allele frequency.

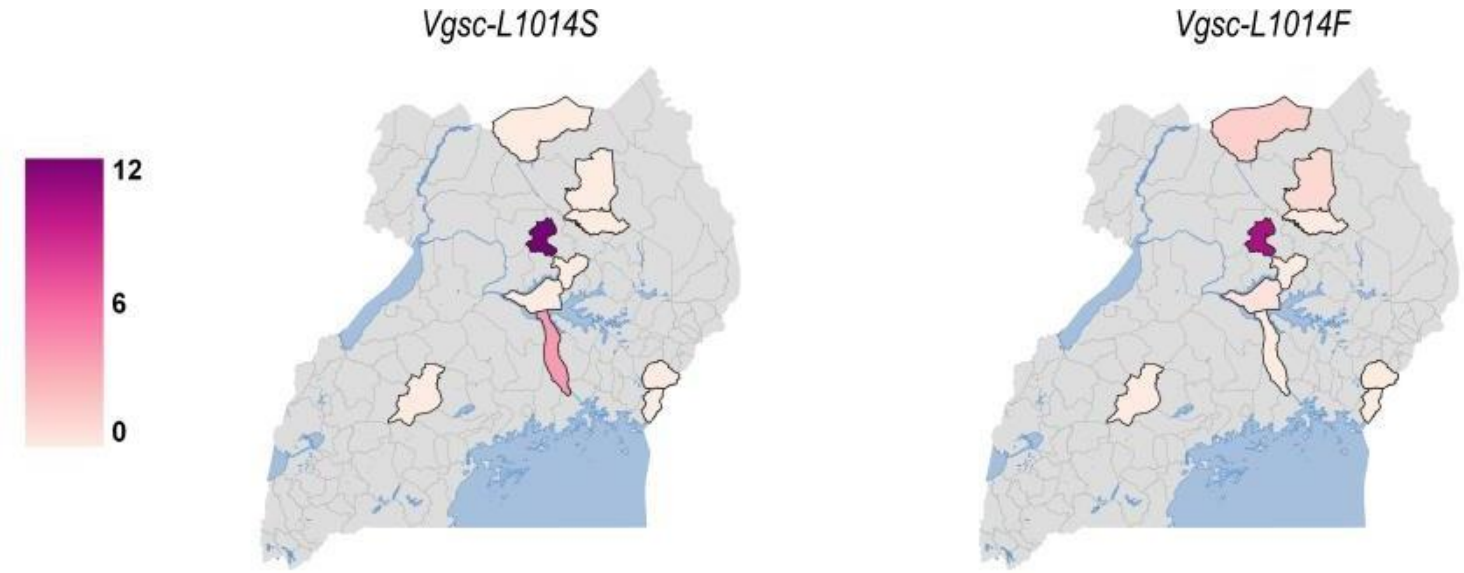

Fig. 6.4 Heat maps showing the frequency of target site mutation *Vgsc-L1014S* and *Vgsc-L1014F* in *An. arabiensis*. The color scale ranges from white (0%) to dark purple (12%); the darker the shade, the higher the resistant allele frequency.

CHAPTER 7 DISCUSSION

7.1 Chapter introduction

This chapter summarizes the main findings from this thesis, discusses their implications, highlights the study strengths and limitations, and provides overall conclusions and recommendations. This chapter comprises 8 sections. The first section discusses the systematic literature review findings and gaps in insecticide resistance patterns in East Africa; section 2 summarizes the study findings for each thesis objective; section 3 discusses the impact of different mosquito collection methods on indicators of anopheles vectors in Uganda; section 4 discusses the impact of seasonality and malaria control interventions on *Anopheles* density and species composition; section 5 discusses pyrethroid resistance and mechanisms in *Anopheles gambiae* s.s. and *An. arabiensis* collected from 11 districts in Uganda; section 6 discusses the implications of the thesis findings; section 7 presents the thesis strengths; section 8 addresses the limitations of the thesis; and section 9 provides the overall conclusion from the thesis.

7.2 Systematic literature review

The systematic review of insecticide resistance patterns in East Africa presented in Chapter 2 showed wide ranging variation in the outcomes from phenotypic assays with high levels of pyrethroid resistance (2% mortality) reported in some areas [1] and pyrethroid susceptibility (100% mortality) in others [2]; these differences were also observed between sympatric *Anopheles* species [2, 3]. Although 97% (31 of 32) of selected studies reported phenotypic outcomes for pyrethroid resistance, only 44% (14 of 32) reported outcomes on synergist assays with PBO. Overall, PBO increased mortality to pyrethroid exposure and restored full susceptibility in 3 of 7 (43%) studies in *An. gambiae* s.s., and in 6 of 7 (86%) studies in A*n. arabiensis* and 4 of 7 (57%) studies in *An. funestus*. However, none of the selected studies

measured associations between genotypes and survival of *Anopheles* mosquitoes after exposure to PBO and pyrethroids. Examining genotypic markers of insecticide resistance showed that 35 of 40 studies (88%) reported outputs on target site resistance markers (*Vgsc-L1014S, Vgsc-L1014F, Vgsc-N1575Y, Ace-1R*), but only 14 of 40 studies (35%) recorded outcomes for metabolic resistance, and only 3 of 40 studies (8%) assessed DNA-based metabolic resistance markers (*Cyp4j5, Coeae1d, Cyp6p4, Cyp6aa1, ZZB-TE*). This thesis aimed to address some of these knowledge gaps, conducting phenotypic and genotypic assays, and assessing the association between genotypic markers of insecticide resistance and survival in mosquitoes following exposure to PBO plus pyrethroids using DNA-based metabolic resistance markers, which is an underutilized method for monitoring metabolic resistance variants in sub-Saharan Africa [4, 5].

7.3 Summary of research findings

Findings from this thesis are summarized in Table 7.1, aligning with the research objectives, and are discussed further in detail in the subsequent sections.

Table 7.1: Thesis objectives and summary of key findings

THESIS OBJECTIVE	KEY RESULT FINDING
Objective 1: To compare the proportion of *An. gambiae* s.s. and *An. arabiensis* and *An. funestus* s.l. mosquitoes collected using different sampling methods	• Compared to indoor HLCs, vector density was lower using CDC light traps and prokopacks. • Sporozoite rates were similar between indoor methods, although precision was limited. • Compared to outdoor HLCs, vector density was higher using pit trap collections,while the sporozoite rate was lower. • *An. funestus* were predominant in the prokopack collections (75.0%) comparedto the indoor HLCs (25.8%) • *An. arabiensis* were predominant in the pit trap collections (84.3%) compared to the outdoor HLCs (36.9%).
Objective 2: To describe malaria vector species composition in areaswith differing vector control interventions.	• In Walukuba (low malaria transmission site), LLIN distribution was associated with a decline in *Anopheles funestus* vector density (0.07 vs 0.02) mosquitoes per houseper night, density ratio, but not *Anopheles gambiae* s.s. nor *Anopheles arabiensis*. • In Kihihi (moderate malaria transmission site), LLIN distribution was associated with a decline in *An. gambiae* s.s. vector density • In Nagongera (high malaria transmission site), the combination of LLINs and multiple rounds of IRS was associated with near complete elimination of *An. gambiae* s.s. (28.0 vs 0.17), and *An. funestus* s.l., with a less pronounced decline in *An. arabiensis*.
Objective 3: To determine insecticidesusceptibility of malaria vectors in areas with differing vector control	• Overall, mortality following exposure to permethrin and deltamethrin was 18.8% in *An. gambiae* s.s. and 74.6% in *An. arabiensis*. • Mortality was significantly lower in *An. gambiae* s.s. than *An. arabiensis* to both permethrin and deltamethrin

interventions **and** **Objective 4: To evaluate the association between genetic polymorphisms (*Cyp6aa1, Cyp6p4, ZZB-TE, Cyp4j5, Coeae1d, Vgsc- L995S/L1014S* and *Vgsc- L995F/L1014F*) and mosquito survivalin pyrethroid exposed *An. gambiae* s.l. adults.**	• Mortality to permethrin was significantly lower than that to deltamethrin • PBO increased mortality to permethrin and deltamethrin for both *An. gambiae* s.s.,and *An. arabiensis*. • *An. gambiae* s.s. had the *Vgsc-995S* frequency ranging from 67.09%-93.13%; *Vgsc- 995F* frequency ranged from 3.43%-28.40% and the *Cyp6p4* resistance allele (87%),while the frequency of *Cyp4j5* and *Coeae1d* were lower (52% and 55%, respectively). • Significantly higher *Vgsc-995F* frequency was observed in *An. gambiae* s.s. from the'IRS stopped' area compared to 'No IRS' area. • The *Vgsc-995S/F* mutation was significantly associated with *An. gambiae* s.s.survival to deltamethrin • *An. arabiensis* were predominantly wildtype for the *kdr* mutation (>89%) *Vgsc-L1014S/F* • The triple mutation (*Cyp6p4, Cyp6aa1, ZZB-TE*) was associated with *An. gambiae* s.s. survival to deltamethrin and to permethrin plus PBO • Cytochrome p450 *Cyp4j5* was associated with survival to deltamethrin plus PBO

7.4 KEY RESULT 1

CDC light traps and prokopack aspirators are alternative methods for sampling mosquitoes indoors, but prokopacks are less efficient in sampling malaria vectors. Pit traps could be a useful alternative for sampling outdoor resting mosquitoes, but target resting not host seeking *Anopheles* vectors.

Alternative mosquito collection methods were compared to HLCs, the gold standard, for both indoor and outdoor collections. Indoors, both CDC light traps and prokopack aspirators had significantly lower vector density compared to indoor HLCs overall. When stratified by species, the density of vectors collected using CDC light traps was not significantly different from indoor HLCs for both *An. gambiae* s.s. and *An. funestus*. This showed that CDC light traps can be used as alternatives to HLCs indoors to generate comparable vector density estimates for highly anthropophilic malaria vectors [6, 7]. Overall, CDC light traps are an attractive alternative to indoor HLCs, given evidence of their comparable sampling efficiency to HLCs, and feasibility for use on a larger scale than HLCs [8].

In a similar study conducted in the same study area Tororo, Kilama et al, [9] reports a positive correlation between CDC light traps and indoor HLCs for estimation of human biting rates (which are a function of mosquito density) [9]. However, this study focused on indoor collections and did not evaluate prokopack aspirators or outdoor collections. In a pooled analysis of the applications and limitations of CDC light traps in comparison to HLCs, Briet et al, [10], recognizes that CDC light traps collected similar vector densities for *An. gambiae* s.l. and *An. funestus* s.l. as compared to HLC indoors, however, the sampling efficiency varied significantly between studies [10]. In Bioko Island, however,

no correlation was observed between CDC light traps and indoor HLCs in collection of *An. gambiae* s.s. with CDC light traps reported to be unreliable in estimating mosquito biting rates [11].

Significantly lower vector density was observed with CDC light traps compared to indoor HLCs in collection of *An. arabiensis*. This finding may be due to the tendency of *An. arabiensis* to bite and rest outdoors in this region of Uganda (Tororo) [12], as well as the zoophilic nature observed elsewhere (Ethiopia) in this species [13]. A combination of these characteristics may limit the number of *An. arabiensis* entering the houses. In contrast, in Macha, Zambia, CDC light traps captured almost twice as many *An. arabiensis* as indoor HLCs [14]. Specific behavioral characteristics of the different *Anopheles* species may affect the sampling efficiency of CDC light traps when compared to HLCs as observed by Briet et al, [10]. CDC light traps have utility in longitudinal studies with repeated measurements [9], however, current CDC light trap models do not have a monitoring module to ascertain whether the trap was active throughout the sampling period.

Prokopack aspirator collections resulted in a higher vector density as compared to indoor HLCs, although this difference was not significant. Overall *An. funestus* were predominant in the prokopack collections accounting for 75.0% of all *Anopheles* collected compared to the indoor HLCs where only 25.8% were *An. funestus.* In comparison, prokopacks had significantly lower density for both *An. gambiae* s.s. and *An. arabiensis.* Prokopacks have been reported to be less efficient in collecting malaria vectors and their sampling efficiency may be affected by the species composition of *Anopheles* vectors in the study population [15].

Compared to outdoor HLCs, vector density was significantly higher overall using pit trap collections however, the sporozoite rate was significantly lower. Pit traps target outdoor resting mosquitoes which may not necessarily be host seeking or could be zoophilic, gravid or unfed mosquitoes [16, 17]; these characteristics may account for the high vector density yet significantly lower sporozoite rates than outdoor HLCs. Stratification of vector density by species revealed significantly higher vector density for *An. arabiensis* in the pit traps compared to HLCs outdoors. This observation is likely supported by findings from Musiime et al, [12], demonstrating increased outdoor biting behavior in *An. arabiensis* (collected from the study area-Tororo) in response to vector control which may naturally align to outdoor resting behavior observed in this species [18]. *An. arabiensis* were predominant in the pit trap collections accounting for 84.3% of all collections compared to the outdoor HLCs where *An. arabiensis* accounted for 36.9%. Despite the general finding that pit traps collections showed higher vector densities than outdoor HLCs, stratification of the data by species showed that vector density for both *An. gambiae* s.s. and *An. funestus* was significantly lower in the pit traps than outdoor HLCs, highlighting the importance of analyzing species specific differences. *Anopheles gambiae* s.s. and *Anopheles funestus* are are regarded as highly anthropophilic vectors [6, 7, 18, 19] which may explain the higher density of both species observed in outdoor HLCs compared to pit traps. Whereas, HLCs outdoors attract host seeking mosquitoes [20], pit traps primarily attract outdoor resting mosquitoes [16] these inherent differences in the two methods suggest that, each method may be sampling a different mosquito population. Nonetheless, pit traps have been reported elsewhere (Tanzania) to collect higher densities of *An. arabiensis* compared to outdoor HLCs and may be feasible alternatives HLCs in sampling *An. arabiensis* outdoors.

7.5 KEY RESULT 2

The impact of LLINs and IRS on *Anopheles* density and species composition varied in sites with differing malaria transmission intensity.

The impact of LLINs and IRS on malaria vector density and species composition was investigated in three sites with varying malaria transmission intensity in Uganda. LLIN distribution in the low transmission site (Walukuba, Jinja) was associated with a significant decline in *An. funestus,* but not *An. gambiae* s.s. or *An. arabiensis.* Similar observations were reported in Tanzania by Kreppel et al, [21], where *An. funestus* s.l declined 10 fold following distribution of LLINs however, the apparent decline in abundance of *An. arabiensis* was not significant [21]. Conversely, the distribution of LLINs in the moderate transmission site (Kihiihi, Kanungu) was associated with a significant decline in *An. gambiae* s.s. vector density. This was due, in part, to the fact that nearly all mosquitoes (98%) collected from this site were *An. gambiae* s.s. In addition, declines in abundance of *An. gambiae* s.s. following LLIN distribution have been described in Kenya and Senegal [22, 23]. In the high transmission site (Nagongera, Tororo), both LLINs and IRS were deployed. The combination of LLINs and IRS was associated with near complete elimination of *An. gambiae* s.s. and *An. funestus* s.l. but with less pronounced decline in *An. arabiensis*. This finding underscores the fact that the combination of LLINs and IRS resulted in an overall decline of species abundance for all the sympatric vector species. However, the observed decline was less pronounced in *An. arabiensis*. The gradual decline of *An. gambiae* s.s. and *An. funestus* s.s. vector populations in response to vector control has also been documented in studies from neighboring Kenya and Tanzania [21, 22, 24]. In experimental hut trials in Muheza, Tanzania, a change in species composition resulting from more *An. gambiae* s.s. and *An. funestus* s.s. killed by pyrethroid treated LLINs is associated with pyrethroid resistance in *An. arabiensis*, which were found to be more resistant. In the study areas of Tororo and Jinja, evidence of pyrethroid

resistance has been documented in *An. arabiensis* [3, 25]. This however is an unlikely explanation for less pronounced impact of non-pyrethroid IRS (bendiocarb) on *An. arabiensis* vector density, as no carbamate or organophosphate resistance was reported in this species during the study period. Behavioral avoidance of *An. arabiensis* to insecticide based interventions which limits the physical contact of this mosquito species to indoor vector control tools has been reported in Ethiopia [26]. The combination of behavioral avoidance, and the zoophilic nature of *An. arabiensis* is the most plausible explanation for the less pronounced impact of LLINs and IRS on *An. arabiensis* vector density.

In addition, whereas *An. gambiae* s.s. and *An. funestus* sampled from the study area have been reported to be resistant to pyrethroids [3, 25, 27] there was no indication of resistance to the carbamate bendiocarb used for IRS. The increase in relative abundance of *An. arabiensis* may be due to the fact that highly anthropophagic *Anopheles* vectors, including *An. gambiae* s.s. and *An. funestus* s.s., are particularly responsive to indoor based vector control [12, 18, 28]

7.6 KEY RESULT 3

Resistance to pyrethroids was widespread across Uganda, and higher in *An. gambiae s.s.* than *An. arabiensis*. Where IRS was active, *An. arabiensis* dominated. Addition of PBO to pyrethroids increased mortality. Most *An. gambiae* s.s. had the target site mutation *Vgsc-9*95S and the *Cyp6p4* resistance allele, while the frequency of *Cyp4j5* and *Coeae1d* was lower. Most *An. arabiensis* were wild type for *kdr*.

Phenotypic and genotypic assays were performed in 11 sites stratified by IRS status, namely 'No IRS' (Mubende, Busia, Kayunga); IRS stopped (Lamwo, Amuru, Agago, Kole) and IRS active (Amolatar, Dokolo. Otuke, Tororo). Pyrethroid resistance to permethrin and deltamethrin was widespread in all the 11 study sites similar to observations in other studies from Uganda [25, 29] and elsewhere [1, 30-

34].). Species-specific differences were observed in the resistant phenotype and showed that *An. gambiae* s.s. had significantly lower mortality than *An. arabiensis* in exposure to both permethrin and deltamethrin. Similar observations were reported by Abeku et al, [35] in Uganda and by Ochomo et al, [34] and Mathias et al, [36] in Western Kenya, where *An. gambiae* s.s. were more resistant than *An. arabiensis* to permethrin and deltamethrin. Prior exposure of pyrethroid insecticides to PBO (piperonyl butoxide), significantly increased mortality to permethrin and deltamethrin exposure regardless of species. This was reported in several studies from Uganda [25, 29] and elsewhere [32, 37-41]. Increase in mortality with PBO exposure was indicative of involvement of cytochrome p450 monooxygenases in the resistance phenotype [42, 43] and provided evidence to support the use of PBO LLINs in study areas. Target site mutation *Vgsc-995S* was found to be approaching fixation in *An. gambiae* s.s. but at very low frequency(<11%) in *An. arabiensis*. Patterns of high knock down resistance (*kdr*) frequency are reported by Lynd et al, [44] from a cross- sectional survey of 48 districts in Uganda [44]. The alternative *kdr* mutation *Vgsc-995F* was found at a frequency <30%, particularly in Northern Uganda, in districts where 'IRS was stopped'. Additionally, the frequency of *Vgsc-995F* in the 'IRS stopped' sites was significantly higher than in the 'No IRS' sites. This suggests significant insecticide selection pressure selecting for resistance genotypes associated with pyrethroid resistance in the 'IRS stopped' stopped sites. In *An. gambiae* s.s. from the 'IRS stopped' sites mortality to permethrin was significantly lower than to deltamethrin when compared to the 'No IRS' sites. This may partly be explained by the presence of the *Vgsc-995F* mutation at appreciable frequencies (<30%) in the 'IRS stopped sites. The *Vgsc-995F* mutation is associated with strong phenotypic resistance conferring greater resistance to permethrin (type-1 pyrethroid) compared to deltamethrin (type-2 pyrethroid) [45]. In West Africa, another mutation in the voltage gated channel, *Vgsc-N175Y* found on the *Vgsc-1014F* (now *Vgsc-995F*) haplotype background in *An. gambiae* s.s. is shown to confer a stronger resistance phenotype to pyrethroids [46]. In genotype and phenotype measures of associationtarget site mutations *Vgsc-995S/F* were associated with resistance to deltamethrin. Ramphul et al,[3] in samples collected from eastern

Uganda reports a significant relationship between *Vgsc-1014S* in *An. gambiae* s.s. and resistance to permethrin. Whilst, the recently described triple mutation (*Cyp6p4, Cyp6aa1, ZZB-TE*) was found at a frequency of 87% in the study areas, Njoroge et al, [47] describes a triple mutant frequency of 29.7% in a pyrethroid resistant colony from Busia, Uganda. In this study *Cyp6p4* was used as the marker of reference for the triple mutation due to the very high level of correlation (Spearman's rank correlation >70%). *Cyp6p4* was associated with resistance to deltamethrin, as observed by Njoroge et al,[47]. However, the triple-mutant was associated with *An. gambiae* s.s. survival to permethrin following PBO exposure, a novel observation that was unexpected given the expected blocking effects of PBO on P450 enzyme activity [42]. Of note, it is argued by Feyereisen [48], that PBO does not inhibit all p450 enzymes equally [48]. However, Njoroge et al. [47] demonstrated that PBO LLINs were effective against a pyrethroid-resistant colony (from Busia, Uganda) with a triple-mutant frequency of 29.7%. Another novel finding in this thesis is the association between metabolic resistance marker *Cyp4j5* and mosquito survival following exposure to deltamethrin plus PBO; which similarly was unexpected. Notably, Weetman et al, 2018 found a significant association between *Cyp4j5* and pyrethroid resistance (deltamethrin and permethrin). To the best of my knowledge and from the literature examined the *Cyp6p4* (triple mutant) and *Cyp4j5* resistance marker association(s) relationship with PBO has not previously been evaluated.

7.4 Implications of thesis findings

The method of collecting mosquitoes for entomologic surveillance can impact on outcome measures, including vector density, species composition and sporozoite infection rate estimates. CDC light traps were found to have comparable sampling efficiency with indoor HLCs and are the leading alternative to HLCs for indoor mosquito collections. Prokopack aspirators were found to be less efficient in sampling *An. gambiae* s.s. and *An. arabiensis* in this setting, showing high-representation of *An.*

funestus s.l. However, prokopacks are an attractive alternative for sampling mosquitoes on a large scale. Outdoors, pit trap collections showed a considerably high-representation of *An. arabiensis* and were not efficient in sampling anthropophilic *An. gambiae s.s.* and *An. funestus*. The utility of pit traps may therefore be limited to sampling *An. arabiensis* and could serve as an alternative to outdoor HLCs for examining vector density in this species.

The combination of LLINs and IRS resulted into a steep decline in vector density for both *An. gambiae* s.s. and *An. funestus* s.l. but was less pronounced in *An. arabiensis*. The selective advantage to *An. arabiensis* and subsequent increase in abundance of this species resulted into having *An. arabiensis* as the predominant *Anopheles* species in Tororo post IRS [12]. The ability of *An. arabiensis* to avoid the insecticidal contact with current indoor based vector control tools coupled with increase in abundance of this species is of particular concern, given that increased outdoor biting in this species has been reported in the study area [12]. Outdoor biting *An. arabiensis* present a challenge for current indoor based vector control tools and may increase the threat of outdoor malaria transmission.

Despite high levels of pyrethroid resistance observed in the 11 study sites, prior exposure of *Anopheles* mosquitoes to the synergist PBO increased mortality to both permethrin and deltamethrin promoting the use of PBO LLINs in these areas. Two cluster randomized trials, including 1 in Uganda [49] and 1 in Tanzania [50] showed significant declines in *Anopheles* vector density and *Plasmodium falciparum* parasite prevalence associated with deployment of PBO LLINs. In addition, WHO's recently updated guidelines on malaria control, recommend the use of PBO LLINs in areas with high pyrethroid resistance [51]. As PBO LLINs are scaled-up, there is need for increased surveillance of metabolic resistance, as the novel findings in this thesis suggest.

7.5 Thesis strengths

This thesis has several strengths. First, this thesis provides an overview of insecticide resistance patterns in East Africa from a review of 40 studies dated from 1990 to 2022. The comprehensive assessment and synthesis of available data from selected articles provides a body of knowledge from which several gaps can be identified. Second, comparison of mosquito collection methods accounted for vector density, species composition, sporozoite infection and annual Entomological Inoculation Rates (aEIR). The study area was adequately covered using randomly selected households selected from an enumeration database to eliminate sampling bias. Data collection time points were harmonized so that collections were comparable over time and space. Third, the impact of vector control interventions on *Anopheles* vector density and species composition was assessed using longitudinal data with mosquitoes collected over a nearly 5-year (58 months) period. Absolute numbers of mosquitoes collected were used, as well as relative proportions, increasing the precision of vector density estimates. Moreover, the timing of interventions was taken into account. The impact of control interventions was assessed for the three primary malaria vectors, *An. gambiae* s.s., *An. arabiensis* and *An. funestus* s.l in different study settings, with inclusion of outcomes from the LLIN and IRS combination. This thesis presents a robust evaluation of malaria vector control interventions on species composition and vector density in Uganda. Fourth, the resistance phenotype was determined over a wide geographical area representing the Northern, Central and Eastern parts ofthe country. This thesis reports high levels of pyrethroid resistance particularly in the 'IRS stopped' area, where the presence of resistance mutations was more pronounced, generating an opportunity for subsequent investigations into the underlying markers of metabolic resistance in *Anopheles* survivors of PBO and insecticide exposure. Finally, this thesis explores the contribution of the recently described triple mutation in defining resistance to pyrethroids as well as examining the contribution of metabolic resistance to survivors of PBO plus insecticide exposure.

7.6 Thesis limitations

This thesis had several limitations; briefly outlined in each of the result chapters. The limitations are discussed in more depth here.

For comparison of mosquito collection methods

First, mosquito parameters such as parity, abdominal status and blood meal index, which provided information on mosquito age, blood feeding success and source of blood meal to distinguish mosquitoes feeding on humans and on animals were not measured. Second, not all available methods for indoor and outdoor alternatives were included in the study due to feasibility limitations and cost implications. Third, the study on mosquito collection methods was geographically limited to households located in 3 parishes within 2 districts in Eastern Uganda, and these findings may not be generalizable to other settings. Fourth, the houses used for CDC light trap collections were not the same as those used for other collection methods and variability in household characteristics was not accounted for. Fifth, the data collection period for comparing mosquito collection methods was five months and not a complete calendar year, which may have provided more robust aEIR estimates.

For assessment of malaria control interventions on Anopheles density and species composition.

First, the findings were limited in geographical scope to three sub-counties from three districts, and may not be generalizable to other settings. Notably, however, mosquito collections were conducted in randomly selected households to eliminate bias. Second, mosquito collections were only conducted indoors using CDC light traps, it is unknown if different mosquito collection methods would yield the same results. Third, sporozoite data for each *Anopheles* species were not collected, which limited

assessment of malaria transmission patterns. Fourth, whereas pyrethroidresistance was documented in both *An. gambiae s.s* and *An. arabiensis* [52] within the study area, with evidence of carbamate resistance observed in *An. gambiae s.s.* from Nagongera and Kihihi [52], the extent to which insecticide resistance affected mosquito survival under field conditions was not assessed. Fifth, study sites were not randomized to receive particular interventions; longitudinal measurements of mosquito density were made alongside vector control interventions delivered by the Uganda Ministry of Health. Of note, whereas monthly rainfall measurements were used in the analysis and interpretation of the results, temperature and humidity data were unavailable for the study period.

Characterizing pyrethroid resistance and mechanisms in Anopheles gambiae and An. arabiensis

First, the findings are limited by the cross-sectional sampling done in only 11 districts. This may have introduced bias, however, sampling from several districts provided a snapshot of pyrethroid resistance in geographically distinct areas. Second, the definitions of insecticide resistance are based on WHO cut-offs using diagnostic concentrations of permethrin (0.75%) and deltamethrin (0.05%). Pyrethroid intensity assays to determine the operational significance of insecticide resistance were not conducted due to sample size limitations. Third, sample size limitations reduced the statistical power available to adequately test genotype: phenotype associations, nonetheless there are significant associations between target site/metabolic resistance markers with pyrethroid resistance. Fourth, the concentration of PBO used was 4.0% which may not be directly comparable to the concentration of PBO on LLINs. In a study of PBO LLINs distributed by the Ugandan Ministry of Health in 2017-18, the concentration of PBO at baseline was 26.81 g/kg in PermaNet 3.0, and 8.17 g/kg in Olyset Plus [53] which may not be equivalent to the concentration included in the WHO tube assay. Finally, the absence of historical data before LLIN and/or IRS implementation limited the inferences that could be made on the development and spread of pyrethroid resistance mutations. Moreover, metabolic resistance

mechanisms were not explored in *An. arabiensis* due to resource limitations.

7.7 Conclusion

Overall, this thesis examined alternative mosquito collection methods compared to HLCs, highlighting the importance of considering the mosquito sampling method when designing studies and programs and interpreting entomologic outcomes. In areas of low and moderate malaria transmission, universal distribution of LLINs resulted in substantial reductions in *An. gambiae s.s*. and *An. funestus s.l.*, while deployment of LLINs plus IRS in the highest transmission site resulted in the near collapse of these main vectors, with *An. arabiensis* becoming the predominant malaria vector. In Uganda, resistance to pyrethroids was widespread, and higher in *An. gambiae* s.s. than *An. arabiensis*. The addition of the synergist PBO to pyrethroids, increased mortality supporting the use of PBO LLINs in the study areas. The role of metabolic resistance in understanding the underlying mechanisms of resistance in mosquitoes that survive PBO plus insecticide exposure was identified as a critical knowledge gap.

7.8 REFERENCES

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8.0 Appendices

8.1 List of Appendices

Appendix A-School of Medicine Research Ethics Committee Amendment

Appendix B-London School of Hygiene and Tropical Medicine Ethics Approval

Appendix C- School of Medicine Research Ethics Committee Approval

Appendix D: Uganda National Council of Science and Technology Approval

Appendix A: School of Medicine Research and Ethics Committee Approval

MAKERERE UNIVERSITY
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COLLEGE OF HEALTH SCIENCES
SCHOOL OF MEDICINE

RESEARCH ETHICS COMMITTEE

July 08, 2022

Prof. Moses Kamya
Department of Internal Medicine

Category of review
[ ] Initial review
[ ] Continuing review
[X] Amendment
[ ] Termination of study
[ ] SAEs

Dear Prof. Kamya,

RE: REC REF 2019-134

Title: "Cohort and entomology studies to compare malaria metrics in the Tororo and Busia Districts of Uganda Vs. 8.0 dated 20th June 2022"

Your proposal entitled **"Cohort and entomology studies to compare malaria metrics in the Tororo and Busia Districts of Uganda"** was initially reviewed and approved by the School of Medicine Research and Ethics committee on 14th October 2019.

On 22nd June 2022, you requested for permission to update the protocol version from 7.0 dated 07th April 2022 to 8.0 dated 20th June 2022 to incorporate the following changes;

- To add a Label-free malaria scope, an automated microscopy-based malaria diagnostic tool developed by scientists from the Zuckerberg Bio Hub in San Francisco (section 6.3.17 under laboratory methods added to study protocol). The device operates by imaging fresh blood in liquid form without fixation or staining and uses machine learning to process images of blood cells and detect malaria parasites and quantify parasite density.

- To extend the duration of follow-up for study participants from August 2022 to March 31st 2024 when the funding period for this study will end because since recruitment began in August 2020 participants have been consented to be followed for up to 2 years and some of them would reach this end point in August 2022. Participants enrolled after approval of protocol version 8.0 will be consented using updated forms.

- To update the parental main consent form, adult main consent form and the assent form for the purpose of re-consenting active study participants to allow for the study follow-up period to be extended.

The committee considered these changes on 08th July 2022. On behalf of the committee, I am glad to inform you that these changes have been approved. Please forward regular reports on your study to the committee.

Yours sincerely,

[redacted]

MAKERERE UNIVERSITY
SCHOOL OF MEDICINE
AMENDMENT
★ 08 JUL 2022 ★
RESEARCH & ETHICS COMMITTEE
P.O. BOX 7072,KAMPALA

Dr. Aloysius Gonzaga Mubuuke
Vice Chairperson School of Medicine Research & Ethics Committee

Appendix B: LSHTM Ethics Approval

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
United Kingdom
Switchboard: +44 (0)20 7636 8636

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LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

Observational / Interventional Research Ethics Committee

Prof Sarah Staedke
LSHTM

1 September 2022

Dear Prof Sarah Staedke,

Study Title: Cohort and entomology studies to compare malaria metrics in the Tororo and Busia Districts of Uganda

LSHTM Ethics Ref: 17777 - 09

Thank you for your letter responding to the Observational Committee's request for further information on the above amendment to research and submitting revised documentation.

The further information has been considered *on behalf of the Committee by the Chair.*

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above amendment to research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval for the amendment having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Other	PRISM Border Cohort Study protocol version_8.0_track changes	20/06/2022	8.0
Other	PRISM Border Cohort Study ADULT Main Participation IC Vers 5.0 _track changes	20/06/2022	5.0
Other	PRISM Border Cohort Study ASSENT Main Participation IC Vers 4.0_track changes	20/06/2022	4.0
Other	PRISM Border Cohort Study PARENTAL Main Participation IC Vers 4.0_track changes	20/06/2022	4.0
Local Approval	PRISM SOMREC Approval letter Vs 8	08/07/2022	8.0
Covering Letter	Cover_letter-LSHTM.doc	15/08/2022	1.0

After ethical review

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project by submitting a Serious Adverse Event form.

An annual report should be submitted to the committee using an Annual Report form on the anniversary of the approval of the study during the lifetime of the study.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: http://leo.lshtm.ac.uk

Additional information is available at: www.lshtm.ac.uk/ethics

Yours sincerely,

[redacted]

APPENDIX C: SOMREC APPROVAL

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COLLEGE OF HEALTH SCIENCES
SCHOOL OF MEDICINE

RESEARCH ETHICS COMMITTEE

March 14, 2022

Mr. Henry Ddumba Mawejje
Infectious Diseases Research Collaboration (IDRC)

Category of review
[ ] Initial review
[X] Continuing review
[ ] Amendment
[ ] Termination of study
[ ] SAEs

Dear Mr. Mawejje,

RE:, REC REF 2018-066

Title: "Investigating spatial and localized interactions between insecticide resistance, insecticidal Malaria vector control and Malaria transmission in Anopheles mosquitoes from Uganda"

Renewal Approval Date: 14th March 2022
Project Expiration Date: 27th March 2023

On behalf of the committee, I write to inform you that the **proposed extension has been approved.** The School of Medicine Research and Ethics Committee (REC) initially reviewed and approved the above-referenced protocol on 28th March 2018. Previous approval of this protocol expires on March 27th 2022.

Renewals: REC approval is valid until the expiration date given above. If you are continuing your project, you must submit an Application for renewal at least six (6) to eight (8) weeks before the lapse date. If the project is completed, please submit an application for permanent closure.

Amendments: The REC must review any changes in the project, prior to initiation of the change. Please submit an Application for Amendments to have your changes reviewed and summarize the proposed change and the rationale for it in a letter to the School of Medicine Research and Ethics Committee. If changes are made at the time of renewal, please include an Application for Amendments with the renewal application.

In addition, submit three (3) copies of an updated version of your original protocol application- one showing all proposed changes in bold or 'track changes,' and the other without bold or track changes.

Adverse Events: If issues should arise during the conduct of the research, such as unanticipated problems, severe adverse events or any other problem that may increase the risk to the human subjects, notify the REC Chairman promptly. The forms are available to report these issues.

Please use the REC REF number listed above on any forms submitted which relate to this project/study.

Good luck in your research. If we can be of further assistance, please contact us at (+256) 0414 - 533541 or via email at research@chs.mak.ac.ug/rresearch9@gmail.com. Thank you for your cooperation.

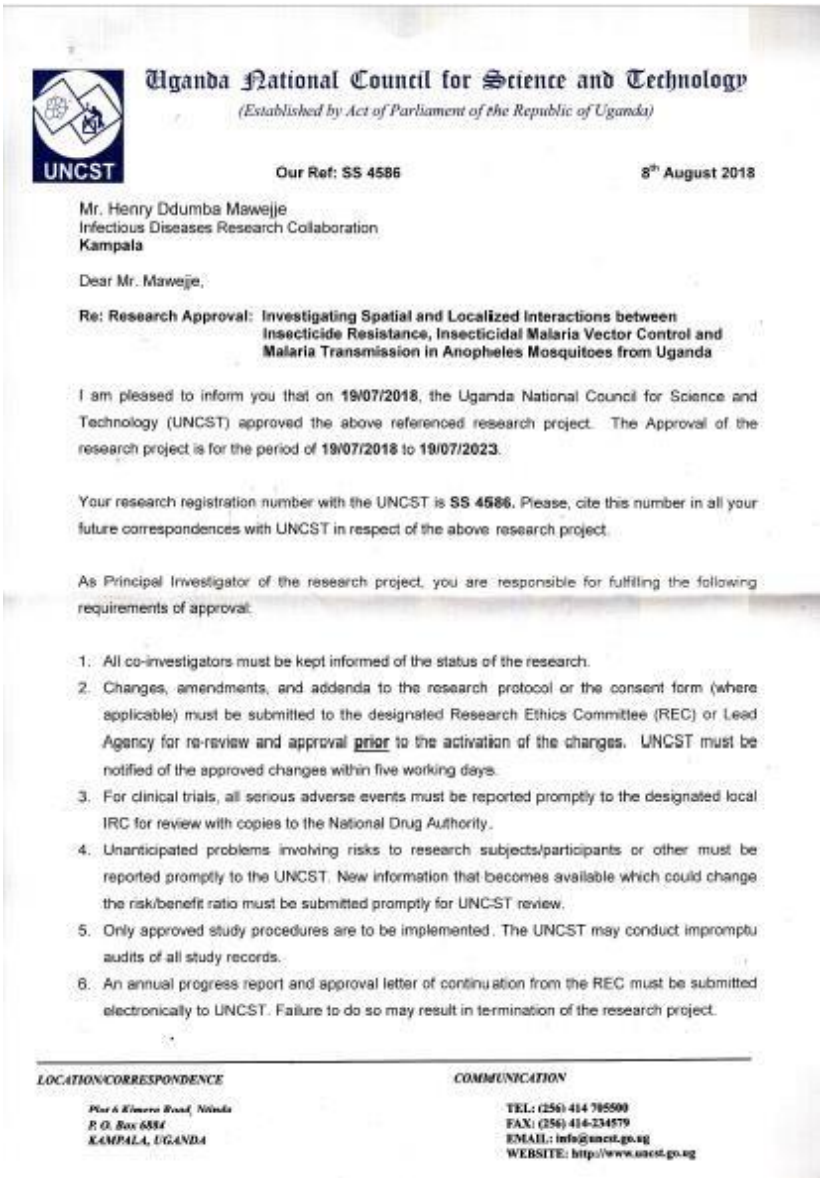
MAKERERE UNIVERSITY
SCHOOL OF MEDICINE
RENEWAL
VALID UNTIL
27 MAR 2023
RESEARCH & ETHICS COMMITTEE
P.O. BOX 7072,KAMPALA

Yours Sincerely,

[redacted]

Assoc. Prof. Ponsiano Ocama
Chairperson School of Medicine Research and Ethics Committee

APPENDIX D: UNCST APPROVAL



Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

UNCST

Our Ref: SS 4586

8th August 2018

Mr. Henry Ddumba Mawejje
Infectious Diseases Research Collaboration
Kampala

Dear Mr. Mawejje,

Re: Research Approval: Investigating Spatial and Localized Interactions between Insecticide Resistance, Insecticidal Malaria Vector Control and Malaria Transmission in Anopheles Mosquitoes from Uganda

I am pleased to inform you that on **19/07/2018**, the Uganda National Council for Science and Technology (UNCST) approved the above referenced research project. The Approval of the research project is for the period of **19/07/2018** to **19/07/2023**.

Your research registration number with the UNCST is **SS 4586.** Please, cite this number in all your future correspondences with UNCST in respect of the above research project.

As Principal Investigator of the research project, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the research protocol or the consent form (where applicable) must be submitted to the designated Research Ethics Committee (REC) or Lead Agency for re-review and approval prior to the activation of the changes. UNCST must be notified of the approved changes within five working days.
3. For clinical trials, all serious adverse events must be reported promptly to the designated local IRC for review with copies to the National Drug Authority.
4. Unanticipated problems involving risks to research subjects/participants or other must be reported promptly to the UNCST. New information that becomes available which could change the risk/benefit ratio must be submitted promptly for UNCST review.
5. Only approved study procedures are to be implemented. The UNCST may conduct impromptu audits of all study records.
6. An annual progress report and approval letter of continuation from the REC must be submitted electronically to UNCST. Failure to do so may result in termination of the research project.

LOCATION/CORRESPONDENCE

Plot 6 Kimera Road, Ntinda
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COMMUNICATION

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Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Below is a list of documents approved with this application:

	Document Title	Language	Version	Version Date
1.	Research proposal	English	2.0	February 2018
2.	Informed consent forms	English, Langi, Lugbara, Rukiga, Runyankore, Lusoga and Acholi	2.0	February 2018

Yours sincerely

[redacted]

For: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

Copied to: Chair, Makerere University School of Medicine, Research Ethics Committee