



**Impact of Exposure to Long Lasting
Insecticide Treated Nets on Mosquito
Survival and Behaviour at the Net
Interface in Insecticide Susceptible and
Resistant Strains of the Afrotropical
Anopheles mosquito**

Thesis submitted in accordance with the requirements of the
University of Liverpool for the degree of Doctor of Philosophy

by Angela Jane Hughes

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Abstract

The central role of long-lasting insecticidal nets (LLINs) in malaria prevention in Africa is threatened by insecticide resistance. Little is known about mosquito behaviour near, or of the importance of sub-lethal or delayed impacts of, the most widely-used LLIN types, and yet these effects are fundamental for developing new net treatments and for understanding the nature of resistance and managing its emergence.

Effects of insecticide exposure on longevity and reproductive output of pyrethroid-resistant and susceptible adult female *Anopheles gambiae* mosquitoes were investigated using standard tests and newly developed video assays. To investigate effects on longevity, mosquitoes were repeatedly exposed to LLINs (PermaNet® 2.0, Olyset®) or untreated netting using WHO cone bioassays, at various intervals between 4- and 16-days post-emergence, to simulate the natural exposure of repeat blood feeding attempts. Effects on reproductive fitness were investigated by quantifying egg production (and hatch rate) in mosquitoes that had bloodfed through an LLIN or bloodfed after repeated LLIN exposure.

On average, over all experiments, longevity of moderate and highly resistant mosquitoes was reduced significantly from 11.3 to 6 days, and 14.6 to 9.2 days, respectively, following exposure to PermaNet. Egg production by mosquitoes exposed to LLINs was not significantly different to those on untreated net. In extremely resistant mosquitoes, this delayed mortality effect was reduced, which would ensure that pyrethroids continued to reduce the transmission potential of insecticide resistant mosquitoes but could eventually be eroded by continued, intense selection for resistance.

Precisely how insecticides affect mosquito behaviour has never been fully elucidated or quantified. Using two simple bench top tests, the Cone Video Test and the Thumb Test, detailed behavioural events associated with insecticide exposure were compared in insecticide susceptible and resistant strains of *Anopheles gambiae* and *Anopheles funestus* at different LLIN types (PermaNet® 2.0, Olyset®, Olyset Duo® and Duranet®).

The Cone Video test employs scan-sampling to quantify a video record of behaviour during a standard 3-min WHO cone test, classifying activity as resting on net, on cone, or in-flight. Throughout all tests, susceptible and resistant strain mosquitoes rested preferentially on untreated and treated netting, except for susceptible mosquitoes on Olyset®, where net resting was significantly lower than the other LLIN types. Notably, this effect was not seen in pyrethroid resistant mosquito strains. In a variant of the same bioassay, when direct LLIN contact was prevented, resting activity was restored, suggesting that the different response to Olyset was a contact-irritancy response rather than non-contact repellency. In Cone Video tests that incorporated a human host, increased net contact occurred in all tests, suggesting that host attraction exceeds any deterrent properties on these LLINs.

The Thumb tests enable quantification of the detailed behavioural events during feeding, or attempts to feed, by a single mosquito at a human-baited LLIN interface. Here, all mosquito strains readily fed through all LLINs tested, but the duration of blood feeding was significantly lower than on untreated net, in all susceptible and resistant mosquito strains.

The study findings also highlight the importance of investigating the impacts of resistance beyond immediate mortality. It is possible that delayed mortality effects, or other fitness costs from LLIN exposure, may be reducing the impact of resistance on LLIN performance. However, as the degree of resistance increases, over the lifetime of resistant mosquitoes, the magnitude of these fitness costs may diminish and eventually disappear.

Declaration

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Table of contents

Abstract.....	i
Declaration.....	ii
Acknowledgments.....	iii
Table of contents.....	v
Table of figures.....	x
Table of tables.....	xiii
1 Background information	1-1
1.1 Thesis themes	1-1
1.2 Malaria - a persistent problem	1-1
1.3 Pathophysiology.....	1-2
1.4 Mosquito disease vectors	1-2
1.5 <i>Anopheles</i> : the primary disease vector.....	1-3
1.5.1 Key elements of the mosquito life cycle	1-4
1.6 Insecticide based vector control.....	1-5
1.6.1 LLINs	1-5
1.6.2 IRS.....	1-5
1.6.3 Modes of action	1-6
1.6.4 Combining LLINs and IRS.....	1-7
1.6.5 Testing efficacy of LLINs and IRS interventions	1-7
1.6.6 Larval control	1-8
1.6.7 Supplementary tools.....	1-8
1.7 WHO recommended products for malaria control.....	1-10
1.7.1 Pyrethroids.....	1-11
1.7.2 Carbamates and Organophosphates	1-12
1.7.3 Neonicotinoids	1-12
1.7.4 Organochlorines.....	1-13
1.7.5 Alternative compounds.....	1-13
1.8 Insecticide resistance	1-15
1.8.1 Mechanisms of IR.....	1-16
1.8.2 Cross resistance	1-20
1.9 Impact of IR on malaria transmission	1-22

1.9.1	Personal protection.....	1-22
1.9.2	Community effect of LLINs.....	1-23
1.9.3	Vectorial capacity.....	1-23
1.10	Behavioural response to insecticides.....	1-28
1.11	Detecting and monitoring IR.....	1-29
1.11.1	Bioassay limitations.....	1-30
1.12	Aims and Objectives of this study.....	1-31
2	Lifelong impact of prolonged exposure to pyrethroids in insecticide resistant <i>Anopheles gambiae</i>	2-32
2.1	Introduction.....	2-32
2.2	Aims and Objectives.....	2-33
2.3	Methods.....	2-34
2.3.1	Mosquito colonies.....	2-34
2.3.2	Long-lasting insecticide treated nets (LLINs).....	2-35
2.3.3	Standard WHO Cone bioassay protocol.....	2-36
2.3.4	Blood feeding.....	2-37
2.3.5	Egg quantification – <i>en masse</i> egg counts (filter paper).....	2-37
2.3.6	Egg quantification – individual egg counts (dissections).....	2-37
2.3.7	Hatch rate assay from <i>en masse</i> egg collections.....	2-38
2.4	Experimental design.....	2-39
2.4.1	Exposure rationale.....	2-39
2.4.2	Survival assays.....	2-40
2.4.3	Reproductive output assays.....	2-41
2.5	Data analysis.....	2-42
2.6	Results.....	2-43
2.6.1	Immediate (24 h) mortality.....	2-43
2.6.2	Longevity and median survival.....	2-47
2.6.3	Magnitude of exposure effects on survival.....	2-55
2.6.4	Reproductive output.....	2-57
2.7	Discussion.....	2-61
2.7.1	Impact of immediate and delayed mortality effect on survival.....	2-61
2.7.2	Do resistant mosquitoes survive long enough to transmit malaria?.....	2-64
2.7.3	Exposure while blood feeding shows no effect on fecundity.....	2-66
2.7.4	Limitations.....	2-67
2.8	Conclusion.....	2-67

3	Characterisation of LLIN contact and subsequent mortality of insecticide susceptible and resistant <i>Anopheles gambiae</i> and <i>Anopheles funestus</i>	3-68
3.1	Introduction	3-68
3.2	Aims and Objectives.....	3-71
3.3	Materials and methods.....	3-72
3.3.1	Mosquito colonies.....	3-72
3.3.2	Long-lasting insecticide treated nets (LLINs)	3-72
3.3.3	HPLC chemical analysis of LLINs.....	3-73
3.3.4	Standard WHO Cone bioassays.....	3-73
3.3.5	Video recording.....	3-73
3.4	Experimental design.....	3-75
3.4.1	Experiment 1 – Modified WHO cone behavioural bioassay	3-75
3.4.2	Experiment 2 – Knockdown (KD) and mortality.....	3-76
3.4.3	Experiment 3 - Timed exposure	3-77
3.4.4	Data analysis	3-78
3.5	Results.....	3-79
3.5.1	Chemical Analysis of Nets	3-79
3.5.2	Experiment 1 - Mosquito behaviour at the LLIN interface	3-79
3.5.3	Experiment 2 - Knockdown (KD) and mortality	3-91
3.5.4	Experiment 3 - LLIN contact time required for knock down.....	3-95
3.6	Discussion.....	3-98
3.6.1	Current WHO cone bioassay	3-98
3.6.2	Current alternatives to the standard WHO cone bioassay?	3-99
3.6.3	Modified video cone bioassay	3-100
3.6.4	Effect of the presence of a host.....	3-103
3.6.5	Absence of evidence for contact irritancy in IR mosquitoes	3-104
3.6.6	How much exposure to an LLIN is required to kill a mosquito?	3-105
3.6.7	Can the cone bioassay be improved?	3-106
3.6.8	Limitations.....	3-107
3.7	Conclusion.....	3-109
4	Investigating feeding behaviour of susceptible and resistant strains of <i>Anopheles gambiae</i> at the LLIN interface.....	4-110
4.1	Introduction	4-110
4.2	Aims and Objectives.....	4-111
4.3	Materials and methods.....	4-112
4.3.1	Mosquito colonies.....	4-112

4.3.2	Long lasting insecticide treated nets	4-112
4.4	Experimental design.....	4-112
4.4.1	Thumb test procedure	4-114
4.5	Behavioural characteristics	4-116
4.6	Data analysis	4-117
4.7	Results.....	4-118
4.7.1	Baseline activity of <i>An. gambiae</i> mosquitoes blood feeding at an untreated net surface	4-118
4.7.2	Activity of <i>An. gambiae</i> mosquitoes blood feeding at PermaNet LLIN surface	4-119
4.7.3	Activity of older <i>An. gambiae</i> at untreated and treated net surfaces.....	4-120
4.7.4	Mean activity for all net types and mosquito strains	4-121
4.7.5	Responders and survivors per experiment	4-123
4.8	Discussion.....	4-124
4.8.1	LLINs reduce net contact and feeding times compared to untreated netting ..	4-124
4.8.2	Susceptible and resistant mosquitoes successfully feed and rest in a similar way	4-125
4.8.3	Older resistant mosquitoes do not behave differently from younger mosquitoes.....	4-126
4.8.4	Limitations.....	4-126
4.9	Conclusion.....	4-128
5	General discussion	5-129
5.1	Overview	5-129
5.2	Delayed mortality effect	5-130
5.3	Behaviour near the LLIN surface.....	5-131
5.4	Impact of a host	5-133
5.5	Insecticide bioavailability.....	5-134
5.6	Improvements towards a better benchtop assay.....	5-136
5.7	Limitations of current WHOPES guidelines.....	5-136
5.8	Current and future work.....	5-137
	Conclusion.....	5-139
6	References	6-140
6.1	WHO references.....	6-175
7	Appendices.....	7-177
7.1	Appendix 1 - KM curves (chapter 2, experiment 4)	7-177
7.2	Appendix 2 – Published paper and paper in prep.....	7-178

7.3	Appendix 3 – Frequency charts (chapter 3)	7-179
7.3.1	Contact - Untreated	7-179
7.3.2	Contact - Duranet	7-180
7.3.3	Contact - Olyset	7-182
7.3.4	Contact - Olyset Duo	7-183
7.3.5	Contact - PermaNet	7-185
7.3.6	Host – Untreated	7-187
7.3.7	Host – Olyset	7-188
7.3.8	Host – PermaNet	7-189
7.3.9	Non-contact Untreated	7-191
7.3.10	Non-contact Duranet	7-192
7.3.11	Non-contact Olyset	7-193
7.3.12	Non-contact PermaNet	7-195
7.4	Appendix 4 – WHO tube bioassay (chapter 4)	7-197
7.5	Appendix 5 – Mean time for each behavioural event for all mosquito cohorts (chapter 4)	198
7.6	Appendix 6 – GEE ANALYSIS results where contact on the net is compared between treatments/strain	199

Table of figures

Chapter 1

Figure 1-1 Map of projected changes in malaria incidence rates.....	1-2
Figure 1-2 Photographs of blood feeding female <i>An. gambiae</i> and <i>An. funestus</i> mosquitoes..	1-3
.....	1-3
Figure 1-3 Diagram showing various effects of LLINs and IRS on mosquitoes	1-6
Figure 1-4 Map of African region showing reports of insecticide resistance in African	
<i>Anopheles</i> malaria vector	1-15
Figure 1-5 Dot plots graphs to show changes in pyrethroid mortality in major African	
malaria vectors over time.	1-16
Figure 1-6 Diagram showing the topology of the sodium channel protein	1-17

Chapter 2

Figure 2-1 Photograph of standard WHO cone bioassay.....	2-36
Figure 2-2 Photograph of unhatched and hatched <i>An. gambiae</i> eggs	2-38
Figure 2-3 Photographs of dissected <i>An. gambiae</i> ovaries.	2-38
Figure 2-4 Exposure schematic for exposure and blood feeding experiments 1-4.	2-40
Figure 2-5 Bar chart of immediate mortality of insecticide susceptible control Kisumu strain	
of <i>An. gambiae</i>	2-43
Figure 2-6 (A-B) Bar charts of immediate mortality in experiment 1 and experiment 2....	2-44
Figure 2-7 Bar chart of immediate mortality in experiment 3.....	2-45
Figure 2-8 Bar chart of immediate mortality in experiment 4.....	2-46
Figure 2-9 Kaplan Meier survival curve and box and whisker plot (Kisumu).....	2-47
Figure 2-10 Kaplan Meier curves for experiment 1 (PermaNet)	2-48
Figure 2-11 Kaplan Meier curves for experiment 2 (PermaNet)	2-49
Figure 2-12 Box and Whisker plots for experiments 1 and 2 (PermaNet).....	2-49
Figure 2-13 Kaplan Meier curves for experiment 1 (Olyset).....	2-50
Figure 2-14 Kaplan Meier curves for experiment 2 (Olyset).....	2-51
Figure 2-15 Box and Whisker plots for experiments 1 and 2 (Olyset)	2-51
Figure 2-16 Kaplan Meier curves for experiment 3 (PermaNet)	2-52
Figure 2-17 Kaplan Meier curves for experiment 3 (Olyset).....	2-53
Figure 2-18 Box and Whisker plots for experiments 3 (PermaNet and Olyset)	2-53
Figure 2-19 Kaplan Meier curves for experiment 4	2-54
Figure 2-20 Box and Whisker plots for experiments 4	2-54
Figure 2-21 Bar chart for experiment 5 showing mean percentage of feeders	2-57
Figure 2-22 Bar chart for experiment 5 showing total egg numbers per female	2-58
Figure 2-23 Bar chart for experiment 5 showing hatch rates.....	2-59
Figure 2-24 Box and whisker plot for experiment 6 showing mean egg counts	2-60
Figure 2-25 Line graph of the possible impact of insecticide resistance on the efficacy of	
LLINs.	2-66

Chapter 3

Figure 3-1 Labelled photograph of experimental set up for WHO cone bioassay and video capture system.	3-74
Figure 3-2 Schematic diagram illustrating modified cone experiments A, B and C.....	3-75
Figure 3-3 Bar chart of experiment 1 - WHO standard cone bioassay results.....	3-92
Figure 3-4 Bar chart of results for experiment 1 (5 versus 10 assay) - Knockdown (KD)...	3-93
Figure 3-5 Bar chart of experiment 1 (5 versus 10 assay) - Mortality	3-93
Figure 3-6 Bar chart of experiment 1 (adapted ball assay).....	3-94
Figure 3-7 Bar chart of experiment 2 (timed exposure assay (Olyset Duo))	3-95
Figure 3-8 Line chart of experiment 2 (timed exposure assay (all nets))-knockdown (KD)	3-96
Figure 3-9 Line chart of experiment 2 (timed exposure assay (all nets))-mortality	3-97
Figure 3-10 Stacked bar chart of experiment 3 behaviour assay	3-79
Figure 3-11 Line graphs of experiment 3 behaviour assay (untreated).....	3-81
Figure 3-12 Line graphs of experiment 3 behaviour assay (PermaNet)	3-82
Figure 3-13 Line graphs of experiment 3 behaviour assay (Olyset).....	3-82
Figure 3-14 Line graphs of experiment 3 behaviour assay (Olyset)	3-83
Figure 3-15 Box and whisker plots of experiment 3 (direct contact assays)	3-84
Figure 3-16 Composite video still images of insecticide susceptible <i>An. gambiae</i> (Kisumu) mosquito activity	3-85
Figure 3-17 Box and whisker plots of experiment 3 (direct contact–Olyset and Olyset Duo)	3-86
Figure 3-18 Box and whisker plots of experiment 3 (direct contact plus host assays).....	3-87
Figure 3-19 Bar chart of experiment 3 (non-host versus host assays)	3-88
Figure 3-20 Box and whisker plots of experiment 3 (non-contact assays)	3-89
Figure 3-21 Box and whisker plots of experiment 3 (direct contact – <i>An funestus</i> – assays).	3-90
Figure 3-22 A diagram to illustrate the size of Olyset net compared with an <i>Anopheles</i> mosquito	3-108

Chapter 4

Figure 4-1 Photographs of thumb assay experimental components.....	4-113
Figure 4-2 Photograph and diagram of thumb assay experimental set up	4-114
Figure 4-3 Photographs of mosquito silhouette taken images during thumb assay	4-117
Figure 4-4 Stacked bar chart of thumb assay behavioural responses (Kis untreated)	4-118
Figure 4-5 Stacked bar chart of thumb assay behavioural responses (Tia untreated).....	4-118
Figure 4-6 Stacked bar chart of thumb assay behavioural responses (Kis PermaNet).....	4-119
Figure 4-7 Stacked bar chart of thumb assay behavioural responses (Tia PermaNet).....	4-119
Figure 4-8 Stacked bar chart of thumb assay behavioural responses (Tia PermaNet).....	4-120
Figure 4-9 Stacked bar charts showing mean time of each defined behavioural event ..	4-121
Figure 4-10 Bar chart showing mean time spent blood feeding	4-122
Figure 4-11 Bar chart showing mean time spent in contact with the net	4-123

Chapter 7

Figure 7-1 Kaplan Meier curves for experiment 4	7-177
Figure 7-2 (a-c) Line graphs for untreated net (contact)	7-179
Figure 7-3 (a-f) Line graphs for Duranet net (contact).....	7-181
Figure 7-4 (a-d) Line graphs for Olyset net (contact).....	7-183
Figure 7-5 (a-d) Line graphs for Olyset Duo (contact).....	7-184
Figure 7-6 (a-f) Line graphs for PermaNet (contact).....	7-186
Figure 7-7 (a-d) Line graphs for untreated net (plus host)	7-188
Figure 7-8 (a-d) Line graphs for Olyset (plus host).....	7-189
Figure 7-9 (a-d) Line graphs for PermaNet (plus host).....	7-190
Figure 7-10 (a-d) Line graphs for untreated (non-contact).....	7-192
Figure 7-11 (a- d) Line graphs for Duranet (non-contact).....	7-193
Figure 7-12 (a-d) Line graphs of Olyset (non-contact)	7-194
Figure 7-13 (a-d) Line graphs for PermaNet (non-contact).....	7-196

Table of tables

Chapter 1

Table 1-1 WHO recommended commercially available long-lasting insecticidal nets.....	1-10
Table 1-2 WHO recommended insecticides for indoor residual spraying against malaria vectors.....	1-10
Table 1-3 Cross-resistance patterns of different classes of insecticide.....	1-21

Chapter 2

Table 2-1 Details of mosquito colonies (survival assays).....	2-34
Table 2-2 Classification of adult mosquitoes	2-37
Table 2-3 Summary of survival experimental components	2-39
Table 2-4 Summary of hazard Ratio (Logrank).....	2-55

Chapter 3

Table 3-1 Details of mosquito colonies (cone assays)	3-72
--	------

Chapter 4

Table 4-1 Thumb assay behavioural events.....	4-116
Table 4-2 Responders and survivors in the thumb feeding behaviour experiment	4-123

Chapter 7

Table 7-1 knockdown (KD) and mortality data of Tiassalé	7-197
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1 Background information

1.1 Thesis themes

The extent by which malaria disease control achieved by insecticides is eroded as insecticide resistance (IR) spreads not only depends on vector densities but the vectorial capacity of individual vectors, which can be dramatically altered by IR. In some instances, IR may impair the ability of the vector to transmit diseases. If this effect is sufficiently large, the impact of IR on disease management may not be as detrimental as previously thought. Alternatively, IR could improve the individual vectorial capacity of insects, further emphasising the urgent need for novel insecticides and IR management strategies (Rivero et al. 2010).

There are limitations in using bioassay data to assess the impact of IR on disease transmission. Current bioassays do not capture the lifetime impact of IR, nor do they provide any data the impact of IR on mosquito behaviour. There are gaps in current knowledge on how physiological IR may affect behaviour, and consequently vectorial capacity. This current study re-examines existing tests, and evaluates newer assays or modifications to existing assays, to determine whether additional relevant data on the impact of IR can be obtained in a laboratory setting.

1.2 Malaria - a persistent problem

In 2016, nearly half of the world's population was at risk from malaria (WHO 2016a) with an estimated 216 million cases of malaria in 91 countries, and deaths reaching 445,000 (WHO 2017a). This year's WHO Malaria report shows that after an unprecedented period of success in global malaria control, progress has stalled. Data from 2015–2017 highlight that no significant progress in reducing global malaria cases was made in this period (WHO 2018a).

The primary obstacle to global eradication remains the parasite's historical stronghold in Africa, where unusually efficient vectors saturate human populations, causing intense transmission (Killeen et al. 2013). Very high transmissibility results from human-dependent speciation processes in *Anopheles* mosquitoes which have had a key influence on the origin of the modern *P. falciparum* from an ancestral, less pathogenic, taxon (Coluzzi 1999).

High malaria prevalence in the Africa region also results from environmental factors relating to climate, as well as socio-economic factors such as land use, agricultural practices, health sector infrastructure and lower socioeconomic status (Stratton et al. 2008). In turn, these

factors lead to less access to vector-control tools such as nets, poor housing and overcrowding.

Approximately 90% of the world's *P. falciparum* infections and deaths occur in sub-Saharan Africa, the latter almost entirely in children younger than 5 years of age (Maitland 2016). Figure 1-1 shows the malaria incidence rates, by country, from years 2000-2015 (WHO 2015a).

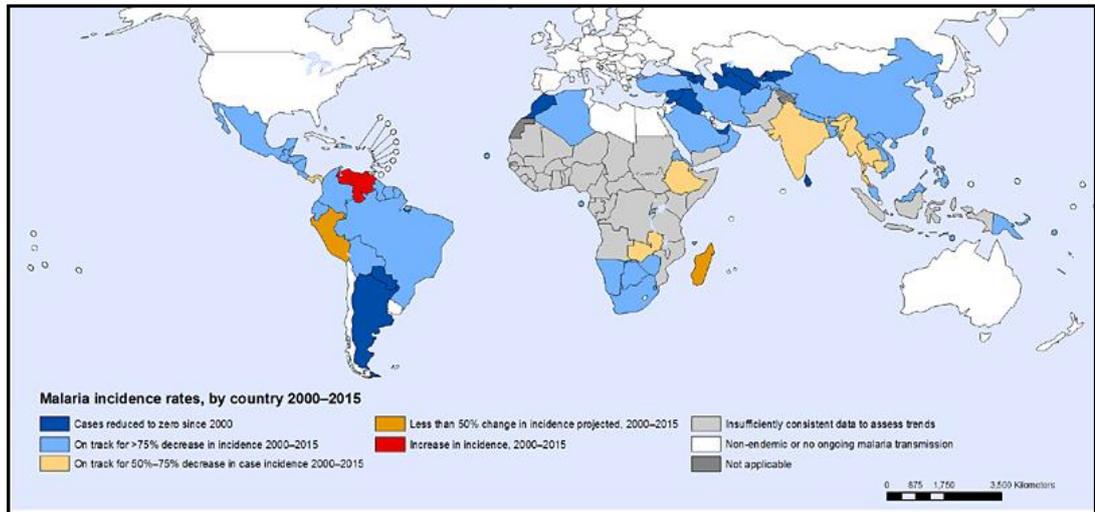


Figure 1-1 Map of projected changes in malaria incidence rates by country 2000–2015. Malaria remains a major burden to people residing in resource-limited areas in Africa, Asia and Central and South America. (Image modified from WHO World Malaria report 2015).

1.3 Pathophysiology

Human malaria, a severe disease caused by the protozoal *Plasmodium* parasite, is transmitted by the bite of a female *Anopheles* mosquito during a blood meal (Phillips et al. 2017). *P. falciparum* and *P. vivax* are the most common species for which humans are the only hosts. The parasite will rapidly invade the host's liver, and proliferate within red blood cells, causing lysis. This erythrocyte rupturing stage occurs 36-72 h post red cell invasion and coincides with the clinical presentations of symptomatic malaria, such as fever, shaking chills, sweating, and headache. At this point, a second mosquito may take a blood meal from the host. Within 7-10 d of this bite, the mosquito will be armed with mature sporozoites and able to infect another human (Phillips et al. 2017).

1.4 Mosquito disease vectors

Found worldwide, mosquitoes are important vectors of diseases, but they also cause a nuisance with non-infectious biting (Service 2008). All mosquitoes belong to the family

Culicidae. This family is subdivided into three subfamilies: Anophelinae (*Anopheles*), Culicinae (*Aedes* and *Culex*) and Toxorhynchitinae.

1.5 *Anopheles*: the primary disease vector

Approximately 41 species of *Anopheles* spp. are regarded as exclusive vectors of human malaria (Sinka et al. 2012). The continent of Africa experiences the bulk of the global malaria burden due in part to the abundant presence of the *Anopheles gambiae* complex, featuring several sibling species. The three main sibling species within this complex are *An. gambiae sensu stricto* (*s.s*) (formally *An. gambiae* Savannah form), *An. coluzzii* (formally *An. gambiae* Mopti form) and *An. arabiensis*. There also exists another dominant vector species in Africa, namely *An. funestus* (Sinka et al. 2011; Coetzee et al. 2013; Wiebe et al. 2017).

An. gambiae complex vectors are so efficient because they attack man in preference over animals (anthropophily compared to zoophily) (Takken & Verhulst 2013). The exception to this is *An. arabiensis* which is zoophilic and frequently feeds on cattle. *An. funestus* is also generally anthropophilic but may opportunistically feed on cattle. Figure 1-2 shows photographs of *An. gambiae s.s.* and *An. funestus* females, blood feeding on a human host. Typical features of anopheline mosquitoes include dark and pale scales on wing veins arranged in distinct blocks and long odour detecting palps (Service 2008). Wing and palp patterns help distinguish between species (Gillies & Coetzee 1987).



Figure 1-2 Photographs of blood feeding female *An. gambiae* (left) and *An. funestus* (right) mosquitoes. Both show typical features of anopheline mosquitoes, including spotted wing detail and long palps. Permission given by Ray Wilson www.raywilsonbirdphotography.co.uk.

An. gambiae s.s. and *An. funestus* are nocturnal and predominantly feed (endophagy) and shelter (endophily) indoors – therefore being most active around sleeping humans late at night (Bayoh et al. 2014). Generally with *An. arabiensis*, although it is a highly efficient vector of malaria, there appears to be an east–west behavioural cline - for populations in western Africa, the majority of bloodmeals are from humans, while, in eastern Africa, a greater proportion feed on cattle (Tirados et al. 2006).

As well as their anthrophilic feeding nature, anopheline mosquitoes are such efficient vectors due to the short gonotrophic cycle (they return to feed on the night on which the eggs are laid) and long life of the females (Haddow & Ssenkubuge 1962). Furthermore, recent studies have shown that during mating, African and Indian *Anopheles* males “plug” females after copulation to stop interloping males from mating (Mitchell et al. 2015). These plugs transfer high levels of the steroid hormone 20-hydroxyecdysone to the females, creating favourable conditions for *Plasmodium* development (Pondeville et al. 2008), and the most elaborate mosquito plugs are found in regions where malaria transmission rates are highest (Mitchell et al. 2015).

1.5.1 Key elements of the mosquito life cycle

After mating and blood feeding, *Anopheles* typically lay between 50-200 small brown or black boat shaped eggs on the water surface. These cannot withstand desiccation and normally hatch within 2-3 d. Larval habitats include sunlit pools, puddles, hoofprints, borrow pits and rice fields. In tropical countries, the larval stage lasts about 7 d, while the pupal period lasts 2-3 d, after which the adult mosquito emerges (Service 2008).

In *An. gambiae*, as in most species of mosquito, mating is initiated in flight, the male being attracted to the female by her flight tone (Charlwood & Jones 1979; Pennetier et al. 2010). Males and virgin females, of the same species, are brought into close proximity within a mating swarm, with the ratio of available males to females being 10:1 on any one night (Charlwood & Jones 1980). Females spend at most 30 seconds in the swarm before flying off *in copulo* where the sperm serves to fertilise all eggs laid during her lifetime.

Most mosquitoes disperse only a few hundred metres from their emergence sites, usually no more than 2km (Service 2008). Both male and female mosquitoes feed on nectar, but the female must take a blood meal to obtain the necessary nutrients for the development of her eggs. As most *Anopheles* adults are nocturnal in their activities, blood-feeding and oviposition normally occur in the evenings, at night or at sunrise. In tropical countries, adult females live an average 1-2 weeks, whereas in temperate climates, longevity is likely to be 3-4 weeks. Due to the long maturation period of the *Plasmodium* parasite, female mosquitoes must survive at least three gonotrophic cycles before being able to transmit malaria (Service 2008). In the laboratory environment (at LSTM) mosquito colonies can exceed 4 weeks.

1.6 Insecticide based vector control

Insecticide based interventions work by disrupting the normal feeding or host-searching behaviour of the mosquito (repellency or deterrence) and/or the insecticide causes mosquito death, affecting age structure of the mosquito population and adult mosquito density (Barreaux et al. 2017). The use of two key interventions - long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) - are crucial to many malaria control programmes, because they are highly effective, have a relatively low cost, and their manufacture and distribution can be rapidly scaled up (WHO 2012).

1.6.1 LLINs

Designed for a minimum lifespan of 20 standard washes or 3 years of usage under field conditions, a net treated with an effective insecticide can reduce contact between mosquitoes and humans by providing both a physical barrier and an insecticidal effect (Takken 2002; Lengeler 2004). LLINs inhibit feeding *before* the mosquito can inoculate the person with sporozoites, and the insecticide component of net provides a lethal effect on the vector (WHO 2015b). In areas where LLINs are used on a large scale, they also provide community-wide protection (Magesa et al. 1991; Hawley et al. 2003; Maxwell et al. 2002).

Across sub-Saharan Africa, household ownership of at least one LLIN increased from 50% in 2010 to 80% in 2016 (WHO 2017a). More people at risk of malaria in Africa are sleeping under an LLIN and subsequently they have been attributed to averting 68% of the malaria cases since 2000 (Bhatt et al. 2015).

1.6.2 IRS

Whereas LLINs provide a high degree of personal protection, the efficacy of IRS relies largely on a vectorial mass effect: the increased mortality of adult vectors following feeding leads to a reduction in transmission (Pluess et al. 2010). For IRS to be effective, there must be high coverage (usually > 85%) of all structures that are potential resting places to obtain the mass effect on the vector population (WHO 2015b). IRS provides some small amount of protection to an individual household by repelling and reducing the number of vectors that come *into* the house. However, the greatest impact of an IRS intervention takes place *after* feeding, when the anopheline mosquito is more likely to rest on a sprayed surface and pick up a lethal dose of insecticide, thus preventing it from going on to transmit the malaria parasite to others in the vicinity.

Carried out between once and three times per year, IRS involves spraying an effective dose of insecticide, with a long residual activity, on indoor walls and ceilings where malaria vectors are likely to rest after biting. To confer significant community protection, IRS needs to be implemented at a high level of coverage with sustained efficacy throughout the malaria transmission season or seasons (WHO 2015b).

1.6.3 Modes of action

When LLIN and IRS coverage is sufficiently high, the number of human-vector contacts drop, thus reducing the daily probability of mosquito survival, interrupting transmission and reducing disease incidence (Teklehaimanot et al. 2007). LLINs and IRS target mosquitoes that enter or attempt to enter the human dwelling, as shown in Figure 1-3 (Okumu & Moore 2011). Both control methods have been extensively shown to confer significant benefits against malaria (Lengeler 2004; Pluess et al. 2010).

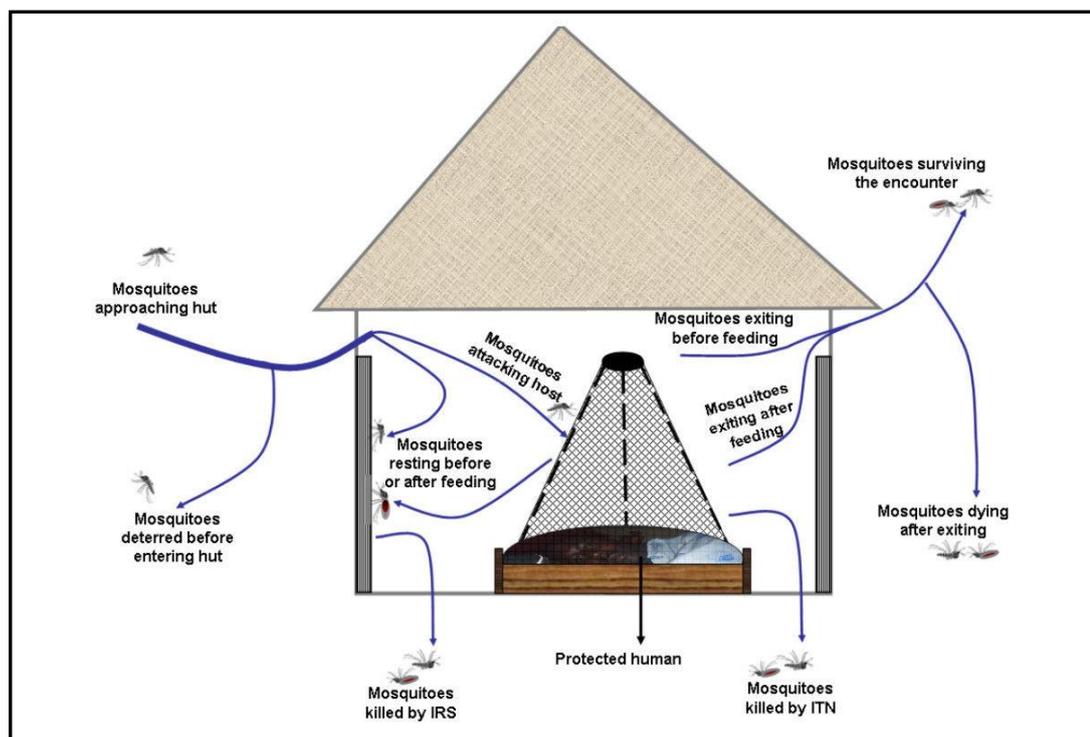


Figure 1-3 Diagram showing various effects of LLINs and IRS on mosquitoes that enter or attempt to enter houses Insecticides used on nets or for IRS effect mosquitoes at different levels along the path towards the individual human inside the sprayed hut. Mosquitoes can be deterred and diverted before they enter houses, killed by the IRS or LLINs, or they can be irritated so that they exit the huts earlier than normal. Exit may occur before or after the mosquitoes have fed, but both the fed and unfed mosquitoes may die later after they have left the huts due to sub-lethal effects of the LLIN or IRS insecticides. The net and the IRS may also inhibit mosquitoes' ability to successfully take blood meals from the hut dwellers. (Image modified from Okumu & Moore 2011)

1.6.4 Combining LLINs and IRS

WHO guidance for countries on combining indoor residual spraying and long-lasting insecticidal nets (WHO 2014) suggests the following: in settings where there is high coverage with LLINs, and LLINs remain effective, IRS may have limited utility in reducing malaria morbidity and mortality. If LLINs and IRS are to be deployed together in the same geographical location, the IRS should use non-pyrethroid insecticides.

Combined use of IRS and LLINs may provide greater protection against malaria than the use of IRS or LLINs alone (Kleinschmidt et al. 2009; Okumu & Moore 2011; Corbel et al. 2012; Protopopoff et al. 2015). In addition, in medium malaria transmission areas, these results suggest that there may be a synergistic effect of using LLINs and IRS together (Fullman et al. 2013). Other studies have shown no improved effects of combining interventions (Corbel et al. 2012; Pinder et al. 2015). The reason for this mixture of findings may relate to differences in the trial settings and methods, including vector species, insecticides used for indoor residual spraying, effective coverage (of each intervention), and IR to one or other of the insecticides used (Lines & Kleinschmidt 2015).

National malaria control programmes often target only selected population sub-groups for IRS; hence, the proportion of the population covered by IRS is generally lower than that for LLINs. Declining IRS coverage may be attributed to a need to change to more expensive insecticide classes (WHO 2015b).

1.6.5 Testing efficacy of LLINs and IRS interventions

To determine whether a new or newly introduced LLIN/IRS product is fit for purpose, *i.e.* can fulfil all modes of actions discussed above, standardised WHO laboratory (phase I), small-scale (phase II) and large-scale (phase III) field studies must be carried out (WHO 2006, 2013a). In Phase I (laboratory) assessment studies, the WHO cone test¹ assesses the efficacy of the impregnated insecticide, wash-resistance and regeneration time of the insecticide on the netting. Nets need to meet the criteria of WHO cone bioassays ($\geq 95\%$ knock-down and $\geq 80\%$ mortality) to continue into phase II and III testing. If the cone test fails, tunnel tests are carried out and if these are passed, the study can continue to phase II. Phase II small-scale (experimental hut) field trials investigate the efficacy of LLINs in terms of blood-feeding

¹ The efficacy of nets and residual activity of IRS surfaces is determined using standard WHO cone bioassays (WHO 2006/2013a). Susceptible, non-blood-fed, 2–5-day-old female *Anopheles* mosquitoes are exposed to each piece of netting for 3 min, or IRS surface for 30 minutes, under standard WHO cones. After which they are held for 24 h with access to sugar solution. Knock-down is recorded 60 min after exposure and mortality after 24 h.

inhibition, deterrence, induced exophily and mortality using susceptible, free-flying, wild mosquitoes. Phase III trials are large-scale field trials, measuring parameters including long-lasting efficiency, community acceptance, physical durability and safety features. Similarly, for IRS, parameters include intrinsic insecticidal activity and irritant/repellent qualities, efficacy and persistence under different ecological settings, and efficacy and residual activity and community acceptance (WHO 2006).

1.6.6 Larval control

Unlike LLINs and IRS, which target the adult mosquito vector, larval source management (LSM) targets immature, aquatic stages of the mosquito (the larvae and pupae), thereby reducing the outdoor abundance of adult vectors (WHO 2013b). Anopheles larvae are 'sitting ducks': they are relatively immobile and often readily accessible. By targeting the larval stages, mosquitoes are killed 'wholesale' before they disperse to human habitations (Fillinger & Lindsay 2011). LSM is conducted by permanently or temporarily reducing the availability of larval habitats (habitat modification and habitat manipulation), or by adding substances/predators to standing water that either kill or inhibit the development of larvae (larviciding and biological control) (WHO 2013b).

Costs for LSM compare favourably with costs for IRS and LLINs, especially in areas with moderate and focal malaria transmission where mosquito larval habitats are accessible and well defined (Worrall & Fillinger 2011). In Africa and Asia, LSM is considered as another policy option alongside LLINs or IRS, or both, for reducing malaria morbidity in both urban and rural areas where a sufficient proportion of larval habitats can be targeted (Tusting et al. 2013).

1.6.7 Supplementary tools

LLINs and IRS target only female mosquitoes inside the domestic dwelling, leaving activities such as sugar feeding, mating, outdoor biting, host searching and house entry, alternative host feeding and outdoor resting, untouched (Barreaux et al. 2017). Moreover, alternative control methods could target both females and males, at multiple points across their life cycle. The following examples of prospective tools which are under investigation, have a mode of action that complement current tools, e.g. target different mosquito behaviour/populations than LLINs and IRS, and are/close to being field ready:

- Attractive toxic sugar baits use a mixture of an oral toxin, natural sugars and floral attractants to lure the mosquito either inside or outside into traps/bait stations (Müller et al. 2010; Beier et al. 2012; Qualls et al. 2015; Fikrig et al. 2017)

- Housing improvements aim to prevent house entry to protect users without LLINs (Lindsay et al. 2002; Ogoma et al. 2009; Knols et al. 2016; Spitzen et al. 2016; Sternberg et al. 2016; Killeen et al. 2017; Tusting et al. 2017)
- Treatment of livestock and livestock dwellings to address the problem of zoophilic feeding behaviour (Hewitt & Rowland 1999; Chaccour et al. 2013; Massebo et al. 2015; St. Laurent et al. 2016; Chaccour & Killeen 2016)
- Spatial repellents potentially protecting users before they go indoors and users without LLINs (Achee et al. 2012; Sheila B. Ogoma et al. 2014; Sheila B Ogoma et al. 2014b; Wagman et al. 2015)
- Ivermectin, a broad-spectrum antiparasitic endectocide used for onchocerciasis and lymphatic filariasis control also kills anopheline mosquitoes that feed on recently treated individuals (Chaccour et al. 2013; Smit et al. 2018). With a different mode of action from other insecticides, Ivermectin could also be effective against mosquitoes that are resistant to insecticides used for LLINs and indoor residual spraying (Foy et al. 2011).
- Other tools such as mating swarm sprays (Diabate & Tripet 2015; Sawadogo et al. 2017), sterile insect technique (Alphey et al. 2010), topical repellents (Wilson et al. 2014), transinfection with *Wolbachia* (Hughes & Rasgon 2014), population suppression and population replacement strategies (James 2005) are also being investigated.

1.7 WHO recommended products for malaria control

The WHO Pesticide Evaluation Scheme (WHOPES) coordinates the testing and evaluation of pesticides for public health and makes recommendations on vector-control tools such as LLINs, insecticides for IRS, and other vector-control interventions as appropriate. As of 1 January 2017, the WHO evaluation of vector control products has been transitioned from WHOPES to the Prequalification team (WHO 2018b).

Table 1-1 and 1-2 show the range of WHO recommended mono-treated and combination LLINs and IRS treatments currently available. Table 1-2 is the current WHO list of IRS products available, however as of November 2017, WHO prequalified² Clothianidin (SumiShield 50WG, not included in the table) as an indoor residual spray intended to kill malaria-carrying mosquitoes.

Table 1-1 showing WHO recommended commercially available long-lasting insecticidal nets (WHO 2015c)

Product name	Product type	Status of WHO recommendation	Status of publication of WHO specification
DawaPlus 2.0	Deltamethrin coated on polyester	Interim	Published
DawaPlus 3.0	Combination of deltamethrin coated on polyester (side panels), and deltamethrin + PBO incorporated into polyethylene (roof)	Interim	Not yet
DawaPlus 4.0	Deltamethrin + PBO incorporated into polyethylene	Interim	Not yet
Duranet	Alpha-cypermethrin incorporated into polyethylene	Full	Published
Interceptor	Alpha-cypermethrin coated on polyester	Full	Published
Interceptor G2	Alpha-cypermethrin and chlorfenapyr coated on polyester	Interim	Not yet
LifeNet	Deltamethrin incorporated into polypropylene	Interim	Published
MAGNet	Alpha-cypermethrin incorporated into polyethylene	Full	Published
MiraNet	Alpha-cypermethrin incorporated into polyethylene	Interim	Published
Olyset Net	Permethrin incorporated into polyethylene	Full	Published
Olyset Plus	Permethrin + PBO incorporated into polyethylene	Interim	Published
Panda Net 2.0	Deltamethrin incorporated into polyethylene	Interim	Published
PermaNet 2.0	Deltamethrin coated on polyester	Full	Published
PermaNet 3.0	Combination of deltamethrin coated on polyester with strengthened border (side panels), and deltamethrin + PBO incorporated into polyethylene (roof)	Interim	Published
Royal Sentry	Alpha-cypermethrin incorporated into polyethylene	Full	Published
SafeNet	Alpha-cypermethrin coated on polyester	Full	Published
Veeralin	Alpha-cypermethrin + PBO incorporated into polyethylene	Interim	Published
Yahe	Deltamethrin coated on polyester	Interim	Published
Yorkool	Deltamethrin coated on polyester	Full	Published

Table 1-2 showing WHO recommended insecticides for indoor residual spraying against malaria vectors (WHO 2017b)³

Insecticide compounds and formulations ¹	Class group ²	Dosage (g a.i./m ²)	Mode of action	Duration of effective action (months)
DDT WP	OC	1-2	contact	>6
Malathion WP	OP	2	contact	2-3
Fenitrothion WP	OP	2	contact & airborne	3-6
Pirimiphos-methyl WP, EC	OP	1-2	contact & airborne	2-3
Pirimiphos-methyl CS	OP	1	contact & airborne	4-6
Bendiocarb WP, WP-SB	C	0.1-0.4	contact & airborne	2-6
Propoxur WP	C	1-2	contact & airborne	3-6
Alpha-cypermethrin WP, SC	PY	0.02-0.03	contact	4-6
Alpha-cypermethrin WG-SB	PY	0.02-0.03	contact	up to 4
Bifenthrin WP	PY	0.025-0.05	contact	3-6
Cyfluthrin WP	PY	0.02-0.05	contact	3-6
Deltamethrin SC-PE	PY	0.02-0.025	contact	6
Deltamethrin WP, WG, WG-SB	PY	0.02-0.025	contact	3-6
Etofenprox WP	PY	0.1-0.3	contact	3-6
Lambda-cyhalothrin WP, CS	PY	0.02-0.03	contact	3-6

² A prequalified product has been assessed by WHO and found to be acceptable, in principle, for procurement by UN and other international agencies and countries. Products that meet prequalification requirements are added to the WHO list of vector control products.

³ ¹CS = capsule suspension; EC = emulsifiable concentrate; SC = suspension concentrate; SC-PE = polymer enhanced suspension concentrate; WG = water dispersible granules; WG-SB = water dispersible granules in sealed

1.7.1 Pyrethroids

Pyrethroids are a major class of neurotoxic insecticides. They are synthetic analogues of the naturally occurring insecticidal esters of chrysanthemic acid (pyrethrins I) and pyrethric acid (pyrethrins II), originally found in the flowers of *Chrysanthemum cinerifolius* (Davies et al. 2007). Pyrethroids are widely used in public health because of their relative safety for humans, high insecticidal potency at low dosages and rapid knock-down effects (WHO 2005). Furthermore, they are photostable compounds with low mammalian toxicity and show limited soil persistence (Zaim et al. 2000; Davies et al. 2007). They also have an irritant effect (Evans 1993; Kongmee et al. 2004; Cooperband & Allan 2009; Said et al. 2009). Irritant insecticides can be demonstrated by a strong stimulation to take off and fly, with a high proportion of mosquitoes exiting from a treated house (Pates & Curtis 2005).

Spatial pyrethroids are used for mosquito coils, vaporizer mats and emanators. They confer protection against mosquito bites through the spatial action of emanated vapor or airborne pyrethroid particles (Ogoma et al. 2012; Achee et al. 2012). A recent study (Ogoma et al. 2014a) compared the effect of pyrethroid (transfluthrin and metofluthrin) coils and DDT, on house entry, exit and indoor feeding behaviour of *An. gambiae*. The authors showed airborne insecticides and freshly applied DDT had similar positive effects on deterrence, irritancy and feeding inhibition. Another study (Ogoma et al. 2014b) showed *An. gambiae* mosquitoes exposed to transfluthrin coils inside a chamber were delayed from feeding normally for 12 h, suggesting the airborne pyrethroid minimises human–vector contact through reduced and delayed blood feeding.

Type I pyrethroids (e.g. permethrin) are generally good knockdown agents due to their ability to induce repetitive firing in axons, resulting in restlessness, un-coordination and hyperactivity followed by prostration and paralysis (Davies et al. 2007). Type II compounds (e.g. deltamethrin) cause a pronounced convulsive phase that results in better kill because depolarization of the nerve axons and terminals is irreversible. The differing physiological effects are explained by the duration of modified sodium currents - Type I compounds last

water-soluble bags; WP = wettable powder; WP-SB = wettable powder in sealed water-soluble bags. ²OC = organochlorines; OP = organophosphates; C = carbamates; PY = pyrethroids.

only tens or hundreds of milliseconds, whilst those of Type II compounds last for several seconds or longer.

1.7.2 Carbamates and Organophosphates

Carbamates are used for IRS vector control in the form of bendiocarb, which is highly effective, but has a short residual duration, meaning that multiple spray cycles may be required for optimal protection in areas of prolonged transmission (Bradley et al. 2012). Propoxur is another carbamate insecticide that is currently used for IRS in Ethiopia where it performs better than bendiocarb (Yeebiyo et al. 2016).

Organophosphates are highly effective but do not induce an excito-repellent response from the vector and they tend to have short residual activity (WHO 2012). In 2013, a new formulation of pirimiphos-methyl capsule suspension (CS) (Actellic) showed vastly improved residual duration up to 12 months against *An. gambiae s.s.*, *An. arabiensis* and *An. funestus* and is now one of the most commonly used products for IRS in Africa (Rowland et al. 2013; Chanda et al. 2013; Oxborough et al. 2014; Kanyangarara et al. 2016). The gradual replacement of relatively inexpensive pyrethroids, firstly with bendiocarb and subsequently with pirimiphos-methyl CS, has contributed to the downscaling of most IRS programmes. The most commonly cited reason was increased cost of pesticides, as vector resistance necessitated switching from pyrethroids to organophosphates (Oxborough 2016).

The mode of action is similar for both insecticide classes. Both inhibit acetylcholinesterase (AChE), a class of enzymes that catalyses the hydrolysis of the neuro-transmitting agent acetylcholine (ACh), resulting in neuromuscular overstimulation and death of the insect (Fukuto 1990).

1.7.3 Neonicotinoids

Clothianidin, a neonicotinoid insecticide, represents a class of chemistry not previously used for vector control. SumiShield® 50WG (clothianidin, developed by Sumitomo) is an alternative insecticide formulation for IRS with long-lasting residual activity. A recent study in Benin (Agossa et al. 2018), comparing SumiShield with deltamethrin IRS, showed very low toxicity from deltamethrin against host-seeking resistant *Anopheles* populations, whereas SumiShield gave an overall mean mortality of 91.7% across the eight-month observation period following spraying. A village-wide study in India (Sreehari et al. 2018) found Indoor residual spraying with SumiShield WG to be operationally feasible and safe, and effective for up to 6 months. A second product, Fludora Fusion WP-SB (Bayer CS), is a mixture of

deltamethrin and clothianidin and is currently under WHOPES evaluation for malaria vector control (Hoppé et al. 2016). The neonicotinoids are widely used as systemic insecticides for crop protection against piercing-sucking pests (Tomizawa & Casida 2004) and target nicotinic acetylcholine receptors (nAChRs).

1.7.4 Organochlorines

Chlorinated hydrocarbon insecticide, DDT⁴ (dichloro-diphenyl-trichloroethane), has been popular because of its rapid effect, relative longevity and low cost. DDT has a spatial repellency and an irritant effect on malaria vectors that strongly limit human-vector contact (Kennedy 1947; Roberts et al. 2000). Mosquitoes that are not directly killed by DDT are repelled and obliged to feed and rest outdoors, which contributes to effective disease-transmission control (WHO 2011). DDT is still used for IRS in some regions, but due to the possible adverse human health and environmental effects of exposure, recommendations for use must be evidence based and consider operational, epidemiological, entomological and economic factors (Sadasivaiah et al. 2007).

The target site for DDT is the same as for the pyrethroids, the voltage-gated sodium channel proteins found in insect nerve membranes (Davies et al. 2007). The correct functioning of these channels is essential for normal transmission of nerve impulses. Affecting mainly the peripheral nervous system, initial contact with the insecticide causes neurons to fire spontaneously causing muscles to twitch, with resulting tremors throughout the body and appendages, the so-called 'DDT jitters'. Eventually, over the course of a few hours or days, DDT exposure leads to excitatory paralysis and consequent death of the insect (Davies et al. 2007).

1.7.5 Alternative compounds

Compounds with alternative modes of action are proving to be effective against mosquitoes, when mixed with pyrethroids. Chlorfenapyr has potential for malaria control in treated-nets when combined with a pyrethroid – currently commercially available as Interceptor[®]G2, which combines alphacypermethrin and chlorfenapyr. Studies demonstrate improved efficacy and wash resistance compared to a standard alphacypermethrin LLIN against

⁴ DDT is the best known and most notorious chemical of the 20th century. Due to its unacceptably long persistence in the environment, the production and use of DDT are strictly restricted by an international agreement known as the Stockholm Convention on Persistent Organic Pollutants (WHO 2011). The Convention has given an exemption for the production and public health use of DDT for indoor application to vector-borne diseases, mainly because of the absence of equally effective and efficient alternatives.

pyrethroid resistant mosquitoes (N'Guessan et al. 2014; Djenontin et al. 2015; N'Guessan et al. 2016a). Chlorfenapyr, a member of the pyrrole class, is used commercially for termite control and crop protection. It is a pro-insecticide that is activated by cytochrome P450 monooxygenases to its more active metabolite. Unlike the nerve toxins, chlorfenapyr is a mitochondrial electron transport inhibitor, whose mode of action is to disrupt the conversion of ADP to ATP (oxidative phosphorylation) in mitochondria of cells (N'Guessan et al. 2007).

Pyriproxyfen is an insect growth regulator that affects the physiology of morphogenesis, reproduction and embryogenesis of insects, and sterilises adult mosquitoes upon direct contact (Harris et al. 2013). Furthermore, it has low mammalian toxicity and environmental impact (Invest & Lucas 2008). The efficacy of Olyset Duo, a newly developed mixture LLIN containing pyriproxyfen and permethrin, was evaluated in experimental huts in southern Benin against pyrethroid resistant *An. gambiae* (Ngufor et al. 2014; Ngufor et al. 2016). The authors showed Olyset Duo was superior to mono-treated permethrin Olyset Net in terms of personal protection provided by improved killing pyrethroid resistant *An. gambiae*, and sterilized surviving blood-fed mosquitoes. A recent clinical trial in Burkina Faso demonstrated that Olyset Duo provided better protection against malaria than standard Olyset nets in areas with very high malaria transmission and very high levels of pyrethroid resistance in the vectors (Tiono *et al*, in press).

Piperonyl butoxide (PBO) is a synergist that inhibits specific metabolic enzymes within mosquitoes and has been incorporated into pyrethroid-treated LLINs to form PBO-combination nets, such as PermaNet 3.0 and Olyset®Plus (Gleave et al. 2017). Synergists are generally non-toxic and act by enhancing the potency of insecticides. PBO inhibits metabolic enzyme families, particularly the cytochrome P450 enzymes. A recent meta-analysis of bioassay studies and experimental hut trials was used to characterise how pyrethroid resistance changes the efficacy of standard bed nets, and those containing (PBO), and assess its impact on malaria control (Churcher et al. 2016). The transmission dynamics model used in the study indicated that switching to PBO bed nets could avert up to 0.5 clinical cases per person per year in some IR scenarios. In addition, a recent clinical trial in Tanzania (Protopopoff et al. 2018) looked at the effectiveness of a long-lasting PBO insecticidal nets against malaria transmitted by pyrethroid-resistant mosquitoes. The authors showed malaria infection prevalence after 9 months was lower in the two groups that received PBO long-lasting insecticidal nets than in the two groups that received standard long-lasting insecticidal nets.

1.8 Insecticide resistance

For more than a decade, mosquito vectors of malaria have been targeted with monotherapy, as pyrethroids are the only class of insecticide recommended by WHO for use on LLINs. The scaling up of pyrethroid IRS in sub-Saharan Africa between 2006 and 2010 was attainable as pyrethroid insecticides were inexpensive and had a relatively long residual action (Oxborough 2016). This increased coverage of pyrethroid IRS, often in parallel with pyrethroid LLIN distribution and agricultural use, led to the spread and intensification of pyrethroid resistance across most of malaria endemic sub-Saharan Africa (Ranson et al. 2011). WHO has since recommended that pyrethroid IRS should not be used for IRS in areas of moderate to high LLIN usage, to preserve the effectiveness of pyrethroid LLINs (WHO 2015b).

Insecticide resistance is defined as the ability of mosquitoes to survive exposure to a standard dose of insecticide. This ability may be the result of physiological or behavioural adaptation (WHO 2016b). Inevitably, IR has been selected, and in some parts of Africa pyrethroids no longer kill mosquitoes (Hemingway et al. 2016). Figure 1-4 shows the extensive spread of confirmed IR through many malaria endemic countries in the African region from 2005-2018.

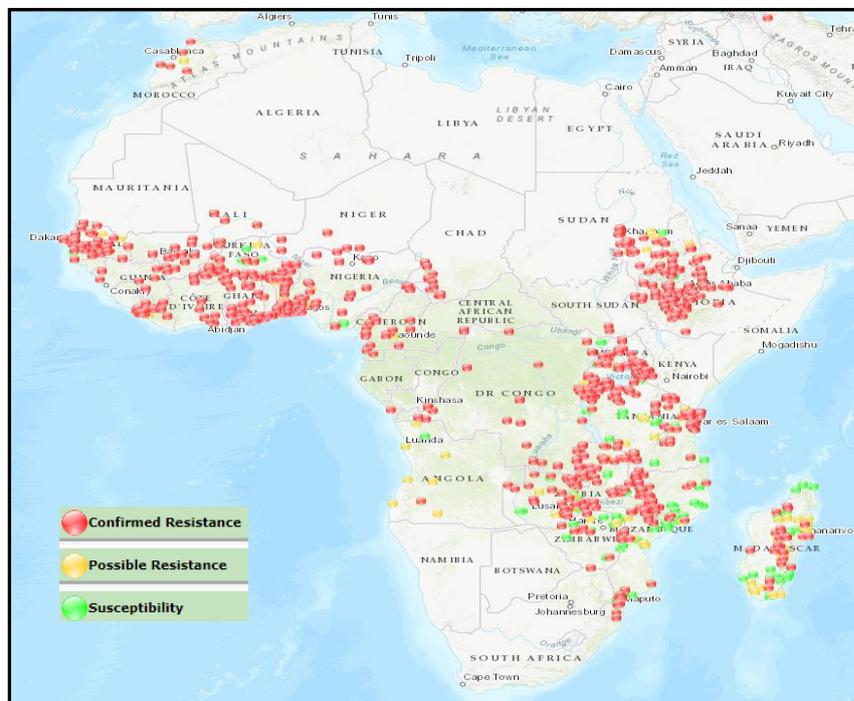


Figure 1-4 Map of African region showing reports of IR in African *Anopheles* malaria vector against all insecticides from 2005 – 2018 (Image reproduced from IR Mapper www.irmapper.com 2018).

Although *An. gambiae* populations fully susceptible to pyrethroids are still present, they are becoming increasingly outnumbered by resistant populations. A similar trend is observed with *An. funestus*. These dramatic increases in pyrethroid mortality over time are illustrated in figure 1-5 (Ranson & Lissenden 2016).

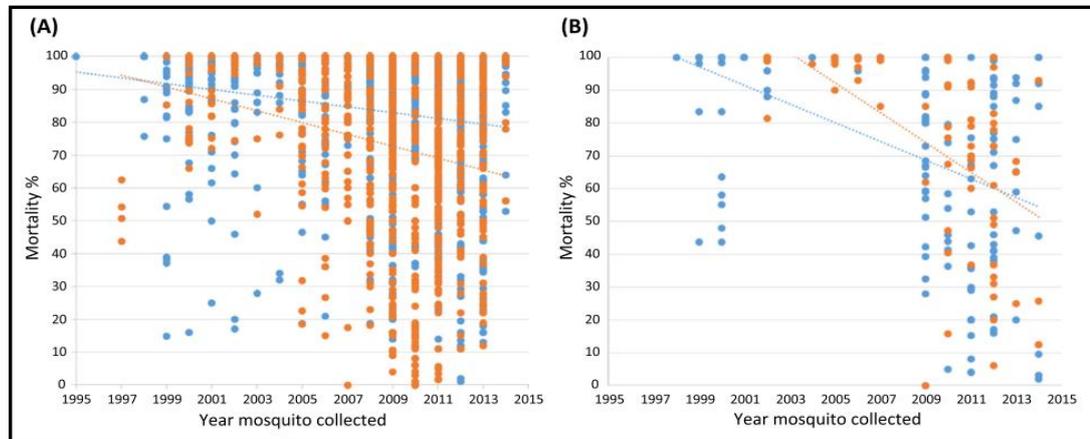


Figure 1-5 Dot plots graphs to show changes in pyrethroid mortality in major African malaria vectors over time. Percentage mortality of (A) *An. gambiae* and (B) *An. funestus* mosquitoes exposed to 0.05% deltamethrin (blue) or 0.75% permethrin (orange) in World Health Organization (WHO) susceptibility bioassays (Image modified from Ranson & Lissenden 2016).

Most *An. gambiae* populations remain susceptible to carbamates and organophosphates although reports of IR to these two classes are increasing and may be expected to rise further in areas where IRS programs are replacing pyrethroids with these insecticide classes in response to pyrethroid resistance (Ranson & Lissenden 2016).

1.8.1 Mechanisms of IR

IR mechanisms are complex and include behavioural and/or physiological changes of mosquitoes leading to insecticide avoidance, altered penetration, sequestration, target site alteration or bio-degradation (David et al. 2013). IR is mainly associated with target site modification and metabolic IR. Two other mechanisms whose importance in malaria vectors has been largely overlooked are behavioural and cuticular IR (Ranson et al. 2011).

1.8.1.1 Mutations in the voltage-gated sodium channel

Target-site IR occurs when the site of action on an insecticide (typically within the nervous system) is modified in resistant strains, such that the insecticide no longer binds effectively, and the insect is therefore unaffected, or less affected, by the insecticide (WHO 2012). In the case of IR to DDT and pyrethroids, the mutation occurs in the 'para' voltage gated sodium channel (VGSC or Na_v) gene of the insect nervous system and confers what is described as "knockdown resistance" (mediated by the *kdr* allele) (Ranson et al. 2000; Davies et al. 2007).

This 'para' gene derives its name from its location in the paralysis (*para*) locus in *Drosophila* (Loughney et al. 1989).

For selection to occur, the resultant amino acid change must reduce the binding of the insecticide without causing a loss of primary function of the target site (Hemingway & Ranson 2000). The most frequent *kdr* mutation in arthropod pests and disease vectors is a leucine to phenylalanine (L1014F in the sodium channel) which is also known as L2i16F using the nomenclature that is universal for sodium channels (Wang et al. 2015). This L1014F substitution in *Anopheles* spp was originally found in strains of *An. gambiae* from Burkina Faso and Cote d'Ivoire (Martinez-Torres et al. 1998). A second substitution at the same position (leucine – serine, L1014S) was found in *An. gambiae* from Kenya (Ranson et al. 2000). More recently, an asparagine-tyrosine mutation found at position 1575 (N1575Y) in the VGSC was identified (Jones et al. 2012). Always found on the 1014F haplotype (same chromosome) background, the mutation compensates for deleterious effects of 1014F and/or confers additional resistance to insecticides. Figure 1-6 shows the positions of these particular mutations on the sodium channel (Wang et al. 2015).

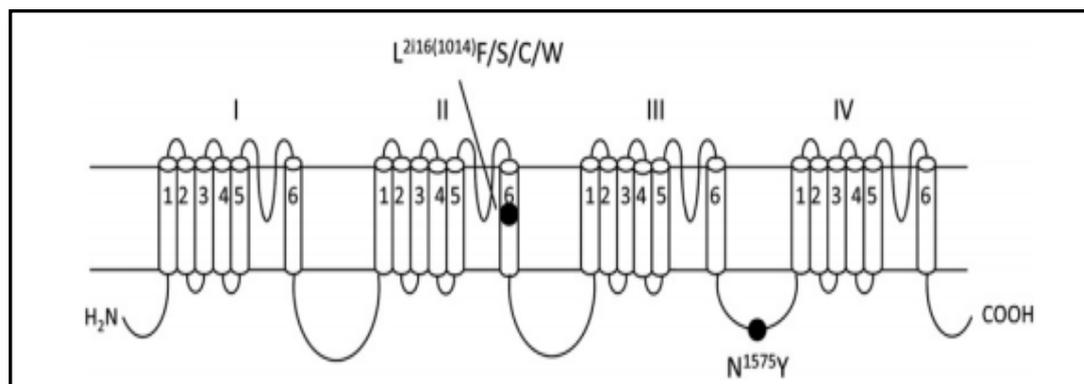


Figure 1-6 Diagram showing the topology of the sodium channel protein indicating the position of L(1014)F/S/C/W and N1575Y mutations. The sodium channel protein consists of four homologous domains (I–IV), each formed by six transmembrane segments (S1–S6) connected by intracellular and extracellular loops. Residue positions L1014F and N1575Y correspond to the housefly sodium channel. L2i16F is labelled using the nomenclature that is universal for P-loop ion channels (Image modified from Wang et al. 2015).

Within the same gene, the V1016I (valine – isoleucine) and F1534C (phenylalanine – cysteine) substitutions are strongly correlated with pyrethroid resistance in *Ae. aegypti* (Saavedra-Rodriguez et al. 2007; Harris et al. 2010).

kdr alleles are found at high frequency in *An. gambiae* (Fanello et al. 2003; Reimer et al. 2005; Pinto et al. 2006; Tripet et al. 2007; Ramphul et al. 2009; Dabiré et al. 2009; Djègbè et al. 2011; Chouaïbou et al. 2017), *An. arabiensis* (Matambo et al. 2007; Balkew et al. 2010; Kawada et al. 2011), *An. coluzii* (Kwiatkowska et al. 2013; Ibrahim et al. 2014; Okorie et al. 2015), *Culex* spp (Martinez-Torres et al. 1999; Xu et al. 2006; Corbel et al. 2007; Tmimi et al.

2018) and *Aedes* spp (Bregues et al. 2003; Da-Cunha et al. 2005; Yanola et al. 2010; Lima et al. 2011; Marcombe et al. 2012; Brito et al. 2013; C.-X. Li et al. 2015; Kushwah et al. 2015; Ishak et al. 2015; Aguirre-Obando et al. 2016; Sayono et al. 2016; Chen et al. 2016; Plernsub et al. 2016). There are no *kdr* resistance mechanisms in *An. funestus* (Coetzee & Koekemoer 2013; Irving & Wondji 2017).

1.8.1.1.1 Insensitive acetylcholinesterase

In the case of carbamates and organophosphates, IR is conferred by a glycine–serine substitution at codon 119 of the *Ace-1* gene. This G119S mutation is responsible for insensitive acetylcholinesterase IR and often entails a large fitness cost which may be ameliorated by gene duplication (Djogbénu et al. 2009). This mutation is largely distributed in several countries of West Africa, sometimes at high frequencies (Weill et al. 2004; Alout et al. 2007; Djogbenou et al. 2008; Dabiré et al. 2008; Ramphul et al. 2009; Alou et al. 2010; Edi et al. 2012; Essandoh et al. 2013; Liebman et al. 2015).

1.8.1.2 Metabolic resistance

Metabolic IR arises when over-expression of enzymes that detoxify insecticides occur (through genetic amplification or hyperactivation of gene expression) or through mutations in the coding gene portion of the enzyme (Silva et al. 2014). In mosquitoes, three major enzyme groups are responsible for metabolically based IR : glutathione S-transferases (GSTs), carboxylesterases (COEs) and cytochrome P450 monooxygenases (Ranson et al. 2002).

Representing a major group of detoxification enzymes, GSTs help to protect cells from oxidative stress (Hayes & Pulford 1995). IR is attributed to increases in the amount of one or more GST enzymes, either as a result of gene amplification or more commonly through increases in transcriptional rate (Enayati et al. 2005). In insects, local gene duplications, particularly within the insect-specific Delta and Epsilon classes, have resulted in expansions of the GST family (Ranson et al. 2002). Elevated GST activity has been associated with DDT resistance in strains of *An. gambiae* (Prapanthadara et al. 1993; Ranson et al. 1997; Ranson et al. 2001; Ortelli et al. 2003; Aravindan et al. 2014) and DDT and pyrethroid resistant strains of *An. funestus*, where *GSTe2* has been demonstrated to directly metabolise the pyrethroid permethrin (Riveron et al. 2014).

Hydrolysing numerous endogenous and exogenous ester-containing compounds (Wheellock et al. 2005), COEs either produce broad spectrum IR through rapid-binding and slow turnover of insecticide (*i.e.* sequestration through gene amplification), or narrow spectrum IR through increased metabolism of a very restricted range of insecticides. This latter mechanism is

almost always found in association with malathion resistance and is associated with single point mutations in the structural genes (Hemingway 2000). Esterase-based resistance to organophosphorus and carbamate insecticides is common in a range of different insect pests (Hemingway & Karunaratne 1998). A number of *Anopheles* species confer IR specifically to malathion through increased rates of metabolism (Hemingway 1985; Malcolm & Boddington 1989).

A superfamily of haemoproteins, cytochrome P450 monooxygenases are important in the metabolism of endogenous compounds and xenobiotics (David et al. 2013). They are the primary enzyme family associated with resistance to most insecticides, including pyrethroids. There are 111 genes encoding cytochrome P450s in *An. gambiae* (Ranson et al. 2002). Overexpression of P450s conferring IR has been found in *Anopheles spp.* (Nikou et al. 2003; Rodpradit et al. 2005; Amenya et al. 2008; Muller et al. 2008; Wondji et al. 2009; Edi et al. 2014; Toé et al. 2015; Djouaka et al. 2016; Ishak et al. 2016), *Cx. quinquefasciatus* (Komagata et al. 2010; Liu et al. 2011; Gong et al. 2013) and other insect species including house flies, honey bees and planthoppers (Liu & Scott 1998; Kasai & Scott 2000; Scharf et al. 2001; Johnson et al. 2006; Daborn et al. 2007; Zhang et al. 2016). Overproduction of P450s leads to increased bio-degradation of insecticide, with the CYP6 group widely implicated in toxin metabolism. Studies have shown metabolism of insecticides by P450s CYP6M2 and CYP6P3 in *An. gambiae* (Müller et al. 2008; Stevenson et al. 2011; Mitchell et al. 2012) and CYP6P3 and CYP6P9 in *An. funestus* (Riveron et al. 2013; Ibrahim et al. 2016). More recently, the insect growth regulator, Pyriproxyfen, has been shown to be metabolised by the same P450s associated with pyrethroid resistance in *An. gambiae* (Yunta et al. 2016).

1.8.1.3 Behavioural (adaptive) resistance

Behavioural IR refers to any modification to mosquito behaviour that facilitates avoidance or circumvention of insecticides (Gatton et al. 2013). Adaptive behavioural responses to insecticides have been reported, such as a shift from endophilic to exophilic behaviour (Russell et al. 2011; Reddy et al. 2011; Killeen et al. 2016; Meyers et al. 2016), changes in the time of feeding (Moiroux et al. 2012; Moiroux et al. 2014) and shifts among primary vector species (Kitau et al. 2012; McCann et al. 2014).

The degree of heritability of behavioural genes is currently unclear but is key to understanding the potential impact of behavioural IR (Gatton et al. 2013). If insecticide interventions are selecting for a heritable trait, such as earlier outdoor biting, the effectiveness of LLINs in reducing malaria infection rates will decrease over time as the

susceptible, indoor-feeding vectors are removed from the population, leaving predominantly the early outdoor feeders. However, if early outdoor feeding is adaptive and a consequence of unsuccessful feeding on the prior evening, mosquitoes will retain their inherent feeding preferences (e.g., location, host, and time) (Gatton et al. 2013).

1.8.1.4 Cuticular resistance

Cuticular IR describes modifications in the insect cuticle and/or digestive tract linings that prevent or slow down the absorption or penetration of insecticides (Ranson et al. 2011). Where insecticides are delivered on bed nets or walls surfaces, uptake of insecticides is through appendage or tarsal contact. Thickening of the cuticle has been reported in association with IR in *An. funestus* (Wood et al. 2010), *Triatoma infestans* (Yadav et al. 2010) and bed bugs (Koganemaru et al. 2013; Lilly et al. 2016). Reduced penetration of insecticide has been demonstrated in DDT resistant strains of *Drosophila melanogaster* (Strycharz et al. 2013).

The insect cuticle is composed of the polysaccharide chitin, proteins, and lipids that form two primary layers. Insects prevent desiccation by depositing cuticular hydrocarbons (CHCs) on their epicuticle as effective waterproofing agents (Bass & Jones 2016). Two of the 243 structural cuticular proteins of *An. gambiae*, CPLCG3 and CPLCG4, have been implicated in IR to date - the location of CPLCG3/4 in the endocuticle may contribute to the thickness of the cuticle (Vannini et al. 2014).

In mosquitoes, the final step of CHC synthesis is catalysed by cytochrome P450s - in particular, the cytochrome P450 *cyp4g16* and *cyp4g17* genes - which are overexpressed in resistant populations of both *An. gambiae* and *An. arabiensis* (Ingham et al. 2014; Jones et al. 2013). A recent study (Balabanidou et al. 2016) demonstrated reduced penetration of radiolabelled deltamethrin in a highly resistant strain of *An. gambiae*, suggesting that cuticular modification may affect insecticide uptake. The authors illustrate the role of CYP4G16 in IR, via enrichment of the CHC content, thus reducing pyrethroid uptake.

1.8.2 Cross resistance

All four IR mechanisms can occur simultaneously in resistant populations with cumulative phenotypic effects leading to resistance to single or multiple insecticides (Corbel et al. 2007; Awolola et al. 2009; Hardstone et al. 2009; Edi et al. 2012; Nwane et al. 2013; Edi et al. 2014), thus providing additional challenges to IR management. Cross-resistance is where resistance to one insecticide confers resistance to another, even if the insect has not been exposed to

the second insecticide (WHO 2018c). General patterns of cross-resistance have been established for the four insecticide classes in common use and for five IR mechanism types (Table 1-3).

Table 1-3 Cross-resistance patterns of different classes of insecticide (adapted from GPIRM 2012). Size of dot indicates anticipated relative importance of the mechanism type in conferring IR to the specified insecticide class. Work is ongoing to update this table to include insecticide classes recently available or soon to be available for malaria vector control (e.g. neonicotinoids and pyrroles) (WHO 2018).

	Metabolic			Target-site	
	Esterases	Mono-oxygenases	GSH S-transferases	<i>kat</i>	Altered AChE
Pyrethroids	●	●●●		●●	
DDT		●	●●	●●	
Carbamates	●	●●●			●●
Organophosphates	●●●	●			●●

AChE, acetylcholinesterase; DDT, dichlorodiphenyltrichloroethane; GSH, glutathione; *kat*, knockdown resistance

1.9 Impact of IR on malaria transmission

1.9.1 Personal protection

A systematic review and meta-analysis of published results on the impact of pyrethroid resistance on the efficacy of LLINs against anopheline mosquitoes, showed that LLINs can remain effective even when IR has developed (Strode et al. 2014). Studies using cone tests showed reduced levels of knock-down associated with higher levels of IR, and tunnel test⁵ results demonstrated a reduced effect of LLINs in resistant mosquitoes in terms of blood feeding, mosquito mortality, and passage through the nets. In experimental hut trials however, mortality for LLINs compared to untreated nets showed that LLINs continued to have an effect even in the presence of resistant mosquitoes. A key limitation of this analysis is the published results were conducted prior to 2013, *i.e.* before the most potent pyrethroid-resistance mechanisms were established (Edi et al. 2014; Toé et al. 2014; Balabanidou et al. 2016); therefore, the current IR situation across Africa may have been underestimated.

Most recently (Kleinschmidt et al. 2018), investigating the implications of IR for malaria vector control with long-lasting insecticidal nets, a 5-country study revealed that long-lasting insecticidal nets provided protection against malaria despite vector IR. They found no evidence that the amount of protection provided by long-lasting insecticidal nets differed by the frequency of IR as measured by WHO bioassays. Similarly, they found no evidence of an association between infection prevalence or clinical incidence with higher pyrethroid resistance. Interestingly, the authors state their study had several limitations. IR measurements were based on the WHO insecticide susceptibility test using a diagnostic dose, which measured the frequency of resistant individuals in the mosquito population, but not the intensity or strength of IR in those individuals. Measures of IR intensity based on a dose-response relationship would have been more informative, but for their study the standard WHO bioassay test was considered best suited for the scale and variety of settings in which insecticide susceptibility status had to be assessed. However, a reduction in vector control effectiveness that was not detected might have occurred in their evaluation. Resistance to pyrethroids (by WHO definition mortality <90%) was observed in 78% of study clusters. Therefore, IR might have an impact on malaria prevalence and incidence across all study clusters, but the insufficient number of susceptible mosquito populations might have

⁵ The tunnel test is used to measure the mortality and blood feeding success of host-seeking mosquitoes in an experimental host baited chamber (WHO 2013c)

rendered any impact undetectable. Conversely, IR intensity might not have reached a level that resulted in a detectable epidemiological effect (Kleinschmidt et al. 2018).

Another recent meta-analysis of bioassay studies and experimental hut trials data sheds light on how current pyrethroid resistance changes the efficacy of LLINs (Churcher et al. 2016). Results show that bed nets will continue providing substantial personal protection until high levels of IR develop in the vectors. It is only when mosquito populations are highly resistant (>60% survival) that an increasing proportion of mosquitoes appear to successfully feed through the LLIN.

1.9.2 Community effect of LLINs

As bed net coverage increases, more people gain personal protection from malaria (Thomas & Read 2016). Mosquitoes seeking those people are killed by insecticides on the nets, and so even people not sleeping under nets experience less exposure to mosquito bites, giving the combined population-wide protective effect of LLINs against a susceptible mosquito population. If IR renders the insecticide completely ineffective, the community benefit is lost and LLINs provide physical protection only. The loss of LLIN-induced mosquito mortality, which will occur when IR is low or moderate, is likely to decrease the community impact of LLINs, increasing average mosquito age and the likelihood that people are infected whilst unprotected by a bed net. This impact is likely where community protection is the greatest, *i.e.* in areas of moderate to high but incomplete LLIN coverage (Thomas & Read 2016).

1.9.3 Vectorial capacity

Using their transmission dynamics model (Churcher et al. 2016), the authors assume that mosquitoes which survive 24 h after LLIN exposure are indistinguishable from unexposed mosquitoes. If this is not the case and mosquitoes exposed to LLINs have reduced fitness, then hut trials data alone will be insufficient to predict the public health impact of pyrethroid resistance and current models will over-estimate its impact. Similarly, if the mosquito population exhibits additional behavioural mechanisms to avoid LLINs together with increased tolerance of pyrethroid insecticides, the predictions will likely underestimate the public health impact as this behaviour change has not been incorporated into the assays (Churcher et al. 2016). Hence, to make predictions on the impact of IR on malaria transmission, based on entomological endpoints from experimental hut studies, it is important to understand how insecticide exposure affects the mosquito's vectorial capacity.

Vectorial capacity (C) describes the rate at which future infections arise from a currently infected host (provided that all female mosquitoes become infected) and provides a measure of the transmission potential of a vector population. It is defined as:

$$C = \frac{ma^2bp^n}{-\ln(p)}$$

where m is vector density (ratio of adult mosquitoes to humans), a is the daily probability of a human host being fed on by a vector, n is the extrinsic incubation period of the parasite, p is the daily probability of adult vector survival, and b is the proportion of mosquitoes with sporozoites disseminated in their salivary glands. $\ln p$ is the natural logarithm of p . The mosquito longevity is $1/p$ (Cohuet et al. 2010; Moller-Jacobs et al. 2014).

For successful transmission of the malaria parasite, mosquitoes must live long enough to become infected, survive the pathogen latent period, and then survive long enough to give some number of infectious bites (Smith et al. 2012). As a result, choice of vector control interventions have been largely guided by the longevity aspect of vectorial capacity, prioritising methods that shorten the lifespan of adult mosquitoes, hence, LLINs and IRS are usually recommended over other interventions (Brady et al. 2016).

If IR affects one or several of the vector capacity variables, its effect on transmission becomes more complex. Indeed, aside from the effect on mosquito population density, IR can impact all the main mosquito-related parameters of the basic reproductive rate⁶ (R_0) including vector longevity, vector competence, and vector feeding behaviour (Rivero et al. 2010).

1.9.3.1 Reproductive/longevity fitness costs

In *An. gambiae*, the *kdr* mutation has been associated with reduced fecundity but an increase in adult survival (Alout et al. 2016). Conversely, the *ace-1* gene has been associated with increased pupal mortality, increased development time, reduced survival to adulthood and reduced male competitiveness (Djogbénou et al. 2010; Assogba et al. 2015). A recent study (Platt et al. 2015) shows *kdr* heterozygote males were more likely to mate than homozygote resistant suggesting a negative impact of *kdr* on *An. coluzzii* mating ability. In mating competition and predator avoidance experiments (Rowland 1991a), homozygotic dielidrin resistant *An. gambiae* and *An. stephensi* males were less successful than homozygotic

⁶ The basic reproductive rate (R_0) is the product of the vectorial capacity, the infectiousness of vectors to humans and humans to vectors, and the human infectious period. R_0 is the total number of malaria cases derived from one infective case that the mosquito population would distribute to man. R_0 must equal at least 1 for the disease to persist or spread. For values less than 1, the disease will regress (Cohuet et al. 2010).

susceptible and heterozygote individuals. Similar fitness costs in metabolic resistant *Culex* mosquitoes showed increased carboxylesterases induced mating competition, where susceptible males had a mating advantage when competing with resistant males, and increased predation costs, associated with decreased locomotion (Berticat et al. 2002; Berticat et al. 2004). Similarly, a study on peach-potato aphids (Foster et al. 2000) shows aphids with higher levels of IR, due to either target site or metabolic mechanisms, tended to move at slower rates, reduced overwintering ability and reproductive success.

Adult female longevity, relative amount of blood ingested by females, number of egg laying females, number and viability of eggs were investigated in *Ae. aegypti* populations exhibiting different levels of resistance to pyrethroids and organophosphates (Martins et al. 2012). Findings reveal shortened longevity, reduced ingested blood meal, reduced egg laying females, egg number and viability amongst resistant strains. Another study (Kumar & Pillai 2011) showed a correlation between reproductive potential and IR in filarial vector, *Cx. quinquefasciatus*, where a reproductive disadvantage was seen in resistant individuals, such as decreases in gonotrophic cycle duration, egg production and hatch rate.

Metabolic IR mechanisms may lead to resource depletion/energetic costs of IR (Rivero et al. 2011). Insecticide resistant *Cx. pipiens* mosquitoes, through the overproduction of esterases, contain on average 30% less energetic reserves than their susceptible counterparts. Another study (Hardstone et al. 2010) looking at differences in lipid and glycogen content in insecticide resistant *Cx. pipiens*, showed calorific content was significantly higher in susceptible compared to resistant adult females. They also found slower emergence time and smaller body size associated with the IR allele of P450-mediated detoxification.

1.9.3.2 Oxidative stress

Senescence (aging), a process associated with a decline in fitness and performance with advancing age, occurs in all multicellular organisms and is a consequence of oxidative stress (cellular damage caused by reactive oxygen species (ROS)) (Hughes & Reynolds 2005). Several antioxidant enzymes such as catalase, peroxidase and superoxide dismutase, mitigate oxidative stress in cells. Pyrethroid tolerance in pyrethroid resistant *An. arabiensis* females from Cameroon was associated with increased levels of several genes with antioxidant roles, including superoxide dismutases, a glutathione S-transferase and a thioredoxin-dependent peroxidase, and a cytochrome P450 (Muller et al. 2008). Increased Glutathione S-transferases (GSTs) levels with peroxidase activity have previously been shown to be involved in protection against pyrethroid intoxication in the

brown plant hopper *Nilaparvata lugens* (Vontas et al. 2001). A conflicting study (Otalı et al. 2014) showed that compared to permethrin susceptible *An. gambiae* females, insecticide resistant females, containing the *kdr* mutation allele, and increased P450 and beta-esterase activities, displayed reduced metabolic rate and mitochondrial coupling efficiency and high mitochondrial ROS production. Furthermore, despite high levels of GSTe3 and catalase transcripts, resistant females had a shorter adult life span than susceptible females, because of excess ROS production. Increased levels of P450 activity has been associated with high levels of ROS production as by-products of the detoxification processes (Murataliev et al. 2008). As such, the authors speculate that the resistant strain may exhibit higher levels of ROS produced by P450 than the permethrin-susceptible strain.

Blood feeding affects oxidative stress and may influence the ability of resistant mosquitoes to withstand insecticide exposure; expression of several ROS detoxification enzymes increases in the midgut after a blood meal (Graça-Souza et al. 2006). A recent study (Oliver & Brooke 2016) showed multiple blood feeding in *An. arabiensis* and *An. funestus* led to reductions in oxidative stress in insecticide resistant females, and also reductions in the oxidative burden induced by DDT and pyrethroids, through increased antioxidant glutathione peroxidase production. Furthermore, it was found that the taking of multiple bloodmeals offered a greater advantage in terms of extending the lifespan of resistant females compared to their insecticide susceptible counterparts. Increases in insecticide tolerance has been shown in a pyrethroid resistant strain of *An. funestus* following a blood meal (Spillings et al. 2008). Authors suggest that insecticide detoxification mechanisms involved in IR are stimulated by the presence of a blood meal prior to insecticide exposure, leading to enhanced expression of the IR phenotype.

1.9.3.3 Parasite competence

Malaria transmission depends on the competence of *Anopheles* mosquitoes to sustain *Plasmodium* development (Kumar et al. 2003). Some susceptible refractory strains (which block plasmodium development by melanising and encapsulating the parasite) are in a chronic state of oxidative stress, which is exacerbated by blood feeding, resulting in increased steady-state levels of reactive oxygen species. As previously discussed (section 1.8.3.2), the ability to tolerate oxidative stress is one potential cause or consequence of IR. Therefore, are insecticide resistant individuals, who can tolerate oxidative stress, better or worse vectors of malaria than susceptible individuals?

Mutations of IR have been shown to affect vector competence of *An. gambiae* for *P. falciparum* isolates (Alout et al. 2013). Strains with target site mutations had increased parasite prevalence than a susceptible strain after feeding on the same gametocyte carriers. Other studies show the presence of the *kdr* increases susceptibility to *Plasmodium* (Ndiath et al. 2014) and presence of *P. falciparum* sporozoites (Kabula et al. 2016). However, while insecticide-resistant mutations may increase vector competence, insecticide exposure may have the opposite effect on mosquitoes carrying those mutations (Alout et al. 2014). *An. gambiae* strains with the *kdr* mutation had significantly reduced prevalence of parasite oocysts after insecticide exposure. Similarly, another study (Kristan et al. 2016) showed sub-lethal doses of pyrethroids against resistant *An. gambiae* reduced prevalence and intensity of *Plasmodium* infection compared to unexposed mosquitoes, after blood feeding with *Plasmodium* infected blood. Therefore, importantly, insecticide applications can still affect transmission of malaria despite IR.

1.10 Behavioural response to insecticides

A side effect of IR is often a reduction in the behavioural responsiveness to the insecticide (Gatton et al. 2013). Earlier studies show an absence in irritability in insecticide resistant mosquitoes compared to susceptible individuals (Rowland 1990; Hodjati & Curtis 1997; Chandre et al. 2000; Hougard et al. 2003). More recently, studies have shown insecticide resistant *An. gambiae* visit insecticide treated netting more frequently than susceptible strains (Siegert et al. 2009). Similarly, after exposure to high concentrations of permethrin, resistant field populations of *Cx. quinquefasciatus* exhibit significantly reduced avoidance behaviour compared to the susceptible strain (Boonyuan et al. 2016). Different repellent actions were shown to exist in resistant strains of *An. gambiae*, *An. arabiensis* and *An. funestus* where authors suggest the *kdr* resistant individuals lose repellency whereas those with metabolic IR do not (Kawada et al. 2014). Furthermore, a study looking at repellent insensitivity showed increased insensitivity to transfluthrin with increased *kdr* frequency and decreased insecticide susceptibility (Wagman et al. 2015).

In mosquitoes, sodium channels are involved in the olfactory signal transduction pathway (Zweibel and Takken 2004). *kdr* homozygotes are less successful than heterozygotes at navigating through the holes to blood-feed and authors suggest the reduced efficiency is due to reduced host-seeking activity (Diop et al. 2015). Resistant gravid females have been shown to be less responsive to oviposition-site stimuli and took to flight least readily than their heterozygote and susceptible counterparts (Rowland 1991b). A recent study (Porciani et al. 2017) found *kdr* homozygous mosquitoes were more attracted by a host behind an LLIN than an untreated net, while the presence of insecticide on the net did not affect the choice of susceptible mosquitoes. The authors suggest the *kdr* mutation modulated the host choice of mosquitoes in the presence of an LLIN, and *kdr* influenced the transmission of an odorant signal in the resistant mosquito resulting in a reduction of neuronal excitability compared to susceptible individuals.

1.11 Detecting and monitoring IR

Resistance arises when insects survive exposures to insecticide, especially at concentrations used in operational vector control which can become problematic if the underlying mechanism has a heritable basis (Weetman & Donnelly 2015). Selection causes a progressively higher proportion of individuals in the population to survive exposure, *i.e.* prevalence of IR increases. Consequently, a rise in prevalence of IR should act as a warning sign of future loss of insecticidal efficacy. The prevalence of IR can be monitored with three complementary methods that provide different types and depths of information: susceptibility testing, biochemical assays and molecular testing (WHO 2012).

The standard WHO susceptibility tube bioassay is a simple direct response-to-exposure test; filter papers are coated with 'discriminatory doses' of insecticide and mosquitoes are exposed to a single dose for a predefined time. Aiming to distinguish between baseline susceptibility and IR, mosquito mortality in the range 98–100% indicates susceptibility and mortality of less than 98% is suggestive of the existence of IR and further investigation is needed (WHO 2013c). As an alternative to the WHO tube bioassay, the CDC (Center for Disease Control and Prevention) bottle assay was developed (Brogdon & McAllister 1998). Even though the two assays can detect IR, they may not be used interchangeably because while the diagnostic dose in the WHO susceptibility test does not allow for detecting shifts at low or extreme IR levels, time-to-knockdown measured in the CDC bottle assay is a poor predictor of 24 h mortality (Owusu et al. 2015). Yet, one study compared the two methods and found that both bioassays give similar results with regards to the susceptibility of mosquitoes to insecticides (Aïzoun et al. 2013).

Biochemical assays detect the presence of a particular IR mechanism or an increase in enzyme activity which may not be detected using bioassays (Brogdon 1989). These have largely been replaced with molecular approaches. Standard polymerase-chain reaction (PCR) molecular assays can be used to test for target-site mutations e.g. *kdr* and *ace-1* (Martinez-Torres et al. 1998; Ranson et al. 2000; Weill et al. 2004), however these are largely being replaced by high-throughput assays such as TaqMan assays for more rapid IR diagnostics (Bass et al. 2007; Bass et al. 2010). Microarray assays have been developed to detect changes in gene expression associated with metabolic IR in *An. gambiae* and *An. funestus* (David et al. 2005; Riann N Christian et al. 2011), with real-time quantitative PCR used to validate these candidate genes (Livak & Schmittgen 2001; Bustin et al. 2009).

1.11.1 Bioassay limitations

Although susceptibility bioassay tests are intended to act primarily as a warning signal, they often remain in use for monitoring long after the arbitrary threshold for defining a population as resistant has been passed (Weetman & Donnelly 2015). Yet this standardised methodology does not provide information on the strength of this IR or its impact (Bagi et al. 2015). The concentration of insecticide used [on filter papers] has no relationship to the quantity of insecticide used in field applications and, using prevalence of IR as the metric, it is not possible to identify regions where IR is likely to be posing the greatest threat to malaria control (Bagi et al. 2015).

Bioassay methodology has further limitations, e.g. with the WHO tube bioassay, only one concentration of one insecticide at one exposure time can be tested; no information on IR mechanism is provided; and IR cannot be detected at low frequency especially where susceptible insects survive exposure due to deterioration of papers or by resting on netting of exposure chambers (Brogdon 1989). They are also sensitive to variations in temperature (Glunt et al. 2014), time-of-day (Balmert et al. 2014), and physiological state and humidity (WHO 2016b), controlling for which necessitates standardised rearing and testing conditions, and makes direct testing of wild-caught adult female mosquitoes problematic.

In addition, these bioassays do not capture the lifetime impact of insecticide exposure, as routine surveillance typically assesses IR on the basis of 24 h mortality responses (Ranson & Lissenden 2016). If resistant mosquitoes have reduced fitness after surviving exposure to LLINs, the impact of parasite transmission may be reduced (Rivero et al. 2010; Viana et al. 2016; Alout, Roche, et al. 2017).

With the aim to provide a stronger focus on producing operationally meaningful data, an expanded three-step WHO intensity bioassay protocol was developed (WHO 2016b) recommending: 1) the detection of the presence of IR phenotypes in a population using discriminating concentration bioassays; 2) the assessment of the strength of phenotypic IR by performing bioassays using 5× and 10× the discriminating concentrations of insecticides, and 3) the determination of the involvement of metabolic IR mechanisms by assessing the effect of a synergist such as PBO on the IR phenotypes detected.

Using this revised WHO protocol, a recent study (Venter et al. 2017) assessed the intensity of pyrethroid resistance in a range of insecticide resistant *Anopheles* strains with known IR mechanisms. All strains tested were classified as pyrethroid resistant based on the use of

discriminating concentrations, the use of follow-on intensity assays showed that the response to pyrethroid intoxication was measurably variable between strains and species, and between type I (permethrin) and type II pyrethroids (deltamethrin) and they were able to distinguish between low to moderate and high intensities.

DNA-based assays have the major advantage over bioassays by being applicable to any mosquitoes collected, including wild-caught adult females (Weetman & Donnelly 2015). More importantly, IR can be detected at low frequencies allowing efficient monitoring of vector control populations. However, molecular methods require sophisticated equipment, and interpretation of results requires strong technical skills. Bioassay tests are needed to compliment molecular based methods as the absence of detectable IR mechanisms does not necessarily mean that IR does not exist (WHO 2012).

1.12 Aims and Objectives of this study

This study explores whether bench top assays can be used or adapted to answer key questions: How does survivorship after exposure to insecticides change throughout the lifetime of IR mosquitoes as compared to susceptible mosquitoes? How do susceptible and resistant mosquitoes behave near the LLIN surface? Does IR make resistant mosquitoes less likely to avoid the LLIN? Does IR affect blood feeding behaviour?

To test the null hypothesis that the impact of insecticide exposure on mosquito populations is confined to acute toxicity and there are minimal long-term consequences to fitness or variation in behaviour for mosquitoes surviving this exposure, the specific objectives of the current study were to determine the long-term (beyond first 24 h as adults) effects of IR on:

- a) Mosquito longevity
- b) Behavioural responses to contact with LLIN netting and behavioural avoidance of contact with LLIN netting (i.e. non-contact effect)
- c) Behavioural responses to contact with LLIN netting with a host
- d) Blood-feeding behavioural response after contact with LLINs

2 Lifelong impact of prolonged exposure to pyrethroids in insecticide resistant *Anopheles gambiae*

2.1 Introduction

The first chapter introduced the African anopheline malaria vector, vector control methods and IR, with reference to the impact of IR on both mosquito vectorial capacity and disease transmission. In this chapter, the impact of insecticide exposure on lifelong fitness cost in insecticide-resistant mosquitoes and its implication for parasite transmission will be examined for the first time.

The longer a female mosquito lives, the more likely she is to encounter an infected host and transmit the infection during subsequent feeding attempts. The extrinsic incubation period (EIP), required for *Plasmodium spp* to develop and the mosquito to become infectious, is a minimum of 9 d (Beier 1998) and typically ~10-16 d (Vaughan 2007). Yet, even in the absence of control interventions, most mosquitoes do not live long enough to transmit the parasite (Gillies & Wilkes 1965; Lines et al. 1991; Charlwood et al. 1997). So, targeting adult mosquitoes with insecticide further reduces the daily survival rates of malarial vectors and thereby reduces transmission.

The future impact of current levels of IR on malaria control strategies is difficult to predict but most experts consider that IR will likely have significant operational impacts if no pre-emptive action is taken (Churcher et al. 2016). This prediction assumes that mosquitoes possessing IR traits will survive repeated exposures to a treated net or a sprayed house, and as a result, the ability of the local vector population to transmit disease will not be reduced (Jones et al. 2012). Thus, IR increases the number of vectors but critically also increases the mean lifespan of the population and thereby increases the proportion of older, potentially infectious mosquitoes. The detection of sporozoite infected mosquitoes alive inside LLINs in some areas is a worrying indication that IR is impacting transmission (Ochomo et al. 2013).

Managing IR is complex and a lack of data on levels of IR impedes decision making. WHO has developed standard test procedures for monitoring IR in a range of disease vectors, including mosquitoes (WHO 2013), whereby young adult female mosquitoes (3-5 d) are exposed to known concentrations (diagnostic doses) of an insecticide for a fixed period, typically one

hour. Assessment of mortality is made 24 h after the exposure. However, the value of data from diagnostic dose assays for malaria control programmes to make decisions on the most appropriate selection of insecticides is limited; diagnostic doses are not representative of effective doses in the field, nor are they predictive of the performance of insecticide-based products in the field, due to a range of environmental factors that reduce reliability of achieving target doses.

Another major limitation of these assays is that, by only considering short-term mortality, they do not measure delayed mortality and/or effects on reproductive output that might result from insecticide exposure (Ferguson & Read 2004; Martins et al. 2012; Viana et al. 2016). Furthermore, by monitoring for IR only in young mosquitoes, the impact of the reduction in IR associated with mosquito ageing, reported by several groups is not accounted for (Lines & Nassor 1991; Mourya et al. 1993; Read et al. 2009; Christian et al. 2011; Rajatileka et al. 2011; Chouaibou et al. 2012; Kulma et al. 2013; Jones et al. 2012; Saddler et al. 2015; Mbepera et al. 2017).

2.2 Aims and Objectives

The immediate aim of this chapter was to determine the long-term impact of periodic exposure to an LLIN on longevity and reproduction rate in IR *An. gambiae*. Three different exposure regimes were used to reflect typical conditions in which mosquitoes are affected by insecticides. The ultimate aim is to surmount the shortcomings of the WHO standard bioassay.

The specific objectives of this chapter were to:

- Determine the effect of repeated exposure of pyrethroid resistant populations to LLINs on mosquito longevity.
- Determine the survivorship of IR mosquitoes when exposed beyond the average 14-day EIP for *P. falciparum*.
- Determine the effect of exposure to LLINs on reproductive output (i.e. eggs) over several gonadotrophic cycles.

2.3 Methods

2.3.1 Mosquito colonies

Four IR strains and one susceptible strain of *An. gambiae sensu lato (s.l.)* were used in this study. The IR profile of each strain is shown in Table 2-1. The Tororo strain is considered ‘moderately’ resistant, the Tiassalé strain is ‘highly’ resistant and the VK7 and Banfora stains are ‘extremely’ highly resistant. The Kisumu reference strain is susceptible to insecticides (Bagi et al. 2015; Toé et al. 2014).

Table 2-1 Details of mosquito colonies including colony name (strain), species, country of origin, pyrethroid resistance phenotypes and profile of the *An. gambiae s.l.* strains used in the survival experiments. Green shading shows susceptible strain(s) and blue shading shows resistant strains. Diagnostic dose (DD) is used to discriminate the proportions of susceptible and resistant phenotypes in a sample of a mosquito population and are determined by exposing mosquitoes (tarsal contact) to insecticide deposits on filter-paper (WHO 2006).

Strain (colony name)	Species	Origin	Resistance phenotype (pyrethroids)	0.75% permethrin mortality DD
Kisumu	<i>An. gambiae</i>	Kenya	Susceptible	100%
Tororo	<i>An. gambiae</i>	Uganda	Resistant	38%
Tiassalé	<i>An. gambiae</i> + <i>An. coluzzii</i>	Cote d’Ivoire	Resistant	3%
Banfora	<i>An. coluzzii</i>	Burkina Faso	Resistant	0%
VK7	<i>An. coluzzii</i>	Burkina Faso	Resistant	0%

The susceptible **Kisumu** colony of *An. gambiae s.s.*, originating from Kenya, was colonised in 1953 (Shute 1956). It has been maintained at the Liverpool School of Tropical Medicine (LSTM) since 1975.

The **Tororo** strain of *An. gambiae s.s.* was colonised from Eastern Uganda in 2013 and maintained at LSTM without selection pressure. It is resistant to DDT, pyrethroids and bendiocarb and contains the 1014S *kdr* mutation (Ramphul et al. 2009). There is no clear evidence for metabolic IR in this strain.

The **Tiassalé** strain was colonised from Southern Côte d’Ivoire in 2013 and maintained at LSTM under six-monthly selection pressure with deltamethrin. This strain, which contains both *An. gambiae* and *Anopheles coluzzii*, is resistant to all public health insecticides (Edi et

al. 2012). It has a high frequency of 1014F *kdr* and *ace-1* mutations and expresses elevated levels of key P450s known to metabolise pyrethroids (Edi et al. 2014).

The **VK7** and **Banfora** strains of *An. coluzzii* originated from Burkina Faso in 2014 and are currently maintained at LSTM. They have high frequency of 1014F *kdr* mutations plus additional pyrethroid resistance mechanisms including elevated P450s and suspected cuticular IR (Toé et al. 2014; Grisales et al. submitted).

Mosquito rearing, and bioassays were performed in the insectaries at LSTM. All life stages were reared at 27°C±2°C, 80±10% relative humidity with a 12 h photoperiod. Larvae were maintained in distilled water in 3L food trays (Solent Plastics, UK) and fed on ground flaked fish food (Tetramin, Tetra GmbH, Germany). Adults were maintained in 30cm x 30cm x 30cm rearing cages (Bugdorm, Megaview Science, Taiwan) and fed on 10% sugar solution. Colony female adults were fed on human blood (National Health Service Blood Transfusion, UK) using an artificial membrane feeding apparatus (Hemotek, UK). Experimental mosquito cohorts were not blood fed prior to use. All experiments were started 4 d post eclosion.

2.3.2 Long-lasting insecticide treated nets (LLINs)

New unwashed LLINs from two manufacturers were used: Olyset® (Sumitomo Chemical Ltd, Japan) and PermaNet® 2.0 (Vestergaard Frandsen, Switzerland)⁷. These nets were purchased locally in Africa.

Both nets use incorporation technology, where a pyrethroid insecticide is incorporated directly into the material from which then the netting is made. Facilitated by certain reagents the insecticide will migrate to the surface of the fibre and will be regenerated from the reservoir after the surface insecticide is washed off or otherwise lost (Kilian et al. 2008). There is no evidence that net material effects insecticide exposure, i.e. effective dose of insecticides.

Olyset is made of polyethylene with 2% w/w permethrin incorporated into the fibres corresponding to 20g/kg ± 3g/kg. PermaNet is a polyester net coated with 55 mg/m² ±25% deltamethrin. An untreated polyester net was used as a control. All LLINs were hung for 1 week, stored at 4°C and removed from 4°C the day before testing, to allow the net to fully acclimatise to insectary room temperature. HPLC chemical analysis of these nets is detailed in Chapter 3.

⁷For all LLINs, after the registered trade mark has been shown for the first time, it will not be shown subsequently.

2.3.3 Standard WHO Cone bioassay protocol

The WHO cone bioassay was carried out in accordance with current standard protocols (WHO 2013a). Individual pieces (15cm x 15cm) of LLIN or untreated netting were sandwiched between 2 identical Perspex boards (LSTM, UK). So multiple assays can be performed at one time, six holes (10cm diameter) were cut into each board to accommodate six plastic cones (WHO, Malaysia). Each net overlapped a hole on the lower board and was secured with adhesive tape. The cone was rested upon the netting and the upper board placed over the cone through the corresponding hole. Both boards were secured together firmly with bulldog clips and placed on a support board at a fixed 45° angle. Figure 2-1 shows the assay set-up.

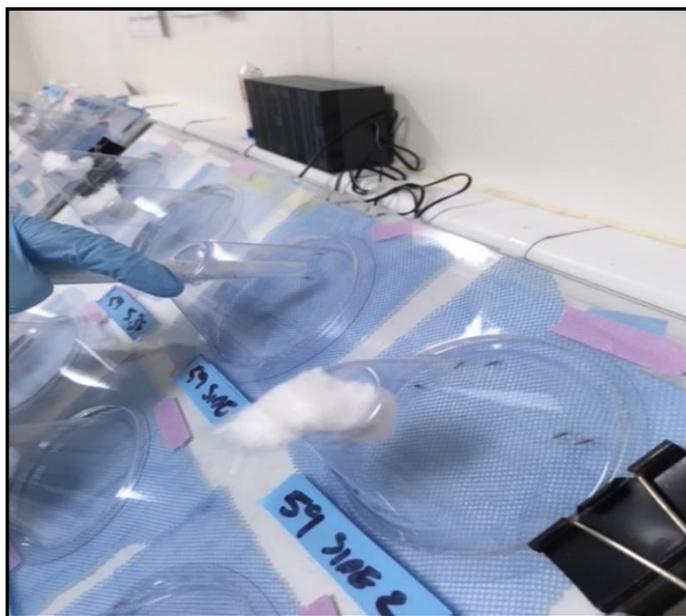


Figure 2-1 Photograph of standard WHO cone bioassay. Each cone contains five mosquitoes which are exposed to netting underneath for 3 min. Cotton wool prevents escape. Mouth aspirators are used to add and remove mosquitoes from the cones.

Mosquitoes were mouth aspirated into the cone which was then plugged with cotton wool to prevent escape. A timer was used to record progress and after a 3 min exposure period, mosquitoes were aspirated from the cones into paper cups with access to sugar solution. Knock-down (KD)⁸ was recorded after 3 and 60 min and mortality after 24 h, for all assays. Table 2-2 shows definitions of KD and mortality.

⁸ For insecticide bioassays, the definition of knockdown and mortality involves not only the state of the insect but also the time at which the observation is made. A mosquito is classified as “dead” or “knocked down” if it is immobile or unable to stand or take off. The distinction between knocked down and dead is defined only by the time of observation. The assessment of knockdown is made within 1 h of exposure. Mortality is determined at least 24 h after exposure. The holding container may be tapped a few times before a final determination is made. Control mortality should be measured over the same recovery period. Mortality after 24 h should be recorded; in some cases, repeated observations may be appropriate (WHO 2016b).

Table 2-2 Classification of adult mosquitoes as alive, knocked down or dead in bioassays is summarised below (adapted from WHO 2016b):

ALIVE	KNOCKED DOWN OR DEAD AFTER EXPOSURE	
	MORIBUND	DEAD
Can both stand and fly in a coordinated manner	<ul style="list-style-type: none"> • Cannot stand (e.g. has only one or two legs) • Cannot fly in a coordinated manner • Lies on its back, moving legs and wings but unable to take off • Can stand and take off briefly but rapidly falls down 	<ul style="list-style-type: none"> • No sign of life • Immobile • Cannot stand

2.3.4 Blood feeding

All experimental blood meals were human arm fed, carried out by one volunteer LSTM staff member, who had no recent travel to, or residency in, malaria endemic countries. The arm was placed on untreated and LLIN net surfaces and mosquitoes allowed to feed through the netting for a maximum of 20 min.

2.3.5 Egg quantification –*en masse* egg counts (filter paper)

After oviposition onto a moist egg paper (55m diameter filter paper (Fisher Scientific, UK) resting on a glass slide staining jar lid), the total number of eggs laid were counted in a monolayer on the paper using a stereoscope (Wild Heerbrugg M3Z, Switzerland), a tally counter and a fine paint brush to move the eggs.

2.3.6 Egg quantification – individual egg counts (dissections)

Two days after blood feeding, gravid mosquitoes were killed by being held at -20 for one hour. Mosquitos were placed individually on a glass slide in a drop of distilled water and ovaries dissected by pulling out the last segment of the abdomen with watchmaker forceps (S Murray™ E010/04, Fisher Scientific, UK). When the ovary was ruptured, released eggs were observed at 200x magnification under a light microscope (Leica MZFLIII, Germany) and counted using a tally counter. Figure 2-2 shows examples of an *An. gambiae* ovary (A) and released eggs (B) modified from (Koama et al. 2015) .



Figure 2-2 Photographs of dissected *An. gambiae* ovaries 12 h after blood meal (A) and eggs fully formed showing the sausage shape 36 h after blood meal (B). Both were shot at 200x magnification. Eggs were dissected in experiment 6 (Images modified from Koama et al. 2015)

2.3.7 Hatch rate assay from *en masse* egg collections

After counting, eggs were gently washed into a larval tray containing 1cm depth of distilled water. Walls of the tray were washed to ensure all eggs have contact with water and to stimulate hatching. After 48 h, 100 eggs were transferred onto damp filter paper with a small brush. Using a stereoscope, hatched and unhatched eggs were recorded by observing whether the operculum (hatching lid/cap) was dislodged (Figure 2-3).

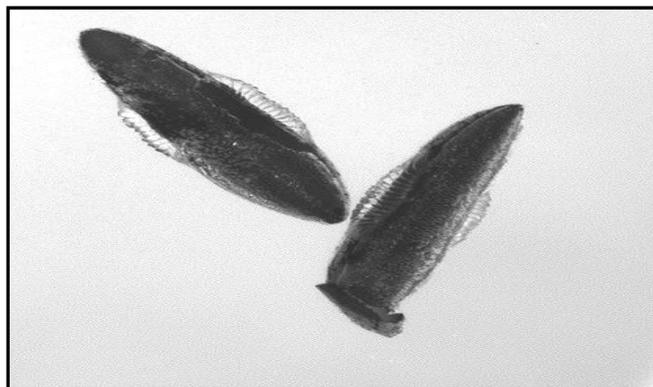


Figure 2-3 Photograph of unhatched and hatched *An. gambiae* eggs from the same mosquito laid on the same day. The dislocated operculum shows the lower egg has hatched (right) compared to the intact operculum in an unhatched egg (left). (Images modified from MR4 Methods in *Anopheles* Research, 2015 Edition)

2.4 Experimental design

2.4.1 Exposure rationale

Over a series of four experiments, the frequency with which mosquitoes were exposed to LLINs varied. Experiment (1-4) are described as follows:

1. Daily exposure for 5 consecutive days
2. Exposure every 4 d, for a maximum of four exposures
3. Exposure and blood fed every 4–6 d for a maximum of 3 exposures
4. Tiered daily exposures, where mosquitoes were exposed either once, twice or three times, over 3 consecutive days.

Tororo and Tiassalé strains were used for experiments 1-3, while extremely resistant VK7 and Banfora strains were later available to test in experiment 4. In all experiments, mosquitoes were first exposed to insecticides when they were 4 d old (post eclosion) and then monitored daily to record mortality until no survivors remained.

All experiments were carried out in duplicate excluding Kisumu exposure which had only one replicate. Days of exposure in experiment 3 changed between replicates due to restricted availability of the human blood source. In each experiment one cohort was exposed to untreated nets and one to a LLIN. Table 2-3 shows a summary of experimental parameters and Figure 2-4 shows the exposure schematic for each experiment.

Table 2-3 Summary of experimental components, including experiment number, frequency of exposure (times exposed), day of exposures, LLIN type (U = untreated, O = Olyset, P=PermaNet), *An.gambiae* strains used (TIA=Tiassalé, TOR=Tororo, KIS=Kisumu, BAN= Banfora), number of replicates and blood feed status (No/Yes – at exposure), for all experiments. All days are post eclosion. Blue shading shows non-blood fed and red shading show blood fed experiments.

	LLIN Exposure (times exposed)	Exposure day	Net type	Strain	Total no. per replicate	Blood fed: No/Yes
1	Daily (x5)	4,5,6,7,8	U, O, P	TIA/TOR	100	No
2	Every 4 d (x4)	4,8,12,16	U, O, P	TIA/TOR/ KIS	100	No
3	Every 4-6 d (x3)	4,10,14 or 4,9,13	U, O, P	TIA/TOR	50	Yes
4	Daily (x1-3)	4, 4/5, 4/5/6	U, P	VK7/BAN	70	No

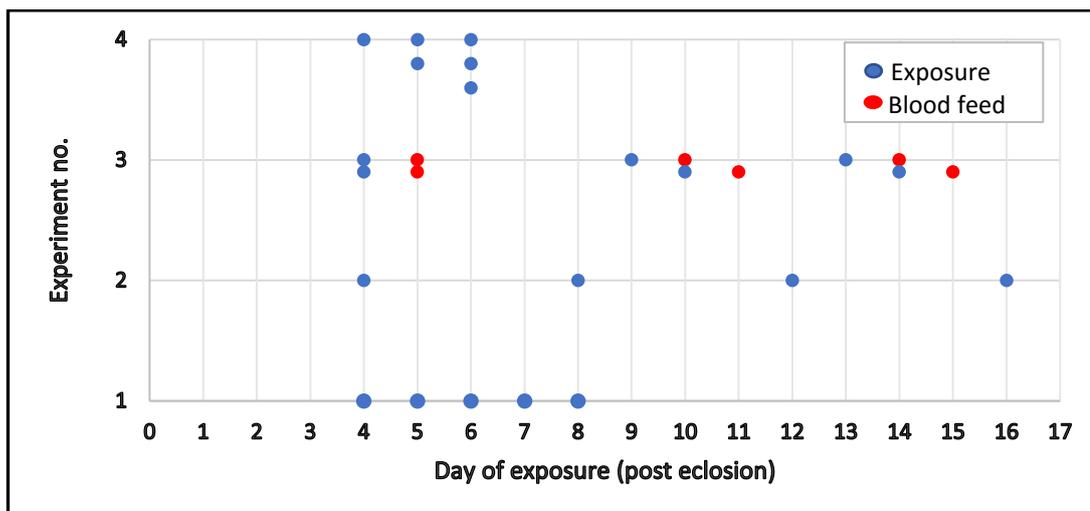


Figure 2-4 Exposure schematic for exposure and blood feeding experiments 1-4. Cohorts of 100 individuals of insecticide resistant Tiassalé and Tororo strains of *An. gambiae s.l* were used in experiments 1-3 and cohorts of 70 individuals of insecticide resistant VK7 and Banfora strains of *An.gambiae s.l* were used in experiment 4. Insecticide susceptible Kisumu strain of *An. gambiae* was tested following experiment 2 method. Blue dots indicate day of exposure day and red dots day of blood meal. Blood feeds occurred 24 h after exposure in experiment 3, where days of exposure were different due to availability of blood meal source.

Together, these regimes cover the likely maximum (daily) and minimum (every 4 d) exposure that *An. gambiae* would expect in areas of high LLIN coverage. These regimes (1-4) were selected to investigate a range of biologically plausible exposures:

1. An unsuccessful blood feed where mosquito that is repeatedly prevented from biting by the presence of a LLIN (thus contacts LLINs on consecutive nights)
2. A theoretical successful blood feed where the mosquito will refrain from feeding until eggs have been laid (~4 d)
3. An actual successful blood feed through an LLIN
4. Unsuccessful blood feed with differing frequency of repeated daily exposures.

2.4.2 Survival assays

2.4.2.1 Experiment 1 - Daily exposure

Experiments measured the effect of five repeated (daily) exposures of insecticides on mosquito survival. Five randomly selected pieces of net were cut from each net and approximately 100 female mosquitoes (in batches of 10) were exposed to either the LLIN or untreated net for 3 min using the WHO cone bioassay (Section 2.3.3). After 24 h, mortality was recorded, and surviving mosquitoes were then exposed again. After the fifth exposure, all surviving mosquitoes were pooled into large labeled paper ‘popcorn’ food containers (2500ml, max 50 mosquitoes per container) covered with hosiery stocking. They were held with access to sugar solution and daily mortality recorded until all mosquitoes were dead.

2.4.2.2 Experiment 2 - 4th daily exposure

Experiments measured the effect of four repeated (4th daily) exposures of insecticides on mosquito survival. The experiment was identical to experiment 1 except mosquitoes were left to recover and exposed every 4 d.

2.4.2.3 Experiment 3 –Simultaneous exposure/feed

The effect of 3 repeated (every 4/5 d) exposures of insecticides, during blood feeding, on mosquito survival was measured. Approximately 50 mosquitoes were starved of sugar solution for 12 h prior to testing then transferred into large containers covered with either PermaNet or Olyset or untreated netting. Mosquitoes were blood fed (Section 2.3.4) and after feeding, mosquitoes were gently transferred into a holding container and offered sugar water. Unfed mosquitoes were discarded, and 24 h mortality recorded. After 48 h, moist filter paper was introduced into each container to allow for egg laying and 24 h later surviving mosquitoes were exposed again as described above. This cycle was repeated until surviving mosquitoes had fed 3 times. After the final exposure, all surviving mosquitoes were held with access to sugar solution. Daily mortality was recorded until all mosquitoes were dead.

2.4.2.4 Experiment 4 – Tiered exposure

This experiment compared the impact of single versus multiple exposures over a three-day period. Three cohorts of 70 mosquitoes (in batches of 10) from the Banfora and VK7 strains were exposed to an untreated or PermaNet LLIN. The first cohort was exposed on a single occasion, the second exposed on two consecutive days and the third on three consecutive days. Daily mortality was recorded as previously.

2.4.3 Reproductive output assays

2.4.3.1 Experiment 5 - Total egg count and hatch rate

Experiments measured the effect of repeated insecticide exposure, during blood feeding, on fecundity and egg hatch rate. 48 h after each simultaneous feed/exposure, surviving mosquitoes from experiment 3 were provided with an oviposition pot. The egg paper was removed the following morning and total eggs were counted and hatch rate established (Section 2.3.5 and 2.3.6). Fecundity was measured as the total number of eggs/total number of females.

2.4.3.2 *Experiment 6 – Individual egg count*

Experiments measured the effect of repeated (daily) insecticide exposure on individual mosquito fecundity. Approximately 100 Tiassalé mosquitoes were exposed to untreated or PermaNet using the standard WHO cone assay three times on subsequent days. Twenty-four hours after the final exposure, remaining mosquitoes were pooled into a large container and offered a blood meal through untreated netting only. 48 h after feeding, gravid mosquitoes were killed and dissected (Section 2.3.7). This cycle was repeated until a total of 100 mosquitoes had been dissected for each exposure. Fecundity was measured by counting the number of fully formed eggs per female.

2.5 Data analysis

Comparisons between each exposure in immediate mortality were determined by non-parametric Kruskal-Wallis test (plus Dunn's multiple comparisons test). Where mortality data was missing (due to total mortality in previous exposure) a comparison could not be made. All analyses were performed using GraphPad Prism 7.03 (GraphPad Software Inc. USA).

Long-term longevity data was determined by Kaplan Meier survival curves, and curve comparisons were determined by the Gehan-Breslow-Wilcoxon⁹ test (Chi square). Hazard ratios (log-rank) were determined from the KM survival curves data for each experiment. Individual egg counts comparisons (experiment 6) were determined by multiple pairwise T-Tests using the Holm-Sidak method (alpha 0.05). Box and whisker plots were prepared on survival data using IMB SPSS Statistics 22.

Further analysis on these current data was carried out using a multivariate Bayesian nonlinear state-space model (SSM), designed and performed by colleagues in Glasgow, as described in the collaborative PNAS paper (Viana et al. 2016), Appendix 2 and section 2.7.1.1.

⁹ Log-rank/Wilcoxon tests are generally used to compare magnitude of effects of exposure on survival. The logrank test is often used for checking the validity of proportional hazards (PH), with the Wilcoxon test being the fallback methods when the PH assumption fails (Martinez & Naranjo 2010). In this study, the Wilcoxon test was used to provide the P value, as it gives more weight to deaths at early time points and is appropriate for our experiments where insecticide exposure was carried out early.

2.6 Results

The effect of repeated exposures to LLINs on immediate and long-term mosquito survival was measured. Experiments 1 and 2 compared exposure to insecticide with exposure to untreated net in moderately resistant (Tororo) and highly resistant (Tiassalé) strains of *An. gambiae*. Experiment 3 looked at the effect of insecticide exposure on blood feeding and subsequent longevity in the same strains. Experiment 4 investigated mortality between repeated exposures to insecticide in two very highly resistant strains (Banfora and VK7). The duration spent on the net in experiment 3 was 20 min compared to the 3 min in the cone assay in experiments 1, 2 and 4.

2.6.1 Immediate (24 h) mortality

In all survival experiments, no mortality was observed 24 h after exposure to untreated netting (data not shown). Kisumu susceptible strain showed 100% immediate mortality against PermaNet, yet after all exposures to Olyset, there were still 7 survivors after exposure 4 and an average mortality of 21.5% (± 7.1 SEM) over all exposures (Figure 2-5).

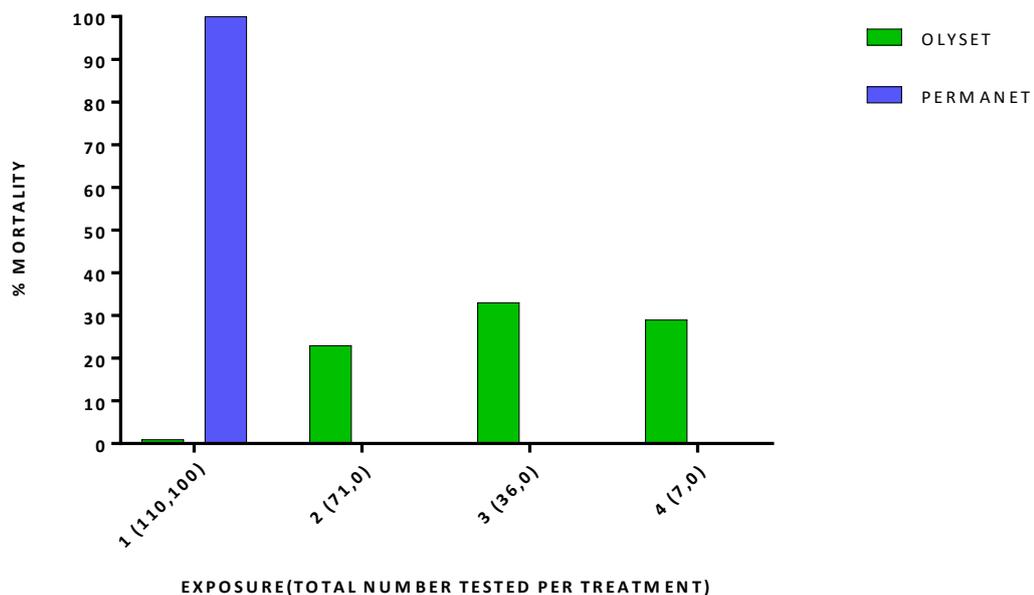


Figure 2-5 Bar chart of immediate mortality of insecticide susceptible control Kisumu strain of *An. gambiae*, following 4 episodes of exposure to both PermaNet and Olyset compared on days 4, 8, 12 and 16 using WHO cone bioassays. Total number tested per treatment relates to number of mosquitoes tested at each exposure. Mortality assays were carried out one time only so mean data is unavailable

Generally, after repeated daily and fourth daily exposures to PermaNet or Olyset in experiments 1 and 2, there was no significant difference in mortality between subsequent exposures in both Tororo and Tiassalé strains (Figure 2-6 A-B). Although the pattern of

mortality increased across exposures, the distribution of mortality was the same across exposures. There was an exception to this rule in experiment 2 which is discussed below.

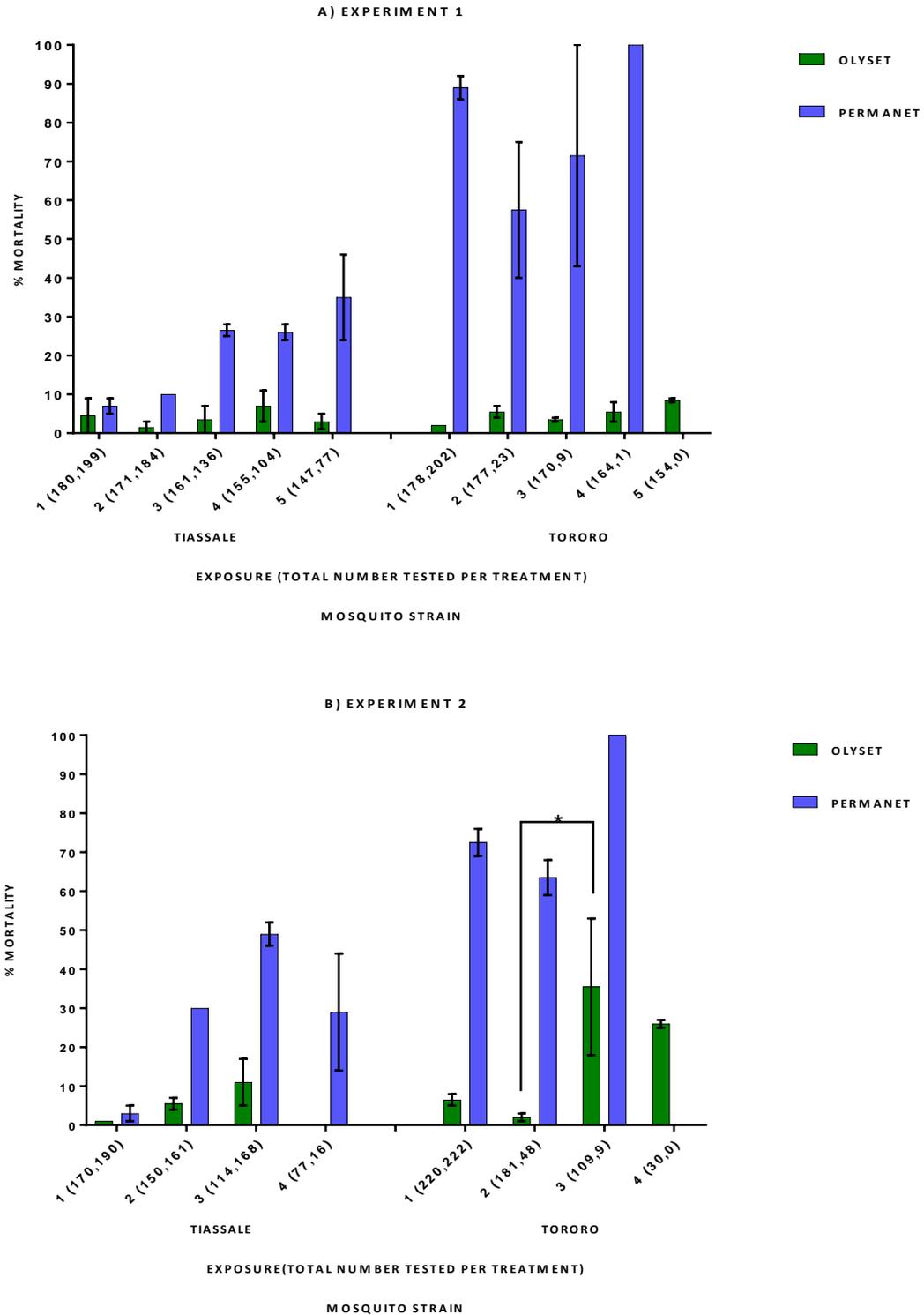


Figure 2-6 (A-B) Bar charts of immediate mortality in experiment 1 and experiment 2 showing mean percentage 24hr mortality of Tiassalé and Tororo insecticide resistant strains of *An. gambiae*. After five daily repeated exposures (1-5) on 4,5,6,7, and 8 d post eclosion (experiment 1) and after four repeated exposures (1-4) on 4,8,12,16 d post eclosion (experiment 2) against PermaNet and Olyset using WHO cone bioassays. Total number of individuals tested shown in brackets. Significance *p = 0.013. Error bars show SEM.

In both experiments, Tiassalé mortality did not exceed 50% (± 3 SEM) after repeated exposure to PermaNet, or 11% (± 6 SEM) after repeated exposure to Olyset, while Tororo showed $>50\%$ mortality after repeated exposure to PermaNet in both experiments. After exposure to Olyset, Tororo mortality was $<8.5\%$ after all exposures in experiment 1 and the first two exposures of experiment 2. Yet, between exposures 2 and 3 in experiment 2, when mosquitoes were 8 and 12 d old respectively, mortality increased from 2% (± 1 SEM) to 35.5% (± 17.5 SEM) (Figure 2-6B). This increased jump in mortality was weakly significant ($p=0.013$).

In experiment 3, where mosquitoes were blood fed through the LLIN, mortality was higher after exposure to PermaNet than Olyset, and higher for Tororo than Tiassalé (Figure 2-7). When Tiassalé was exposed to Olyset there was no mortality was seen. When Tiassalé was exposed to PermaNet, mortality fell from 41.5% (± 8.5 SEM) to zero between exposures 2 and 3 ($p=0.03$). However, this result needs to be considered with caution as there was only one individual mosquito exposed in exposure 3, which survived 24 h later. After the first exposure to PermaNet, Tororo suffered 93.5% (± 6.5 SEM) mortality and no mosquitoes were available for subsequent exposures. After exposure to Olyset, Tororo showed a non-significant decrease in mortality after exposures 1 and 2.

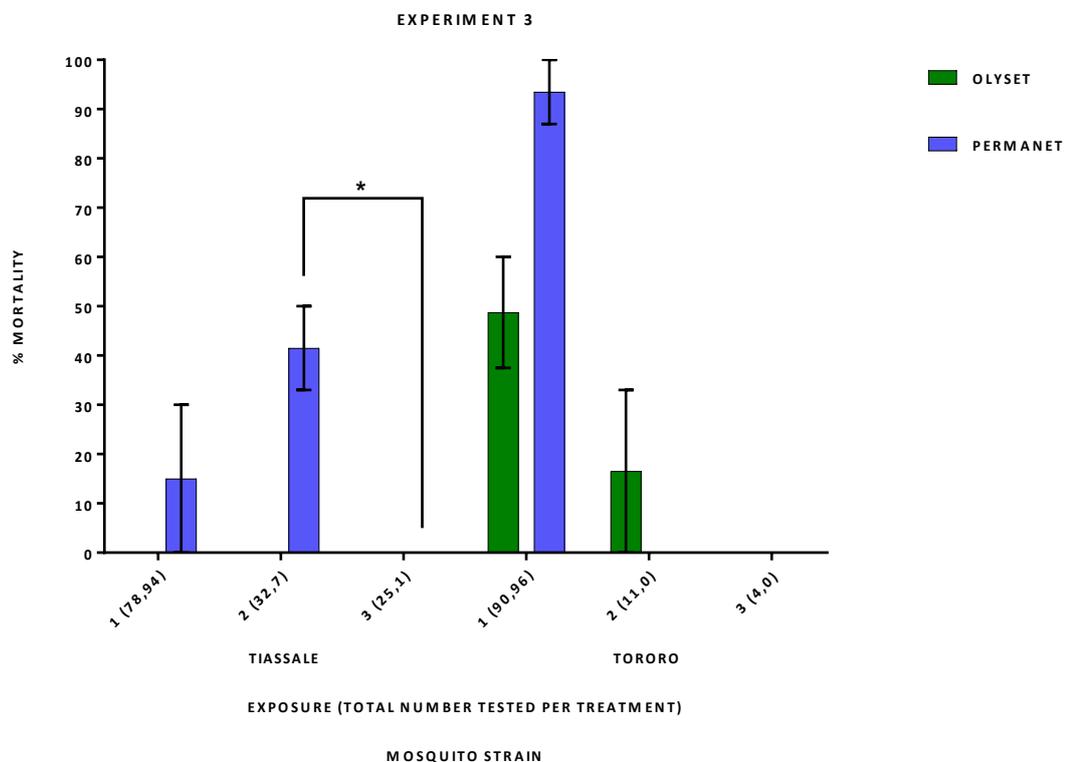


Figure 2-7 Bar chart of immediate mortality in experiment 3 showing mean percentage 24 h mortality of Tiassalé and Tororo insecticide resistant strains of *An. gambiae*. After three repeated exposures/simultaneous feed (1-3) on 4,10,16 d post eclosion against PermaNet and 4,9,13 d post eclosion against Olyset using WHO cone bioassays. Total number of individuals tested shown in brackets. * $p=0.03$. Error bars show SEM.

In experiment 4, after increasing exposure to PermaNet, Banfora mortality did not exceed 12% (± 5 SEM) and VK7 mortality did not exceed 5% (± 2 SEM). There are no significant differences between exposures (Figure 2-8).

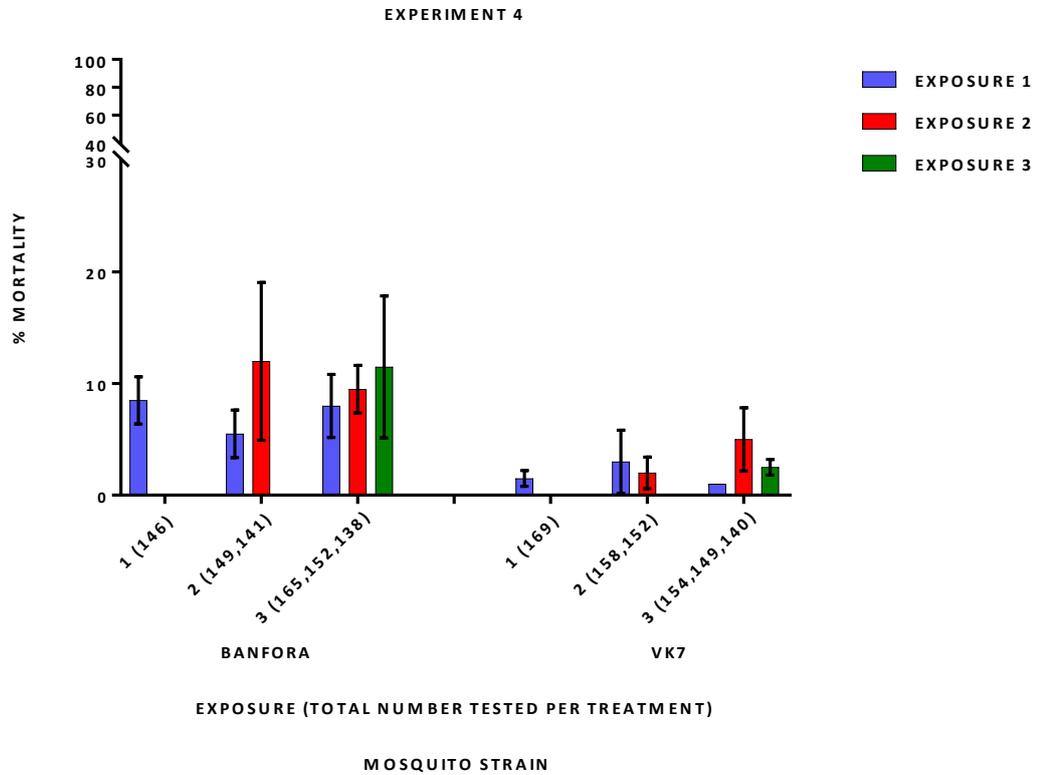


Figure 2-8 Bar chart of immediate mortality in experiment 4 showing mean percentage 24 h mortality of three replicates of Banfora and VK7 insecticide resistant strains of *An. gambiae* after three tiered repeated exposures (1-3) to PermaNet using WHO cone bioassays. Total number of individuals tested shown in brackets. Error bars show SEM.

2.6.2 Longevity and median survival

Kaplan-Meier (KM) daily survival curves were plotted and curve comparisons made to investigate the longer-term effects of repeated exposure to LLINs on post 24 h mosquito longevity. Data from the two replicates in each experiment were pooled. All age references are total days post eclosion (PE) and not time since exposure. In all experiments, the first exposure took place four days PE. The first data point included in the analysis was 48 h post exposure, *i.e.* 6 d PE, to specifically examine the 'delayed mortality'¹⁰ effect.

Box and whisker plots show median survival against both LLINs for each strain in each experiment. A cut-off of 14 d for the average extrinsic incubation period (EIP) of *P. falciparum* is shown in each plot, to illustrate whether, after repeated insecticide exposures, mosquitoes potentially live long enough to allow for parasite maturity and thus disease transmission.

2.6.2.1 Kisumu control - 4th daily exposures

Longevity of the insecticide susceptible strain, Kisumu, after exposure to PermaNet, Olyset and untreated netting is shown in Figure 2-9. Daily survival after exposure to both LLINs compared to untreated netting, is dramatically reduced. Mosquitoes did not survive beyond 24 h after PermaNet exposure but for Olyset, median survival post exposure was 9 d PE compared to 18 d for control nets.

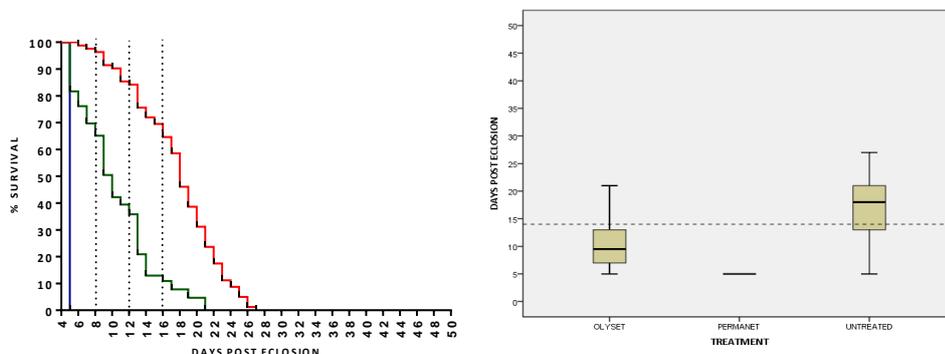


Figure 2-9 Kaplan Meier survival curve and box and whisker plot (Kisumu) showing mean percentage survival (left) and median survival (right) of insecticide susceptible control Kisumu strain of *An. gambiae*, following 4 episodes of exposure to both PermaNet (blue line) and Olyset (green line) compared to untreated (red line) on 4, 8, 12 and 16 d PE. Dotted vertical lines show timings of exposures. Dotted horizontal line shows average 14 d extrinsic incubation period (EIP) of *P. falciparum*. Boxes show median (horizontal line) and upper and lower quartiles (top and bottom edges). The upper and lower whiskers show maximum and minimum values. Statistical difference between survival curves compared to untreated netting: PermaNet and Olyset nets ($p < 0.0001$). Results for one experiments, each with 100 mosquitoes/net.

¹⁰ The term 'delayed mortality' has been used previously in hut trial evaluation of LLINs (Lines et al. 1987; Guillet et al. 2001; Hougard et al. 2003; Asidi et al. 2005) and it refers to the mortality seen at 24 h after an overnight hut experiment. Therefore, in the context of this current study, the use of delayed mortality (>48 h) is different than previously described.

2.6.2.2 Experiment 1 – PermaNet - daily exposures

KM curves for experiments 1 and 2 show repeated exposure to PermaNet resulted in significantly reduced survival compared to mosquitoes exposed to untreated nets in both Tiassalé and Tororo strains ($p < 0.0001$). In experiment 1, after exposure to PermaNet, Tiassalé had a median survival of 8 d PE compared to 14 d against untreated net, while Tororo survived only 6 d compared to 14 d untreated. Tororo did not survive beyond the 5th exposure period (Figure 2-10).

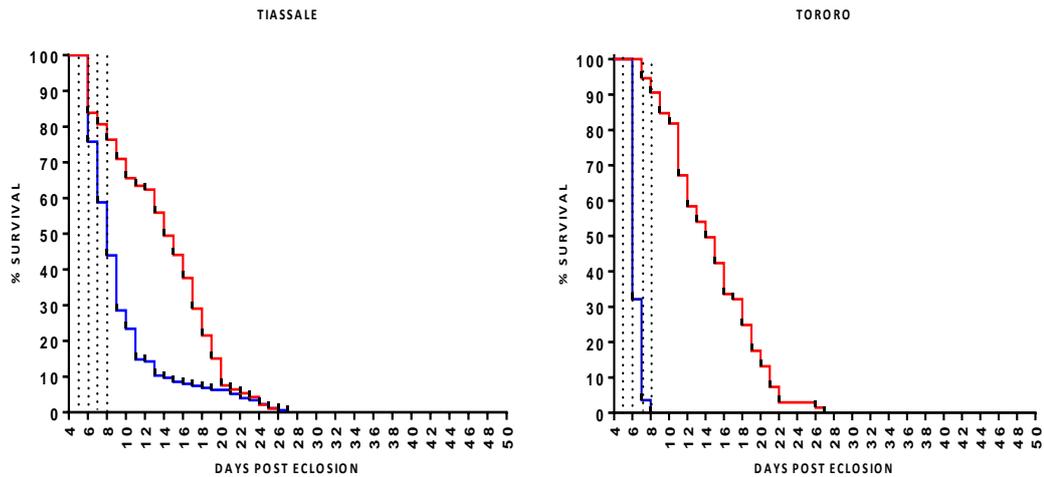


Figure 2-10 Kaplan Meier curves for experiment 1 (PermaNet) showing mean percentage survival for insecticide resistant Tiassalé and Tororo strains of *An. gambiae*, following five episodes (4, 5, 6, 7, and 8 d PE) of exposure to PermaNet (blue line) compared to untreated (red line) net. Dotted vertical lines show timings of exposures. Statistical difference between curves: Tiassalé and Tororo ($p < 0.0001$). Results for two experiments, each with 100 mosquitoes/net/strain were pooled for this analysis.

2.6.2.3 Experiment 2 – PermaNet - 4th daily exposures

In experiment 2 (Figure 2-11), after exposure to PermaNet, Tiassalé had a median survival of 10 d PE compared to 12 d against untreated net, while Tororo survived only 5 d compared to 13 d untreated. Box and whisker plots (Figure 2-12) show that median longevity in both experiments fall below the 14 d cut-off for EIP.

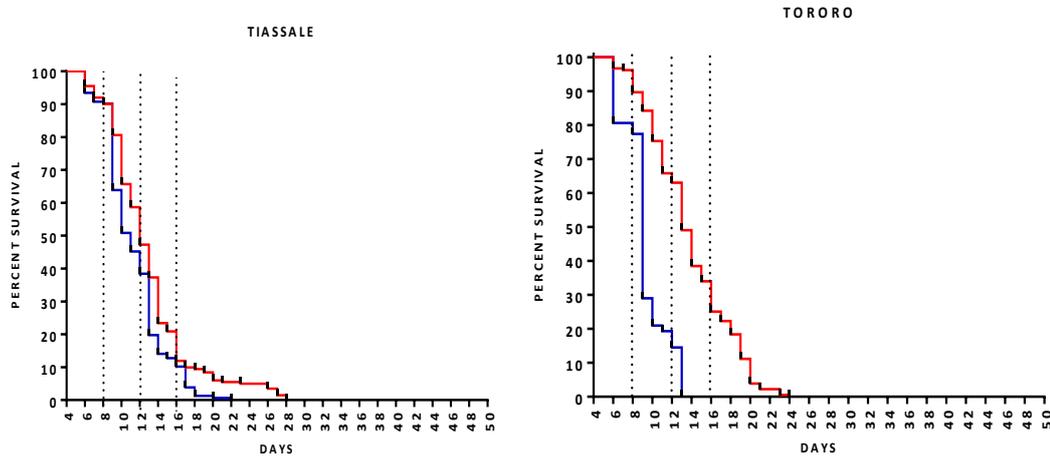


Figure 2-11 Kaplan Meier curves for experiment 2 (PermaNet) showing mean percentage survival for insecticide resistant Tiassalé and Tororo strains of *An. gambiae*, following four episodes (4, 8, 12, 16 d PE) of exposure to PermaNet (blue line) compared to untreated (red line) net. Dotted vertical lines show timings of exposures. Statistical difference between curves: Tiassalé and Tororo ($p < 0.0001$). Results for two experiments, each with 100 mosquitoes/net/strain were pooled for this analysis.

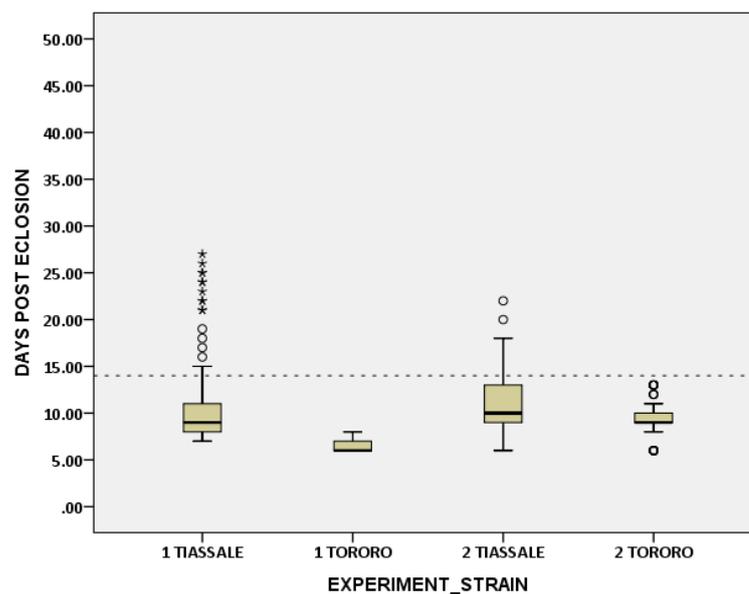


Figure 2-12 Box and Whisker plots for experiments 1 and 2 (PermaNet) showing median longevity of insecticide resistant Tiassalé and Tororo strains of *An. gambiae* after exposure to PermaNet. Dotted line shows average 14 d extrinsic incubation period (EIP) of *P. falciparum*. Boxes show median (horizontal line) and upper and lower quartiles (top and bottom edges). The upper and lower whiskers show maximum and minimum values. Dots and asterisks represent outliers and extreme outliers respectively. Results for two experiments, each with 100 mosquitoes/net/strain were pooled for this analysis.

2.6.2.4 Experiment 1 – Olyset - daily exposures

There was no significant difference in daily survival of the Tiassalé strain after exposure to Olyset compared to untreated netting. Median survival for Tiassalé was 17 d after exposure to both Olyset and untreated net. Median survival for Tororo was 15 d after exposure to Olyset compared to 18 d after exposure to untreated net and there was a significant difference in survival between these treatments ($P < 0.0001$) (Figure 2-13).

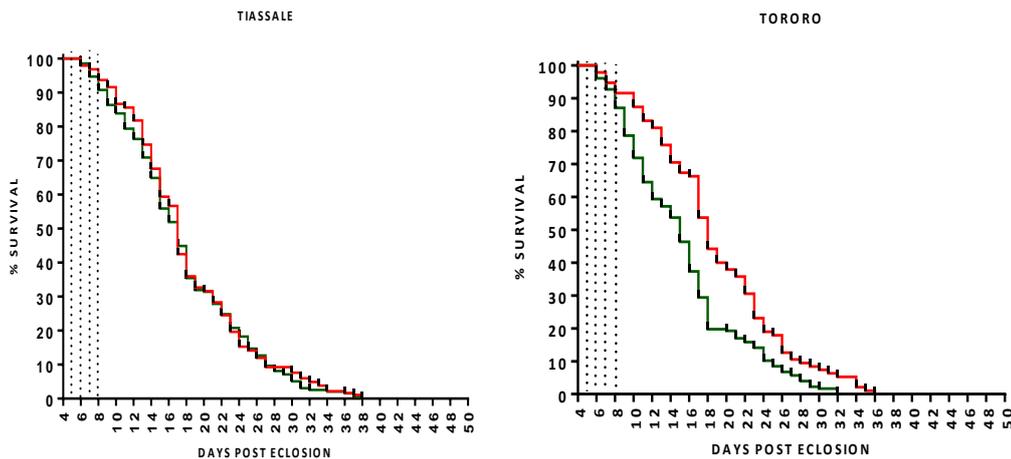


Figure 2-13 Kaplan Meier curves for experiment 1 (Olyset) showing mean percentage survival for insecticide resistant Tiassalé and Tororo strains of *An. gambiae*, following five episodes (4, 5, 6, 7, and 8 d PE) of exposure to Olyset (green line) compared to untreated (red line) net. Dotted vertical lines show timings of exposures. Statistical difference between curves: Tiassalé ($p=0.54$) and Tororo ($p < 0.0001$). Results for two experiments, each with 100 mosquitoes/net/strain were pooled for this analysis.

2.6.2.5 Experiment 2 – Olyset - 4th daily exposures

In both strains, there was no significant effect on daily survival after 4th daily exposure to Olyset (Figure 2-14). Median survival for Tiassalé was 18 d after exposure to Olyset compared to 17 d after exposure to untreated net. Median survival for Tororo was 13 d for both treatments. Box and whisker plots show that the median survival of Tiassalé in both experiments and Tororo in experiment 1 exceeded the EIP (Figure 2-15).

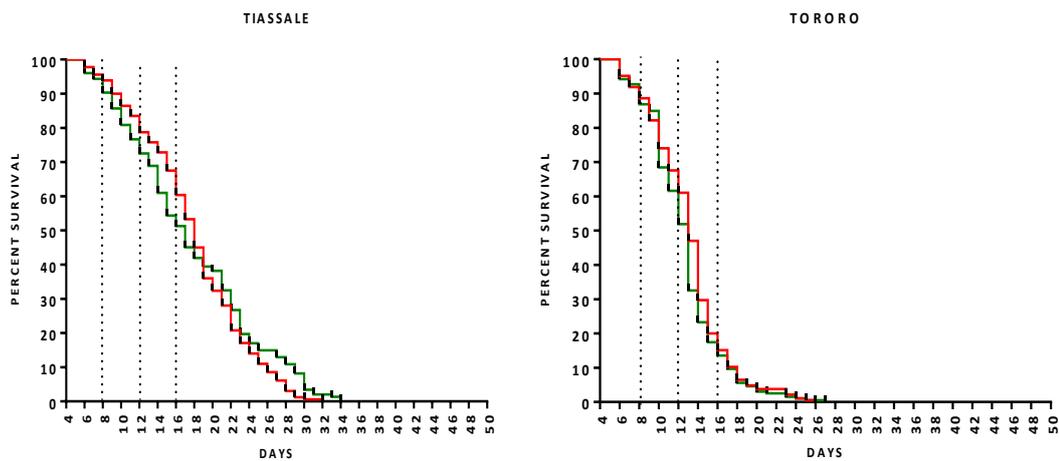


Figure 2-14 Kaplan Meier curves for experiment 2 (Olyset) showing mean percentage survival for insecticide resistant Tiassalé and Tororo strains of *An. gambiae*, following four episodes (4, 8, 12, 16 d PE) of exposure to Olyset (green line) compared to untreated (red line) net. Dotted vertical lines show timings of exposures. Statistical difference between curves: Tiassalé ($p=0.41$) and Tororo ($p=0.08$). Results for two experiments, each with 100 mosquitoes/net/strain were pooled for this analysis.

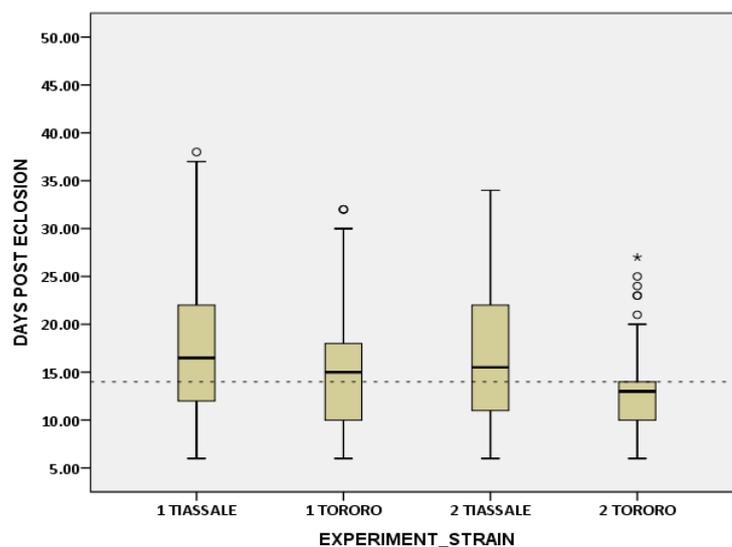


Figure 2-15 Box and Whisker plots for experiments 1 and 2 (Olyset) showing median longevity of insecticide resistant Tiassalé and Tororo strains of *An. gambiae* after exposure to Olyset. Dotted line shows average 14 d extrinsic incubation period (EIP) of *Pl. falciparum*. Boxes show median (horizontal line) and upper and lower quartiles (top and bottom edges). The upper and lower whiskers show maximum and minimum values. Dots and asterisks represent outliers and extreme outliers respectively. Results for two experiments, each with 100 mosquitoes/net/strain were pooled for this analysis.

2.6.2.6 Experiment 3 – PermaNet - simultaneous exposure/feed

Experiment 3 revealed that after exposure to PermaNet during blood feeding, longevity of Tiassalé ($p < 0.0001$) and Tororo ($p = 0.005$) was significantly shorter than after exposure to untreated nets (Figure 2-16). Median survival for Tiassalé was 9.5 d after feeding through PermaNet compared to 30 d after feeding through untreated net. Tororo median survival was 7 d after feeding through PermaNet compared to 17 d after feeding through an untreated net.

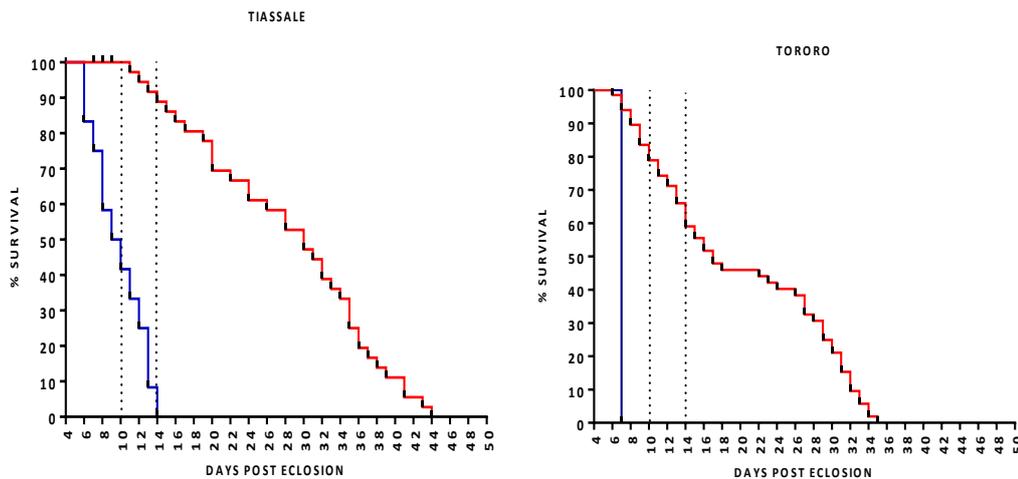


Figure 2-16 Kaplan Meier curves for experiment 3 (PermaNet) showing mean percentage survival for insecticide resistant Tiassalé and Tororo strains of *An. gambiae*, following three episodes of simultaneous blood feed/insecticide exposure (4, 10 14 d PE) to PermaNet (blue line) net compared to untreated (red line) net. Dotted vertical lines show timings of exposures. Statistical difference between curves: Tiassalé ($p < 0.0001$) and Tororo ($p = 0.0005$). Results for two experiments, each with 100 mosquitoes/net/strain were pooled for this analysis.

2.6.2.7 Experiment 3 – Olyset - simultaneous exposures/feed

In contrast to PermaNet, blood feeding through an Olyset net had no impact on survival in either strain (Figure 2-17). Median survival for Tiassalé was 27 after feeding through Olyset compared to 30 d after feeding through untreated net. Median survival for Tororo was 16 d after feeding through Olyset compared with 17 d after feeding through untreated net.

The unusual shape of the Olyset/Tororo curve (Figure 2-17) is because nearly 50% of mosquitoes were killed in the first 24 h preceding the first exposure and by the second exposure, there were only 11 test individuals remaining (Section 2.6.2, Figure 2-7). Furthermore, data plotted from 48 h onwards resulting in a crossed curve which was no longer be proportional. Using the log-rank test under conditions of non-proportional hazards leads to misleading results (H. Li et al. 2015), hence the unreliable p value. Box and whisker plots show median survival beyond the EIP for Tiassalé after feeding through Olyset only (Figure 2-18).

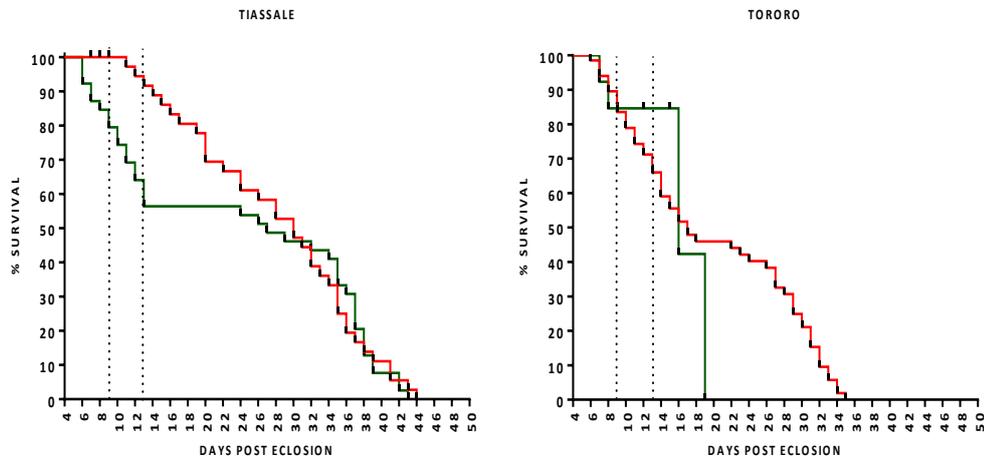


Figure 2-17 Kaplan Meier curves for experiment 3 (Olyset) showing mean percentage survival for insecticide resistant Tiassalé and Tororo strains of *An. gambiae*, following three episodes of simultaneous blood feed/insecticide exposure (4, 9, 13 d PE) to Olyset (green line) net compared to untreated (red line) net. Dotted vertical lines show timings of exposures. Statistical difference between curves: Tiassalé ($p=0.22$) and Tororo ($p=0.8$). Results for two experiments, each with 100 mosquitoes/net/strain were pooled for this analysis.

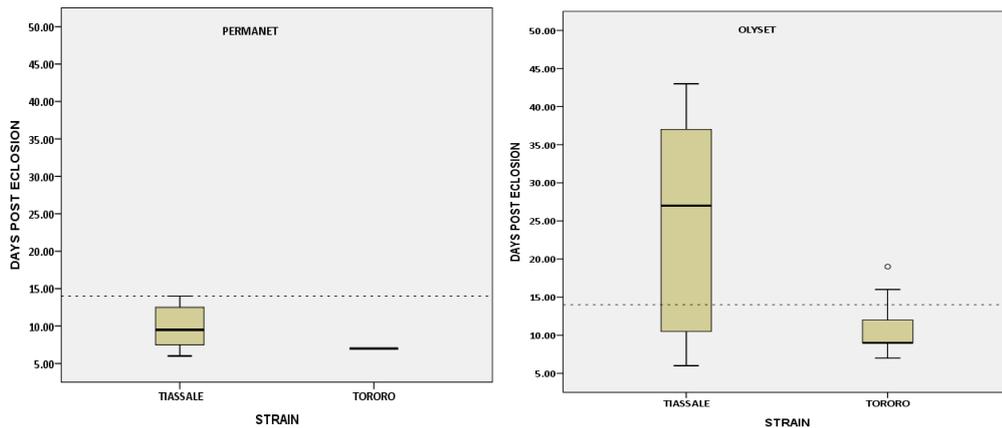


Figure 2-18 Box and Whisker plots for experiments 3 (PermaNet and Olyset) showing median longevity of insecticide resistant Tiassalé and Tororo strains of *An. gambiae* after exposure to PermaNet (left) and Olyset (right). Dotted line shows average 14 d extrinsic incubation period (EIP) of *Pl. falciparum*. Boxes show median (horizontal line) and upper and lower quartiles (top and bottom edges). The upper and lower whiskers show maximum and minimum values. Dots represent outliers and extreme outliers respectively. Results for two experiments, each with 50 mosquitoes/net/strain were pooled for this analysis.

2.6.2.8 Experiment 4 – PermaNet - tiered exposures

In experiment 4, results were pooled as there were no impact of increasing exposure frequency on mortality and survival (Section 2.6.1, Figure 2-8, Appendix 1). Therefore, this experiment compared the effects of multiple exposures to PermaNet versus untreated net in extremely resistant strains of *An. gambiae*. There was a significant difference in survival curves for the Banfora strain ($p<0.0001$) yet these mosquitoes survived equally as long as those exposed to untreated netting (27 d PE). There was no significant difference in survival of the VK7 strain between treatments (Figure 2-19). After exposure to PermaNet, median

survival of Banfora and VK7, was 15 d and 21 d respectively, compared to 16 d and 20 d after exposure to untreated net. Box and whisker plots (Figure 2-20) show median survival exceeding the 14 d EIP in both strains. Olyset nets were not tested on these strains in this experiment.

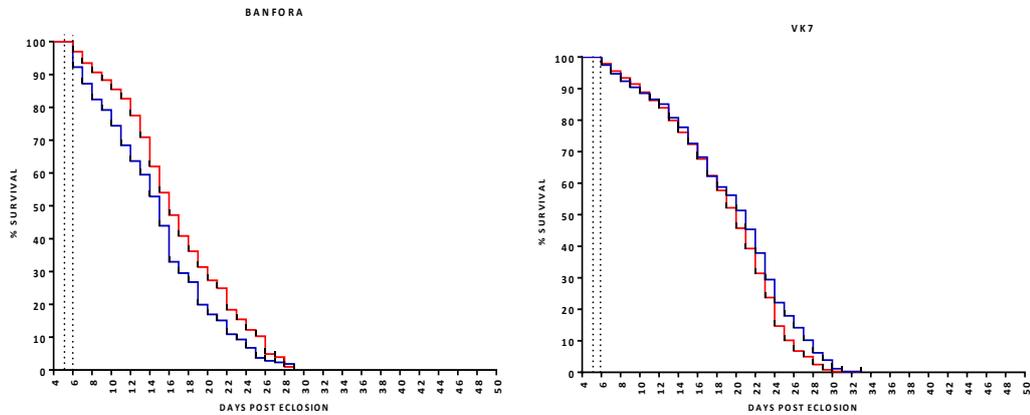


Figure 2-19 Kaplan Meier curves for experiment 4 showing mean percentage survival for insecticide resistant Banfora and VK7 strains of *An. gambiae*, following all three episodes of exposure to PermaNet (blue line) and untreated net (red line) on 4; 4,5 d; or 4,5,6 d PE. Dotted vertical lines show timings of exposures. Statistical difference between curves: Banfora ($p < 0.0001$) and VK7 ($p = 0.131$). Results for two experiments, each with 70 mosquitoes/net/strain were pooled for this analysis.

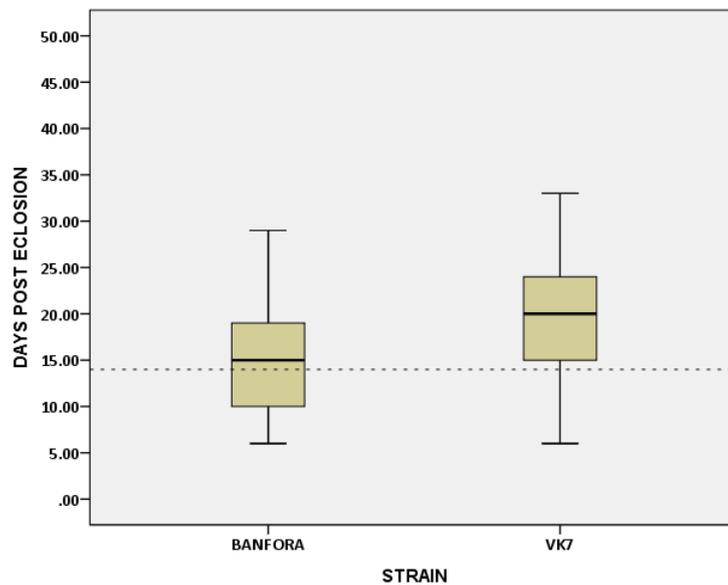


Figure 2-20 Box and Whisker plots for experiments 4 showing median longevity of insecticide resistant Banfora and VK7 strains of *An. gambiae* after exposure to PermaNet. Dotted line shows average 14 d extrinsic incubation period (EIP) of *Pl. falciparum*. Boxes show median (horizontal line) and upper and lower quartiles (top and bottom edges). The upper and lower whiskers show maximum and minimum values. Results for two experiments, each with 70 mosquitoes/net/strain were pooled for this analysis.

2.6.3 Magnitude of exposure effects on survival

Hazard is defined as the slope of the survival curve — a measure of how rapidly subjects are dying. The hazard ratio (HR)¹¹ compares two treatments, where the hazard in the exposed group is divided by the hazard in the unexposed groups, e.g. if the HR is 2.0, then the rate of deaths in one treatment group is twice the rate in the other group.

The hazard ratios of exposed versus unexposed mosquitoes in all experiments is summarised in table 2-4. These ratios quantify the impact or magnitude of insecticide exposure on mosquito survival. For example, in experiment 1, the rate of death of Tororo, after exposure to PermaNet compared to untreated is x5.6, *i.e.* Tororo is 5.6x more likely to die after daily exposure to PermaNet than untreated net. Whereas, exposure of Tiassalé to Olyset nets has a ratio close to 1, suggesting that Tiassalé is equally as likely to survive exposure to Olyset net as exposure to untreated net.

Table 2-4 Summary of hazard Ratio (Logrank) comparisons between KM survival curves of insecticide resistant Tiassalé, Tororo, Banfora and VK7 strains of *An. gambiae* mosquitoes after exposure to LLINs nets compared to untreated nets in experiments 1-4. The hazard ratio shows the rate of deaths seen against PermaNet and Olyset compared to the rate of deaths seen against untreated net. Figures in brackets show 95% confidence interval of ratio. Bold print show significant difference in KM curves, P<0.001 (Gehan-Breslow-Wilcoxon test).

EXPERIMENT	LLIN	STRAIN	HAZARD RATIO (95%CI)
1	PERMANET	TIASSALÉ	1.8 (1.4 – 2.3)
		TORORO	5.6 (2.5 – 12.3)
	OLYSET	TIASSALÉ	1.1 (0.9 – 1.3)
		TORORO	1.6 (1.2 – 2.0)
2	PERMANET	TIASSALÉ	1.4 (1.1 - 1.7)
		TORORO	3.1 (2.1 - 4.8)
	OLYSET	TIASSALÉ	0.9 (0.7-1.13)
		TORORO	1.1 (0.9-1.4)
3	PERMANET	TIASSALÉ	7.2 (1.8 – 28.8)
		TORORO	13.7 (0.01-181)
	OLYSET	TIASSALÉ	1.1 (0.7-1.8)
		TORORO	1.0 (0.4-2.8)
4	PERMANET	BANFORA	1.3 (1.1 – 1.5)
		VK7	0.8 (0.7 – 1.0)

¹¹ The hazard ratio is not computed at any one time point but is computed from all the data in the survival curve. Since there is only one hazard ratio reported, it can only be interpreted if you assume that the population hazard ratio is consistent over time, and that any differences are due to random sampling. This is called the assumption of proportional hazards.

Significant reductions in longevity are seen after exposing both Tororo and Tiassalé to PermaNet in all experiments. Hazard ratios against PermaNet reduce in experiment 2, suggesting daily exposures had a more pronounced impact on survival reduction than exposure every four days. Furthermore, in experiment 3, hazard ratios indicate Tiassalé are 7x and Tororo 13x more likely to die after blood feeding through PermaNet than untreated net. The hazard ratio of VK7 after exposure to PermaNet is less than 1 because treated mosquitoes lived longer than the untreated.

2.6.4 Reproductive output

Both reproductive output experiments show there is no effect of repeated insecticide exposure, on egg number (laid eggs and dissected eggs) or hatch rate, in the insecticide resistant strains of *An. gambiae*.

2.6.4.1 Experiment 5 - Repeated simultaneous feed/exposures

In experiment 3, the first exposure started with approximately 50 ($\pm 10\%$) unfed female mosquitoes, and the second and third exposures were carried out on fed survivors from previous exposures; sample sizes were thus very small for the later exposures. In addition to survival data, mosquito feeding rates, total number of eggs laid per female and egg hatch rate were also measured. The mean percentage of blood fed mosquitoes from the total number exposed is illustrated in Figure 2-21. In both strains, there was no difference in percentage feeding between each exposure to either LLINs. There was a general increase in percentage feeding over exposures, as non-feeders were removed from the experiment.

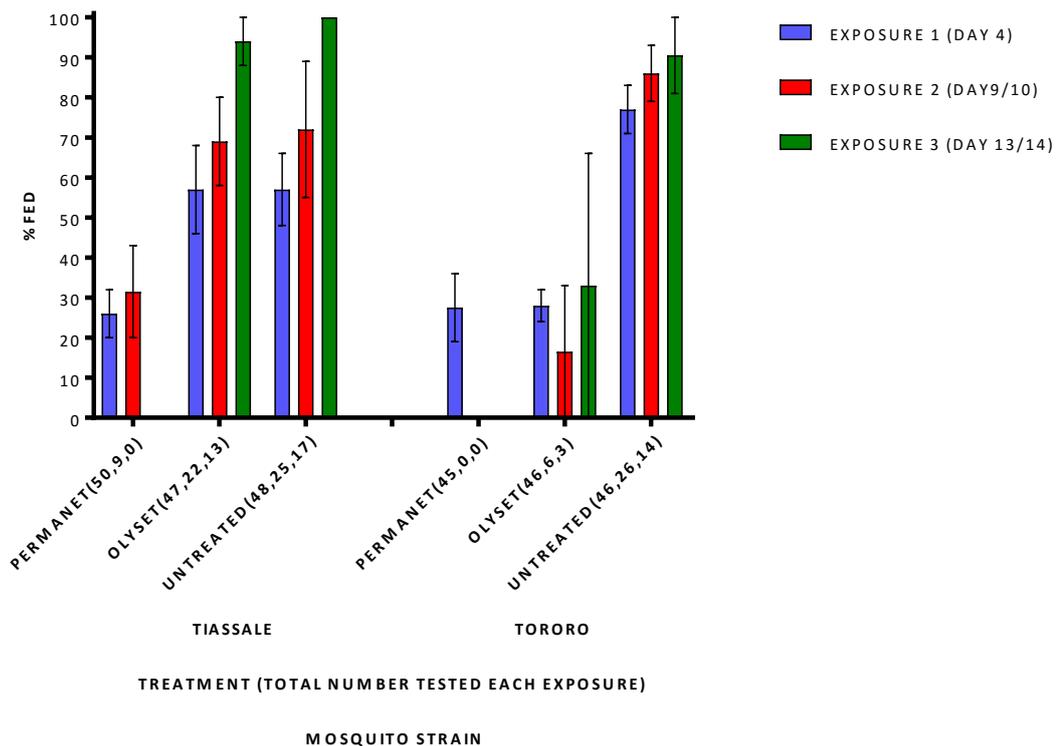


Figure 2-21 Bar chart for experiment 5 showing mean percentage of feeders of insecticide resistant Tiassalé and Tororo strains of *An. gambiae* after each exposure. Both strains are exposed to PermaNet, Olyset and untreated netting in each exposure (1-3). First exposure started with approx. 50 unfed female mosquitoes. Second and third exposures were carried out on fed survivors from previous exposures. Numbers in brackets show total sample size for each exposure. Error bars show SEM.

After exposure to PermaNet, Tiassalé mosquitoes did not survive long enough for a third exposure, while Tororo mosquitoes did not survive long enough for a second exposure. After

exposure to Olyset, 13 Tiassalé individuals (28% of total mosquitoes) survived all three exposures with a 94% (± 6 SEM) successful feed rate in the final exposure. Similarly, 14 Tororo individuals (30% of total) and 17 Tiassalé individuals (35% of total) were tested in the final exposure to untreated net, with 90.5% (± 9.5 SEM) fed rate.

Total egg counts per blood fed female (Figure 2-22) did not statistically differ between strain or treatment. The Tororo strain yielded no eggs after exposure to PermaNet as these individuals suffered immediate mortality after the first exposure (Section 2.6.1, Figure 2-7). Hatch rates generally decreased over the three exposures for all strains and treatments (Figure 2-23) though this trend was not significant.

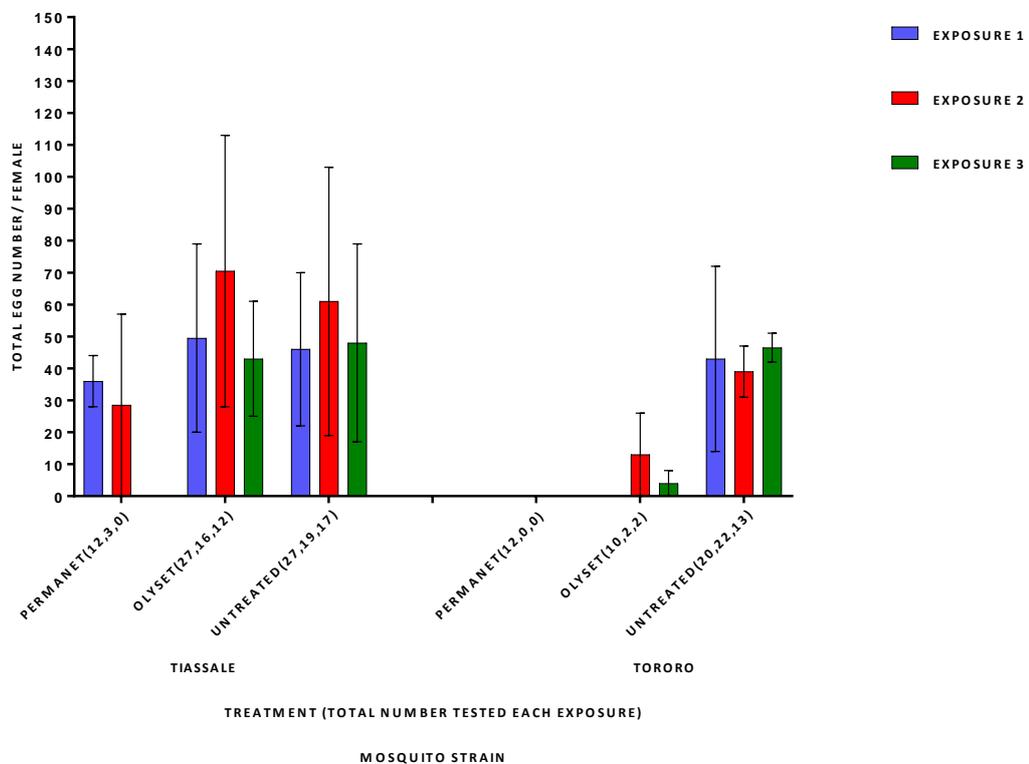


Figure 2-22 Bar chart for experiment 5 showing total egg numbers per female for Tiassalé and Tororo strains of *An. gambiae* following exposure to PermaNet, Olyset and untreated nets after multiple simultaneous/exposures (1-3). Numbers in brackets show mean number of fed females. Error bars show SEM.

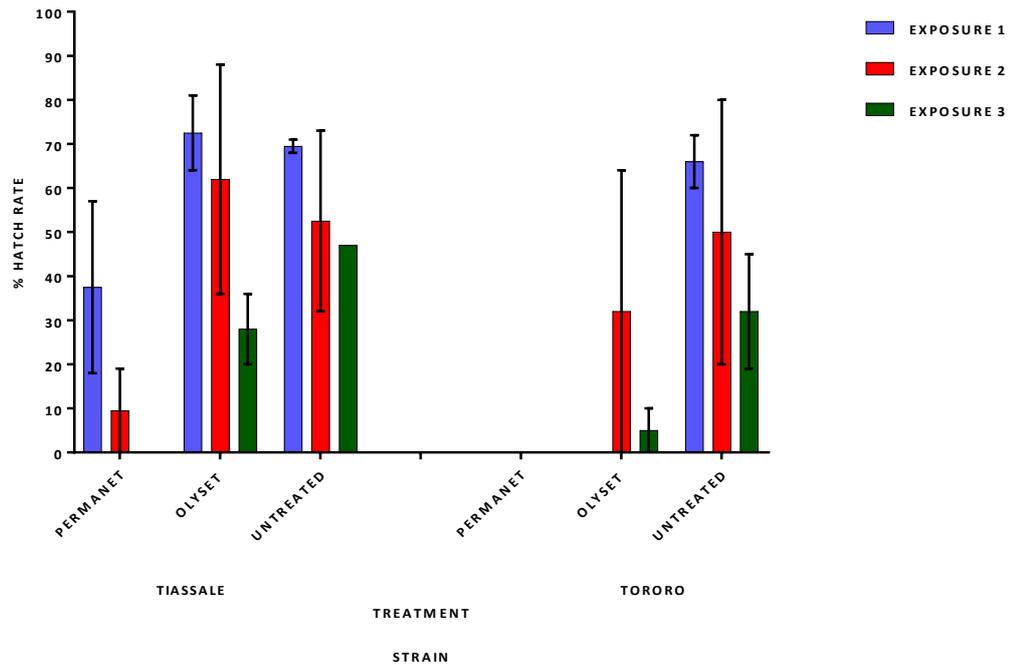


Figure 2-23 Bar chart for experiment 5 showing hatch rates for Tiassalé and Tororo strains of *An. gambiae* following exposure to PermaNet, Olyset and untreated nets after multiple simultaneous/exposures (1-3). Absence of bars indicate zero egg count. Error bars show SEM.

2.6.4.2 Experiment 6 - Egg quantification

In total, 100 individuals from the Tiassalé strain were repeatedly exposed to PermaNet using the WHO cone assay over 3 consecutive days and then blood fed. Gravid individuals were dissected and the number of eggs was measured. Figure 2-24 shows that daily exposure to insecticide had no significant impact on egg numbers, when mosquitoes were offered a blood meal 24 h after the final LLIN exposure (T-test $p=0.51$).

On average, mosquitoes exposed to PermaNet laid $62.88 (\pm 3.96 \text{ SEM})$ eggs, while those exposed to untreated netting laid $66.47 (\pm 3.80 \text{ SEM})$ eggs. In addition to the 100 gravid mosquitoes tested which produced eggs, 15% ($n = 18$) of total fed mosquitoes, exposed to PermaNet, did not yield eggs, compared to 14.5% ($n=17$) of those exposed to untreated netting. These results were not significantly different between treatments.

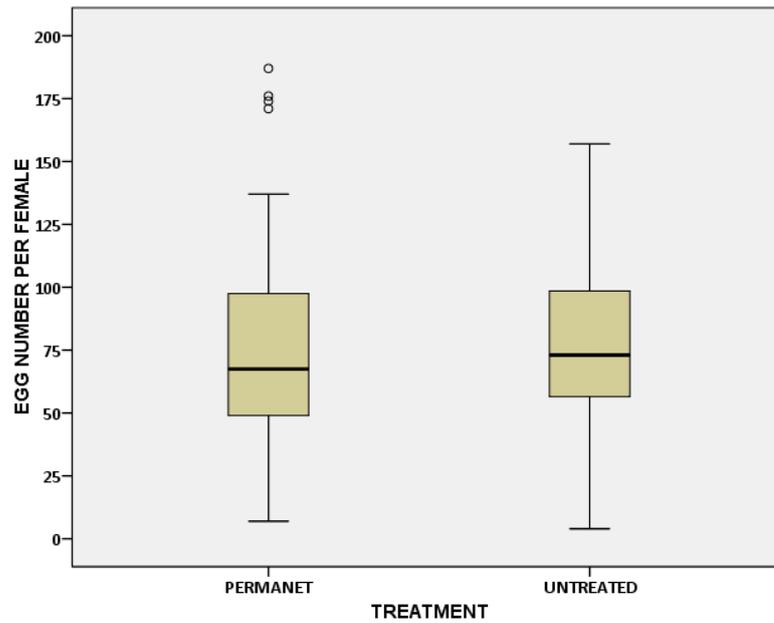


Figure 2-24 Box and whisker plot for experiment 6 showing mean egg counts of insecticide resistant Tiassalé strain of *An. gambiae* following repeated daily exposure to PermaNet (treated, n=119) and untreated netting (n=117) on 4, 5 and 6 d PE . Boxes show median (horizontal line) and upper and lower quartiles (top and bottom edges). The upper and lower whiskers show maximum and minimum values. Dots and asterisks represent outliers and extreme outliers respectively

2.7 Discussion

The immediate aim of this chapter was to determine the long-term impact of periodic exposure to an LLIN on longevity and reproduction rate in IR *An. gambiae*. The specific objectives presented here were to determine the impact of repeated exposure to insecticides on mosquito longevity, assess whether these exposed mosquitoes survive beyond the EIP and determine the effect of exposure to LLINs on reproductive output (i.e. eggs) over several gonadotrophic cycles.

Three different exposure regimes were used to reflect typical conditions in which mosquitoes are affected by insecticides. Mosquitoes were repeatedly exposed to LLINs at different time intervals, to mimic feeding frequency in the field, and immediate (24 h) and longer-term (>48 h) effects of insecticide exposure on survival were examined.

Results suggest that although insecticide resistant strains of *An. gambiae* are not killed upon immediate contact with insecticides, they suffer a longer-term fitness cost from exposure that indirectly reduces their disease transmission potential. In some cases, this delayed mortality effect reduced median survival to an age below that required for parasite maturation in the mosquito. There was no observed effect of repeated insecticide exposure on the reproductive output in insecticide resistant strains of *An. gambiae*.

A delayed mortality effect was evident in all strains but differed in magnitude depending on the strength of IR phenotype. Therefore, delayed mortality impacts may be of most significance in populations where IR has newly arisen and is conferred by a limited range of target site mutations but have minimal impact in populations that have developed both multiple IR mechanisms and compensatory mutations through years of intense selection. Thus, even though delayed mortality impacts of insecticides may be reducing the transmission potential of IR mosquitoes under current conditions, this mitigating effect could become eroded by continued, intense selection for IR in the future. Some of the key implications of these results are discussed below.

2.7.1 Impact of immediate and delayed mortality effect on survival

In the longer term, both Tiassalé and Tororo show reduced longevity after surviving PermaNet exposure (with or without blood feeding) compared to untreated netting. This was not the case after exposure to Olyset, where Tiassalé did not suffer any reduction in

survival. The VK7 strain survived longer after PermaNet exposure than exposure to untreated netting.

Total 100% immediate mortality of the susceptible 'control' Kisumu strain of *An. gambiae* was seen following exposure to PermaNet. The same strain showed only 1% immediate mortality against Olyset but did suffer a significant reduction in long term survival when compared to untreated netting. Importantly, survival persisted in those susceptible mosquitoes exposed to Olyset, with a mean survival of 9.5 d post eclosion. The effectiveness of the WHO cone assay for testing Olyset is a key factor in these findings and is discussed in Chapter 3 of this thesis.

There was no clear impact of repeated exposures on immediate mortality among all strains. Indeed, cumulative toxicity might be expected to increase mortality (Tennekes & Sánchez-Bayo 2013; Rondeau et al. 2014), yet this was not the case in this current study. In all experiments, the moderately resistant Tororo strain was significantly more susceptible to LLIN exposure than the Tiassalé strain. Two strains of mosquitoes from Burkina Faso which show exceptionally high levels of pyrethroid resistance showed that after exposure to PermaNet, neither strain suffered more than 12% (± 5 SEM) mortality after exposure to PermaNet and individuals from the Banfora strain lived as long as those exposed to untreated net (27 d).

Using similar methodology to that used in this current study (Glunt et al. 2011) evaluated the immediate and long-term effects of repeated exposure as mosquitoes aged, using modified WHO resistance-monitoring assays to expose *An. stephensi* females to impregnated paper containing low concentrations of permethrin at 4, 8, 12, and 16 d PE. Authors showed sub-lethal permethrin exposure did not consistently increase mosquito susceptibility to subsequent insecticide exposure, though older mosquitoes were more susceptible.

Studies have shown that older resistant mosquitoes are more susceptible to insecticides than newly emerged females (Hodjati & Curtis 1999; Rajatileka et al. 2011; Jones et al. 2012). A previous study (Hodjati & Curtis 1999) showed pre-exposure of susceptible mosquitoes of *An. stephensi* for a short time to permethrin did not increase tolerance (*i.e.* reduce mortality) when the mosquitoes were re-exposed 24 h later. The authors also show mean knockdown times of pyrethroid resistant strains of *A. stephensi* and *A. gambiae* were significantly shorter if they were 10 d old (either fed or unfed), as compared with those of newly emerged mosquitoes.

2.7.1.1 Glasgow collaboration for publication

The results from this current study were corroborated by collaborators at the University of Glasgow and subsequently published¹². Modellers designed and performed a Bayesian state-space model analysis to quantify the immediate (within 24 h of exposure) and delayed (>24 h after exposure) impact of insecticides, on daily survival and malaria transmission potential, using the experimental data described in this chapter.

This multivariate model revealed that contact with PermaNet reduced the immediate survival of moderately resistant (Tororo) and highly resistant (Tiassalé) *An. gambiae* strains by 60–100% and 3–61% respectively (Viana et al. 2016). Both Tororo and Tiassalé strains experienced a permanent reduction in survival >24h following exposure, *i.e.* the pre-exposure age-independent baseline daily survival levels are never achieved again. The delayed mortality effects of Tiassalé disappear faster because the initial impact on Tororo survival (*i.e.* immediate mortality) was much greater, which resulted in a longer period of recovery back to the baseline daily survival (*i.e.* control daily survival rate). This variation in recovery rate would also apply to differences seen in post-exposure survival in experiment 1 versus 2, where both Tiassalé and Tororo strains survived longer after exposures to PermaNet everyday rather than every four days, *i.e.* mosquitoes were better able to recover after a single batch of early exposures than staggered exposures throughout their lifetime (Section 2.6.2, Figures 2-10, 2-11).

Furthermore, the model demonstrated that delayed mortality effects reduce the overall life span of mosquitoes exposed to PermaNet by nearly 50%. Indeed, median life span of Tororo mosquitoes was reduced by 17–57% in the presence of delayed mortality impacts relative to when they are absent. The median life span in the Tiassalé strain was also estimated to be reduced by 0–40% (depending on exposure regime) in the presence of delayed mortality impacts of insecticides (Viana et al. 2016). However, when longevity data from the extremely resistant VK7 strain in this study were run through this model there was no effect on long term mortality and the delayed mortality was not observed (Dr Viana, personal communication).

These differential impacts on the magnitude of delayed mortality may reflect the mechanisms of IR within the strains. Physiological resistance to insecticides can arise through

¹² This collaborative work published: Viana, M., Hughes, A., Matthiopoulos, J., Ranson, H. and Ferguson, H.M., 2016. Delayed mortality effects cut the malaria transmission potential of insecticide-resistant mosquitoes. *Proceedings of the National Academy of Sciences*, 113(32), pp.8975-8980. See Appendix 2 - Published paper.

target site mutations that interfere with insecticide binding, metabolic IR in which insecticides are detoxified by the overproduction of enzymes, and penetration resistance in which the mosquito cuticle is altered in a way that inhibits insecticide uptake. The Tororo strain exhibits target site IR through the L1014S knockdown resistance (*kdr*) mutation but has shown no clear evidence for metabolic IR. In contrast, the Tiassalé and VK7 strains have both target site IR arising from a high frequency of 1014F *kdr* allele and metabolic IR arising from elevated expression of key P450s. It is likely that the long-term impacts of LLIN exposure on mosquito survival were minimized in the TIA and VK7 strains because of their additional capacity to detoxify residual insecticides (Viana et al. 2016).

2.7.2 Do resistant mosquitoes survive long enough to transmit malaria?

The ability of a mosquito to survive longer than the average EIP following LLIN exposure is key to understanding how the delayed mortality might impact on malaria transmission. In this study, median survival data demonstrated that neither Tororo and Tiassalé strains survived beyond the EIP in any experiment after exposure to PermaNet. Conversely, except for Tororo in experiment 2, median survival in these strains exceeded the 14d cut off after exposure to Olyset. Survival in both the extremely resistant strains, Banfora and VK7, exceeded the EIP against PermaNet.

Using a model to distinguish and quantify immediate and delayed impacts after exposure to PermaNet in this study, Viana et al. 2016, found the proportion of Tiassalé mosquitoes expected to live at least 9 d (minimum necessary time for a mosquito to transmit malaria) following insecticide exposure was predicted to be 25–60% (across different exposure regimes) in the presence of delayed mortality. These differences were even more pronounced within the Tororo strain, where <7% were estimated to survive for 9 d following insecticide exposure when delayed mortality impacts were acting. For each experiment, no Tororo mosquitoes were estimated to be alive at day 9 in the daily-exposure regime compared with 2–7% in treatments where exposures were spaced over 4–5 d. Similarly, 25% of Tiassalé mosquitoes were estimated to survive until day 9 under the daily-exposure regime, compared with 39–60% when exposures were spaced out. For experiment 3, the mean daily survival was ~10% lower in both strains compared with experiment 1 and 2. The authors state that if delayed mortality effects of similar magnitude occur in natural conditions, estimates of transmission potential of insecticide resistant mosquitoes should be reduced by 50% to what would be assumed if insecticides had no impact on their survival.

LLINs would reduce the number of infectious bites delivered by their mosquito strains 3.3- and 7.8-fold for Tiassalé and Tororo respectively, with the delayed mortality accounting for at least half of this reduction.

It is important to interpret the results of this chapter in the context of previous studies that have shown that older mosquitoes are more likely to be killed by insecticide (Jones et al. 2012; Chouaibou et al. 2012). In cases where IR is conferred by over expression of detoxification genes, maintaining the resistant phenotype can impose a significant fitness cost, and investment into enzyme production may not be sustained throughout the life of the mosquito (Rajatileka et al. 2011). Under natural conditions, most mosquitoes do not survive beyond the EIP, and due to the loss of resistant status as mosquitoes age, insecticides work by reducing the number of survivors further (Thomas & Read 2016). In this study, the first exposures in each experiment were carried out on young resistant mosquitoes, however as assays progressed, exposures were carried out on older mosquitoes, especially in experiment 2, where the final exposure was on 16 d old individuals. Results in this study show that mosquitoes' natural mortality varies with age. As immediate mortality does not significantly vary between exposures, results may have been driven by changes in the natural mortality of mosquitoes over time (*i.e.* senescence) rather than increases in susceptibility to insecticide exposure (Viana et al. 2016).

Understanding whether resistant insects are better or worse vectors of diseases than susceptible ones requires further study of the interaction between IR and vectorial capacity. Rivero et al. (2010) suggest increasing numbers of resistant insects need not lead to proportionate increases in transmission: it depends on whether those insects are more permissive transmitters than their susceptible ancestors. In this chapter we have shown that some resistant mosquito populations are negatively impacted by LLIN exposure. If mosquitoes die younger after repeated insecticide exposure, the impact of IR on disease transmission might be less than previously thought. Other effects of LLIN exposure which reduce the ability of mosquitoes to transmit malaria may also reduce the "realised effect" of IR (Thomas & Read 2016, Figure 2-25). These could include delayed mortality (Viana et al. 2016), reductions in feeding, or increased refractoriness to malaria parasites (Alout et al. 2014; Alout et al. 2016; Kristan et al. 2016); such effects could result in a reduced "realised effect size" of IR .

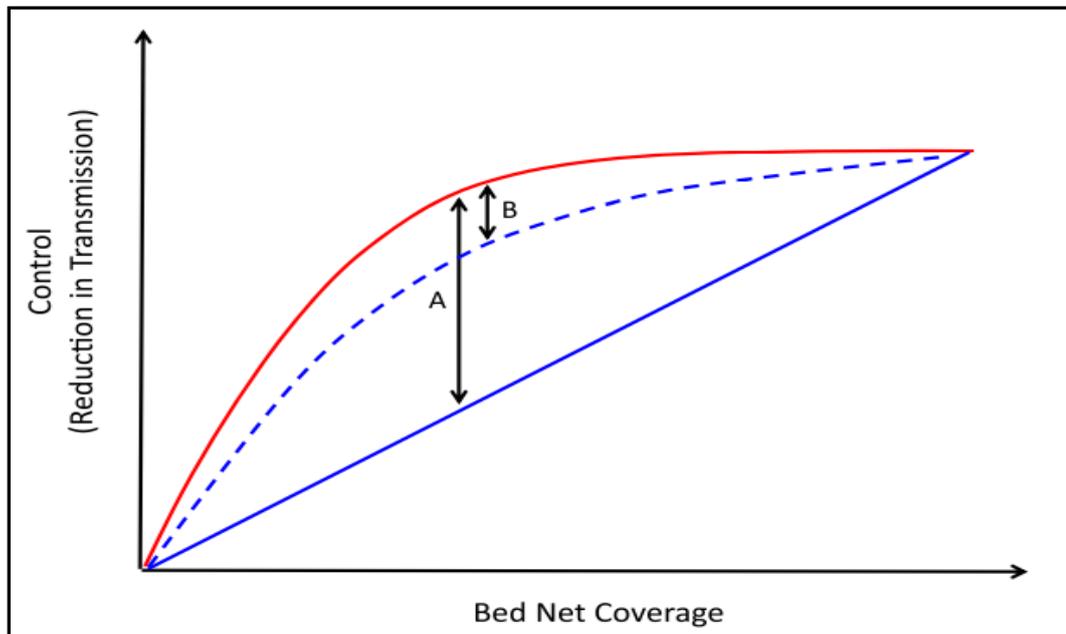


Figure 2-25 Line graph of the possible impact of IR on the efficacy of LLINs. As bed net coverage (proportion of the population using nets) increases, more people gain personal protection from malaria (solid blue line). Mosquitoes seeking those people are killed by insecticides on the nets, and so even people not sleeping under nets experience less exposure to mosquito bites, giving the combined population-wide protective effect of LLINs against a susceptible mosquito population (red line). If IR renders the insecticide completely ineffective, the community benefit is lost and LLINs provide physical protection only (assuming nets are intact and are used effectively). Thus, the difference between the red and blue lines represents the “potential effect size” of IR. At intermediate levels of coverage, the largest effect size is expected, as this is where the mass action effect of the insecticide provides the greatest relative contribution to control (arrow A). However, indirect impacts of insecticide exposure, such as delayed mortality, contribute to reductions in transmission (dashed blue line). Such effects could result in a reduced “realized effect size” of IR (arrow B) (Image modified from Thomas & Read 2016).

2.7.3 Exposure while blood feeding shows no effect on fecundity

In addition to longevity, this chapter also included a preliminary study of the impact of repeated LLIN exposure on reproductive output. Results indicate there is no significant difference in reproductive output (egg numbers and hatch rates) in either strain after exposure to LLINs either during a simultaneous blood feed or repeated exposure prior to blood feeding.

These preliminary results suggest prolonged exposure to LLINs does not act by affecting egg production. Instead, pyrethroids act to quickly produce the symptoms of lost coordination and paralysis (the ‘knockdown’ effect) which is often accompanied by spasm and tremors which can be violent and cause loss of extremities. Consequently, it was noted in this study, that legs were often lost during the 3 min exposure to PermaNet, moreover, in experiment 3 these same mosquitoes were still capable of taking a blood meal. A recent study (Isaacs et al. 2017) explored whether leg loss inhibits mosquitoes from biting and reproducing.

Mosquitoes with one, two, or six legs were evaluated for their success in feeding upon a human, and the authors demonstrate that insecticide-induced leg loss had no significant effect upon blood feeding or egg laying success.

2.7.4 Limitations

After exposure to Olyset LLINs, insecticide susceptible mosquitos showed lower than expected mortality and higher long-term survival compared to PermaNet. The results using Olyset LLINs in this chapter should be taken with caution given that in Chapter 3 it was shown that the cone bioassay is not optimal for evaluating exposure to this LLIN type.

Four different insecticide resistant laboratory reared colonies were tested, which were maintained under insecticide selection to ensure their resistant phenotype is maintained. Ideally, the next step would be to validate these findings in wild populations and assess their relevance to operational control.

The two reproductive output experiments could be improved. Individual mosquitoes from the simultaneous exposure feeding experiments could be isolated into individual tubes provide an egg count per female (in contrast to the pooled egg laying and ovary dissection methods employed here). However, this 'forced' lay technique (MR4 Methods in Anopheles Research, 2015 Edition) may cause stress to the mosquito rendering them less able to subsequently feed.

2.8 Conclusion

Mosquito vectors are defined as "resistant" when insecticides are no longer able to kill them on contact. However, they may suffer longer-term impairment following insecticide exposure that reduces their ability to transmit disease. This chapter used a simple bench top bioassay to highlight the importance of investigating the impacts of IR beyond immediate mortality. The study detailed in the next chapter investigated variations within these bench top assays by elucidating patterns of mosquito activity within the bioassay arena itself. It aimed to investigate potential modifications to improve the performance and scope of applications.

3 Characterisation of LLIN contact and subsequent mortality of insecticide susceptible and resistant *Anopheles gambiae* and *Anopheles funestus*

3.1 Introduction

Chapter 2 revealed the deleterious effect of contact with insecticide treated netting on mosquito longevity, even in resistant strains of *An. gambiae*. Using the WHO cone bioassay, the study showed that resistant strains experienced differing long-term survival after exposure to different net types. Continuing this theme, in this chapter, the cone bioassay is scrutinised to determine whether mosquito behaviour can explain these differing assay outcomes.

The LLIN is a factory-treated mosquito net expected to retain its biological activity for a minimum number of washes and a minimum period under field conditions. To be classified as 'long lasting' by the World Health Organisations' Pesticide Evaluation Schemes (WHOPES) LLIN's must retain their biological activity for at least 20 standard washes under laboratory conditions and 3 years of recommended use under field conditions (WHO, 2013). The WHO cone bioassay is a standardised method used in phase I (laboratory) trials to determine performance and effectiveness of LLINs. The cone bioassay is also employed during quality control testing of LLINs and IRS surfaces in phase II (small) and phase III (large) scale field studies (WHO 2006, WHO 2013a).

Current WHO guidelines, which are largely based on requirements for testing LLINs containing WHO-recommended pyrethroids, recommend that LLINs be tested against susceptible strains of mosquitoes (WHO, 2013a). However, cone bioassays are also used for the evaluation of new LLINs designed to control pyrethroid resistant mosquito populations. The WHO Vector Control Advisory Group (VCAG) state that new formulation nets must be tested against a range of resistant mosquito strains in Phase I studies (WHO 2016c). Resistant strains should be characterised by target site modification (e.g. *kdr*) and the presence of different metabolic IR mechanisms. These guidelines stipulate that the resistant strains must be at least 10-fold more resistant than susceptible stain at LC50 and at least 3

strains should be tested, 2 of which must have metabolic IR. Furthermore, cone bioassays have been used to assess the impact of IR on LLIN performance and to compare the performance of products from different manufacturers against wild caught field populations (Abílio et al. 2015; Agossa et al. 2015; Allossogbe et al. 2017; Glunt et al. 2015; Koudou et al. 2011; Dennis J Massue et al. 2016).

Previous reports suggest that LLINs containing permethrin, such as Olyset nets, often performed badly in cone bioassays and the use of this test alone to compare LLIN products could give misleading results and such anomalous results were observed and discussed in Chapter 2 of this study. Using susceptible strains, Bagi et al.(2015) found Olyset knock down and mortality rates were below the WHO criteria for the susceptible strain, while others (Toé et al. 2014) reported that Olyset achieved the WHO criteria for knockdown only. Similar results have been observed using susceptible strains of *Ae. aegypti* where exposure to new Olyset nets resulted in only 50% knockdown after 60 min and 30% 24 h mortality (Lenhart et al. 2008). A study evaluating LLINs after 2 years of household use (Lindblade et al. 2005) suggested that the WHO cone tests may be unsuitable for testing chemicals like permethrin as it has strong irritant properties that may reduce the time mosquitoes spend on the netting and because Olyset has a large mesh size, mosquitoes can rest on the solid untreated surface of the back board rather than on test netting.

A previous study (Siegert et al. 2009) showed reduced landing attempts and elevated frequency of flights in *An.gambiae*, resulting in reduced mortality, after exposure to Olyset compared to PermaNet. Authors suggest that the onset of behavioural effects modulated mortality, *i.e.* when LLIN contact-induced changes in mosquito behaviour were relatively delayed (e.g. against PermaNet), the mosquito typically knocked down or acquired a lethal dose from the net fabric; however, when contact-induced changes in behaviour occurred early, the mosquito disengaged from the net fabric and thus lethality was reduced (e.g. against Olyset).

Variation in the angle at which the cone test is performed, can significantly affect the amount of time mosquitoes spend resting on nets influencing subsequent mortality (Owusu & Müller 2016). Furthermore, the number of mosquitoes used in the cone differs between tests. For example, WHO guidelines recommend that five individuals be used for LLINs, while ten individuals are used to test insecticide-treated surfaces commonly seen in IRS treatments (WHO 2006). Potentially at higher densities, interactions between mosquitoes could increase, leading to interference and alterations in behaviour within the cone.

In this current study, assays were first performed with either five or ten mosquitoes per assay to examine whether the number of mosquitoes per test could influence test outcomes. An alternative to the WHO cone assay, an adapted wire-ball assay, where 100% of the arena surface is treated with test insecticide and hence all mosquitoes are dosed equally (WHO 2006) was evaluated for comparison.

Furthermore, the possibility of gathering additional data from cone bioassays was explored by using simple modifications with the standard WHO cone bioassay. First, timed cone bioassay experiments were performed to determine the point at which, during the 3 min assay, susceptible mosquitoes become intoxicated (knocked-down) by insecticide. Secondly, video recordings of mosquito activity within the cone with different treated materials and mosquito populations were made to describe the effects of insecticides on mosquito movement and behaviour. Thirdly, cone tests were carried out with a human hand positioned as bait (with blood feeding either possible or prevented), to determine whether repellent properties of nets also occurred with the host present.

3.2 Aims and Objectives

This aims of this chapter were to identify sources of variation in mosquito response to insecticide exposure in the WHO cone bioassay, among insecticide susceptible and resistant strains of *An. gambiae* and *An. funestus*, against various LLINs. Plus, the possibility of gathering additional data was explored using simple modifications to the standard WHO cone bioassay.

The specific objectives of this chapter were to:

1. Evaluate the effectiveness of the current standard WHO cone bioassay method for the determination of performance of a selection of LLINs, based on their ability to knock down and/or kill insecticide susceptible and resistant populations.
2. Investigate alternatives methods to the cone bioassay with a view to improve the knock-down and/or killing ability of LLINs, against susceptible and resistant mosquito strains.
3. Determine the effectiveness of the novel video cone bioassay as a tool for studying mosquito behaviour at the LLIN surface and assessing the repellent and/or contact irritancy quality of an insecticide.
4. Determine which variable (net, strain and/or presence of a host) has significant influence on a) death in the cone (24 hr mortality) and b) time spent on the net surface.
5. Examine variations in observed mosquito activity between susceptible and resistant individuals, after direct and non-direct contact with the LLIN surface, in the absence and presence of a host.

3.3 Materials and methods

3.3.1 Mosquito colonies

Two susceptible and two resistant strains of *An. gambiae s.l* and one susceptible and resistant strain of *An. funestus* were used, as detailed in table 3-1.

Further details of the *An. gambiae* colonies and colony rearing conditions were described in Chapter 2 (Section 2.3.1). The Fumoz *An. funestus* strain is extremely resistant to pyrethroids, resistant to carbamates, and susceptible to organophosphates. It possesses the *rdl* (dieltrin IR) target site mutation and overexpresses GSTE2 and the P450 enzymes, CYP6P9a and CYP6P9b.

Table 3-1 Details of colony name, species, country of origin and pyrethroid resistance phenotypes and profile of the *An. gambiae s.s.* and *An. funestus* strains used in the cone bioassay experiments. Green shading show susceptible strains and blue shading shows resistant strains. Diagnostic dose (DD) is used to discriminate the proportions of susceptible and resistant phenotypes in a sample of a mosquito population and are determined by exposing mosquitoes (tarsal contact) to insecticide deposits on filter-paper (WHO 2006). *kdr* frequency is a measure of the frequency of the common 1014F target site mutation in the population.

Strain (colony name)	Species	Origin	Phenotype (pyrethroids)	0.75% permethrin mortality DD	<i>kdr</i> 1014F frequency
Kisumu	<i>An. gambiae</i>	Kenya	Susceptible	100%	0
Ngousso	<i>An. coluzzii</i>	Cameroon	Susceptible	100%	0
Fang	<i>An. funestus</i>	Angola	Susceptible	100%	0
Tiassalé	<i>An. gambiae</i>	Cote d'Ivoire	Resistant	3%	0.97
VK72014	<i>An. coluzzii</i>	Burkina Faso	Resistant	0%	0.9
Fumoz	<i>An. funestus</i>	Mozambique	Resistant	3%	0

3.3.2 Long-lasting insecticide treated nets (LLINs)

New, unwashed LLINs from three manufacturers were tested: Duranet® (Shobika, India), Olyset® and Olyset Duo® (Sumitomo Chemical Ltd, Japan), PermaNet®2.0 (Vestergaard Frandsen, Switzerland). Duranet was purchased locally in Africa, Olyset Duo was borrowed from an ongoing durability trial. Further details of the Olyset and PermaNet 2.0 were described in Chapter 2 (section 2.3.2). Duranet is made from high-density polyethylene and contains 5.8g/kg alphacypermethrin. Olyset Duo is a polyethylene LLIN containing 2% (w/w) permethrin and 1% (w/w) pyriproxyfen. An untreated polyester net was used as a control. All LLINs were hung for 1 week, stored at 4°C and removed from 4°C the day before testing to allow the net to fully acclimatise to the insectary room temperature.

3.3.3 HPLC chemical analysis of LLINs

High performance liquid chromatography (HPLC) using a Dionex UltiMate 3000 (ThermoFisher Scientific, UK) was performed to confirm that the insecticide concentrations on each LLIN were within the product specifications (assays performed thanks to Ms Rhiannon Logan, LSTM). For one sample, five pieces were cut from the five panels of net used in this current study. Equal sized discs were cut out of the five panels and weighed, this was repeated in triplicate.

Insecticide was extracted in 5ml heptane (and internal standard – Dicyclohexyl Phthalate (DCP)) by boiling at 85°C for 45 min, 1ml (of 5ml total) of the extracted insecticide was removed and the heptane evaporated. Insecticide residue was resuspended in 1ml acetonitrile and samples were filtered to clean and remove contaminants. The HPLC method injected 20µl of the extracted insecticide onto a Hypersil Gold C18 Reverse-Phase column at 24°C. The isocratic mobile phase for each insecticide were as follows: Permethrin – 70% acetonitrile and 30% water, Alpha-cypermethrin – 80% methanol and 20% water, Deltamethrin - 80% methanol and 20% water. The mobile phase flow rate was 1ml/min for all methods. Samples were detected using a variable wavelength detector (VWD) at 226nm. Retention times for *cis*-permethrin, *trans*-permethrin, alpha-cypermethrin and deltamethrin were 25.1 min, 29.1 min, 15.3 min and 16 min, respectively. The insecticide peak areas (mAU*min) were converted to grams of insecticide per kilogram of net (g/kg) using a standard curve produced with analytical grade insecticides.

3.3.4 Standard WHO Cone bioassays

The WHO cone bioassay was carried out in accordance with current standard protocols (WHO 2013) and was previously described in Chapter 2 (Section 2.3.3).

3.3.5 Video recording

Mosquito activity within the cones was captured using video recording mode on an iPhone SE smartphone (Apple Inc, USA). The phone was secured to a rod by clamps at an angle which allowed for visual coverage of the entire cone within the phone screen (see Figure 3-1). The recording was started prior to introduction of mosquitoes into the cone and ended when a timer sounded. Images were uploaded onto a 4TB portable hard drive (Backup Plus, Seagate Technology LLC, USA) before conversion into PC Windows Media Video (WMF) format by Windows Movie Maker software (©2012 Microsoft Corporation, USA). To extract data for analysis the recordings were paused every 5 seconds and each of the five mosquitoes within

the cone were classed as either resting on the net, in flight or resting on the cone. By using this scan sampling approach, data was obtained on the time distribution of each behavioural state in the whole group (Altman 1973). Data were analysed using the event-logging software, EVENT (Bournemouth University, UK) where frequency of activities was recorded every 5 seconds over a 180 second period, totalling 36 events over the 3-min test session.

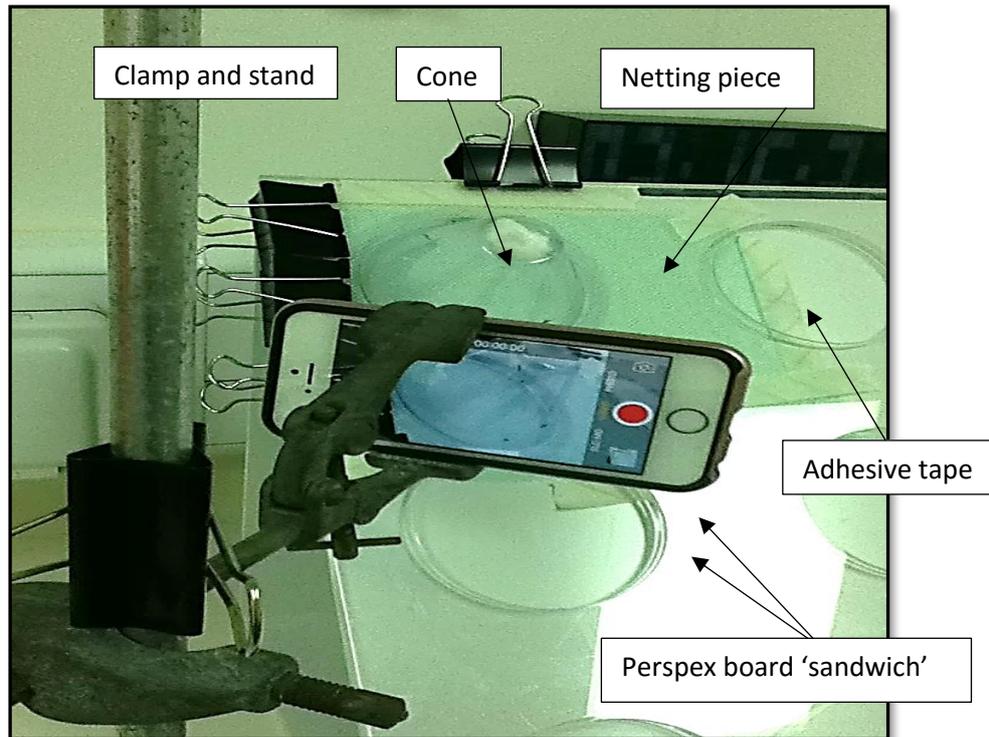


Figure 3-1 Labelled photograph of experimental set up for WHO cone bioassay and video capture system. The phone was clamped to a vertical rod and positioned in front of the cone, to ensure full image coverage. Netting was secured by tape and sandwiched in the top left-hand corner of the testing area in between two Perspex boards with the cone positioned into a hole in the boards over the netting. Cotton wool was positioned over the cone entrance hole. Boards were clamped tightly into place by bulldog clips and rested against a support frame angled at 45°.

3.4 Experimental design

3.4.1 Experiment 1 – Modified WHO cone behavioural bioassay

Three experiments were conducted to determine a potential variation in behaviour of phenotypically different mosquitoes at the LLIN interface, after direct or non-direct contact with a range of LLINs, in the absence or presence of a human host. Modified standard WHO cone assay experiments were set-up as described below (Figure 3-2).

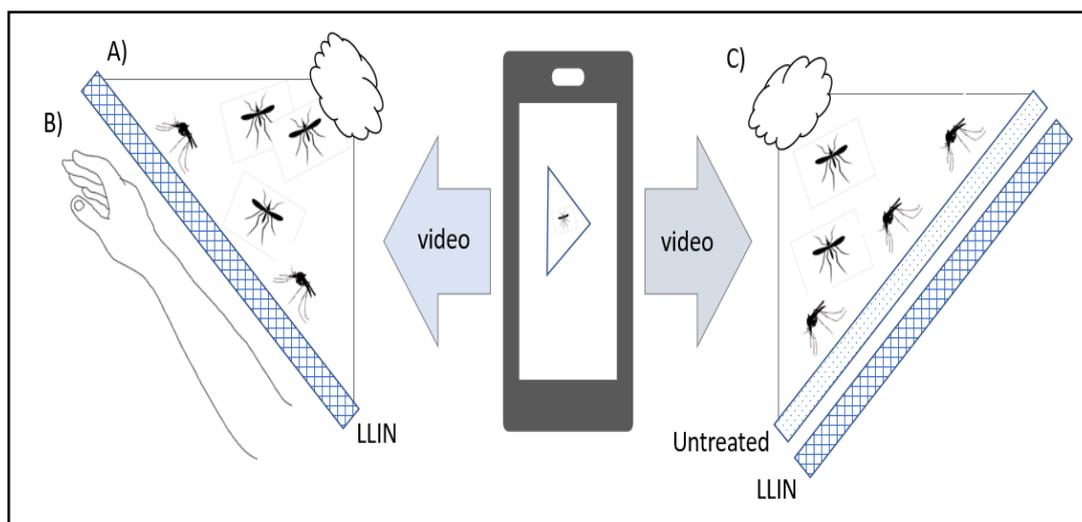


Figure 3-2 Schematic diagram illustrating modified cone experiments A, B and C using insecticide susceptible and insecticide resistant strains of *An. gambiae* and *An. funestus*. Each cone contained five mosquito individuals and was plugged by cotton wool. Each 3 min cone assay was video recorded using a smartphone (centre of diagram). Experiment A) tested mosquitoes in direct contact with an LLIN only. Experiment B) tested mosquitoes in direct contact with an LLIN with the addition of a human host's arm positioned behind the LLIN. Experiment C) tested mosquitoes where direct contact with the LLIN was prevented by an untreated net placed between the LLIN and the mosquitoes.

3.4.1.1 Direct contact bioassay (Fig 3-2A)

The standard WHO cone bioassays were set up as described in Section 2.3.3. One sample of net was tested at a time. Kisumu, Ngousso, Tiassalé and VK7 strains of *An. gambiae* and Fang and Fumoz strains of *An. funestus* were exposed to untreated, Duranet, Olyset, Olyset Duo and PermaNet. Five tests, each with 5 mosquitoes, were carried out on each LLIN type ($n = 25; 5 \times 5$) and video recorded for 3 min.

3.4.1.2 Direct contact bioassay (host present) (Fig 3-2B)

Standard cone assays were set up as described in experiment A, plus a host (human arm) was included. The Perspex boards were slid away from the support board to allow for an arm to be placed behind approximately 5mm from the test netting. Kisumu, Ngousso, Tiassalé and

VK7 strains were exposed to untreated, Olyset or PermaNet. Five tests, each with 5 mosquitoes, were carried out on each LLIN type ($n = 25; 5 \times 5$).

3.4.1.3 Non-contact bioassay (Fig 3-2C)

Modifications to the standard cone assays were set up where the LLIN was attached to the 45° support board and untreated ‘barrier’ netting was sandwiched between the Perspex boards to create a barrier in front of the LLIN. The two nets were 5mm apart and not in contact. Kisumu, Ngouso, Tiassalé and VK7 strains were exposed to untreated, Duranet, Olyset and PermaNet. Five tests, each with 5 mosquitoes, were carried out on each LLIN type ($n = 25; 5 \times 5$).

3.4.2 Experiment 2 – Knockdown (KD) and mortality

3.4.2.1 WHO Standard cone bioassay

Experiments measured KD/mortality obtained by following standard WHO cone bioassay procedures (WHO 2013a). Mosquitoes from all *An. gambiae* and *An. funestus* strains were tested against Duranet, Olyset, Olyset Duo, PermaNet and untreated netting. Five randomly selected samples of netting were cut from each net and attached to the test apparatus. Five individual mosquitoes from all strains were exposed to each LLIN or untreated netting ($n = 25; 5$ individuals \times 5 net samples) as described in Section 2.3.3.

3.4.2.2 Mosquito number variation (5 vs 10)

Data on the effect of varying mosquito number on KD/mortality was obtained in the standard cone bioassay. This variation between five and ten individuals relates to the number of mosquitoes recommended when testing an LLIN or a treated surface (*i.e.* wall after IRS treatment), respectively (WHO 2006). Either five ($n = 25; 5$ individuals \times 5 net samples) or ten ($n = 50; 10$ individuals \times 5 net samples) mosquitoes from Kisumu, Ngouso, Tiassalé and VK7 strains were tested against untreated, Olyset and PermaNet only.

3.4.2.3 Adaptation of wire-ball assay

Experiment investigated an alternative to the standard WHO cone bioassay: the adapted wire-ball assay (see figure 3-3, WHO 2006). Five randomly selected net pieces measuring approx. 30cm \times 30cm were cut and wrapped around five cuboidal wire frames (15 \times 15 \times 15 cm). Each piece of netting was gathered and bound with an elastic band to create a sleeve, through which mosquitoes could be introduced and removed easily with a mouth aspirator. Five individual mosquitoes from Kisumu and Tiassalé strains were introduced into each wire

frame covered by either untreated, Olyset and PermaNet and exposed for 3 min ($n = 25$; 5 individuals x 5 net samples).



Figure 3-3 Photograph of wire-ball assay, bioassay procedure using wire frame for netting material (WHO 2006)

3.4.3 Experiment 3 - Timed exposure

Experiments were undertaken to determine the time required for mosquito knock-down within the 3 min standard cone bioassay period. Three samples of netting were randomly selected and cut from each LLIN. Mosquitoes from the susceptible Kisumu strain were exposed to untreated net and all LLINs for 30, 60, 90, 120, 150 and 180 seconds, using the standard WHO cone bioassay. Five mosquitoes were tested on each piece of netting for each timed exposure ($n = 90$; 5 individuals x 6 exposures x 3 net samples).

3.4.4 Data analysis

In experiments 1 and 2, comparisons in KD and mortality data between the 5 versus 10 mosquitoes, and standard assay versus adapted wire ball assay, were determined by a non-parametric Kruskal-Wallis test (plus Dunn's multiple comparisons test). Analyses were performed using GraphPad Prism Version 7.03 (GraphPad Software Inc. USA).

To establish which independent variables (i.e. strain, treatment) have a significant effect on death in the cone assay (24 h mortality), analysis of mortality data was performed in a standard binary logistic regression model in SPSS Version 24 (IBM Corp. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY, USA). However, analyses revealed a quasi-complete separation problem due to the small sample size (Heinze & Schemper 2002), and therefore an meaningful outcome was not possible. Methods to fit these models include exact logistic regression and Firth logistic regression, a bias-reduction method based on a penalised likelihood, neither of which are available in SPSS. Further analysis of the mortality data in this current study can be done using the R Project for Statistical Computing (R version 3.2 available from <http://www.r-project.org/>) and SAS Statistical Software packages (SAS UK).

To establish which independent variables (i.e. strain, treatment, presence of a host) have a significant effect on the frequency of occasions that mosquitoes spend on the net, analyses using a generalised estimating equation (GEE) model plus Tukey's pairwise comparisons (Appendix 6), was carried out using SPSS Version 24. In the GEE model, the response/dependent variable is binomial (i.e. proportional) rather than binary.

In experiment 3, frequency graphs (Excel© 2010 Microsoft Corporation, USA) showed each sampling point, where each mosquito was categorised into one of three possible events: positioned on the net; positioned on the cone or actively in flight. Box and whisker plots compared flight and net activity in mosquito seconds¹³ and comparisons were carried out using a two-way ANOVA (plus Sidak's multiple comparisons test). Analyses were performed using GraphPad Prism Version 7.03 (GraphPad Software Inc. USA).

¹³Mean observation frequencies for each experiment equate to mean mosquito-seconds for each event, *i.e.* 5 mosquitoes were recorded once every 5s for each replicate. These were converted into single mosquito-seconds by dividing by 5 (total seconds). The sum of the five repeat tests equated the mean observation frequency and was converted to total average mosquito seconds per mosquito by dividing the mean observation frequency by the total number of observations for all repeats (36 x 5 = 180), multiplied by the total sampling time (180).

3.5 Results

3.5.1 Chemical Analysis of Nets

HPLC analysis detected 4.73 g/kg (± 0.16 S.D.) (0.31g/m^2) of alpha-cypermethrin in the Duranet, which is within range of the target dose ($5.8\text{g/kg} \pm 25\%$). The Olyset net contained 18.84g/kg (± 0.33 S.D.) or 0.95g/m^2 of permethrin which is within the manufacturer target range of $20\text{g/kg} (\pm 3\text{g/kg})$. The PermaNet contained 54.7 mg/m^2 of deltamethrin, equating to $1.09\text{ g/Kg} (\pm 0.06\text{ S.D.})$, and is within the target range issued by the manufacturer ($1.8\text{g/kg} \pm 25\%$).

3.5.2 Experiment 1 - Mosquito behaviour at the LLIN interface

3.5.2.1 Frequency observations

Video recordings were analysed to determine the proportion and amount of time mosquitoes spent in contact with the net, resting on the cone walls or in flight (summarised in Figure 3-4). Generally, against untreated, Duranet and PermaNet, the greatest proportion of time is spent in contact with the net, as shown by the grey bars. Increased flight activity is seen against Olyset and Olyset Duo nets, as shown by the blue bars. For each strain and treatment, data from five replicates were averaged to show mean observations of each activity (in flight, on the cone or on the net) over the 180 second assay period. The full data set, excluding those shown below, is provided in Appendix 3.

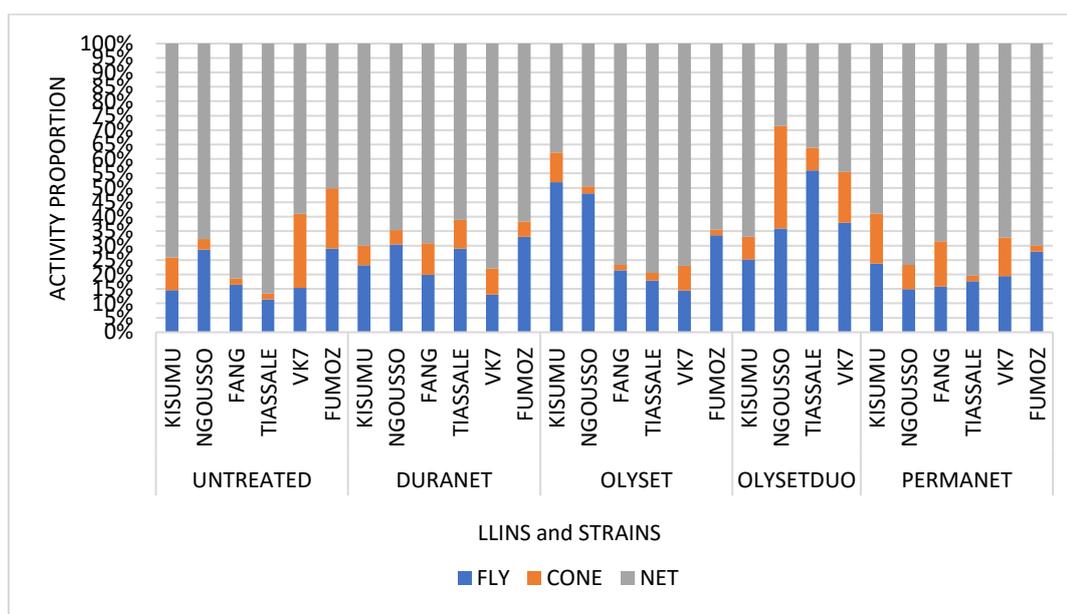
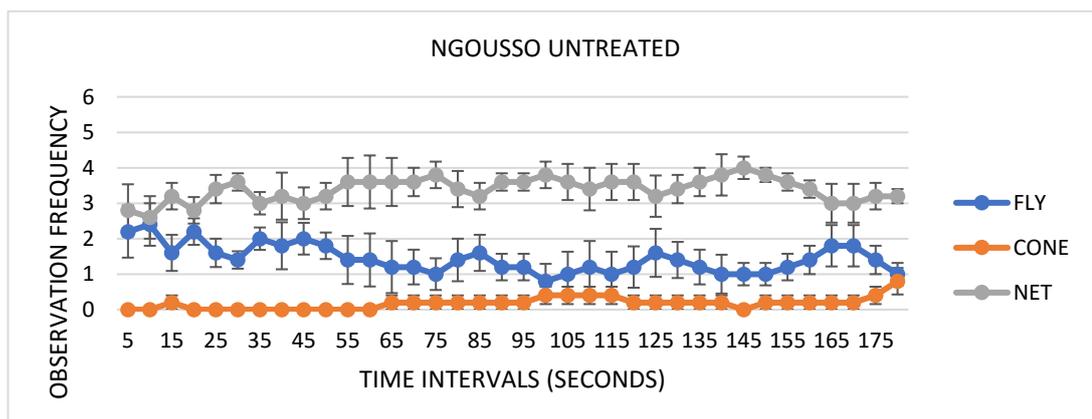
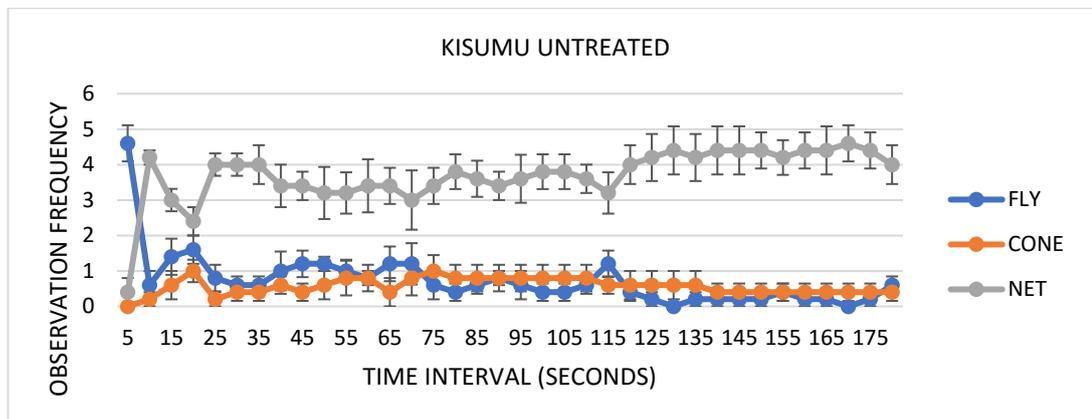


Figure 3-4 Stacked bar chart of experiment 3 behaviour assay (all strains and LLINs) summarising mean percentage proportions of activity at the net (grey bars), at the cone (orange bar) and in flight (blue bar) of insecticide susceptible (KISUMU, NGOUSSO, FANG) and insecticide resistant (TIASSALÉ, VK7, FUMOZ) strains of *An. gambiae* and *An. funestus* after exposure to UNTREATED, DURANET, OLYSET, OLYSET DUO and PERMANET

LLINs using the direct contact WHO standard cone bioassay method in the contact experiment (n = 25, 5 mosquito x 5 net samples).

3.5.2.2 *Baseline response of all three insecticide susceptible mosquito strains to an untreated net in a cone test*

Observation frequency graphs show susceptible strains of *An. gambiae* and *An. funestus* spent considerably more time on the untreated net (grey line) than in flight (blue line) or on the cone (orange line) as seen in Figure 3-5. This pattern of activity remained constant for the assay duration. The mosquitoes do disengage from the net and flight observations increase, but generally there is a preference for net contact over other activities. Insecticide resistant mosquitoes behave in the same way when exposed to an untreated net; individuals are predominantly found on the net with a lower frequency of cone and flight activity (see Appendix 2).



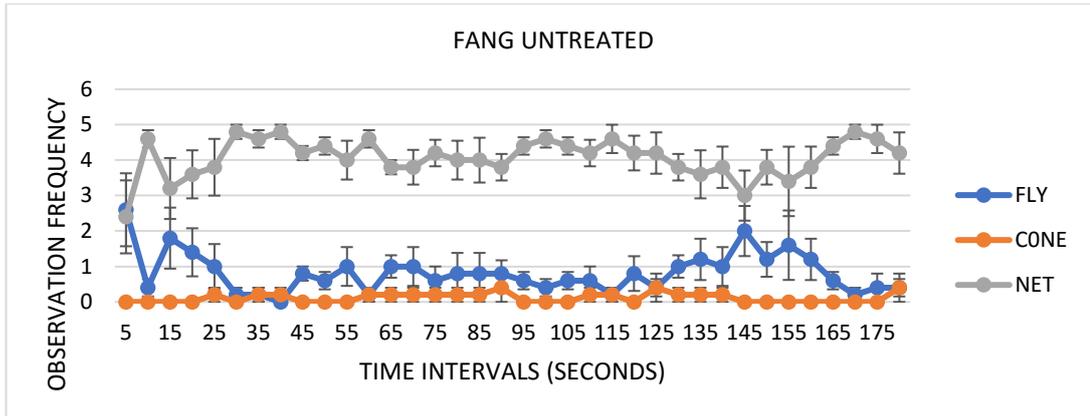
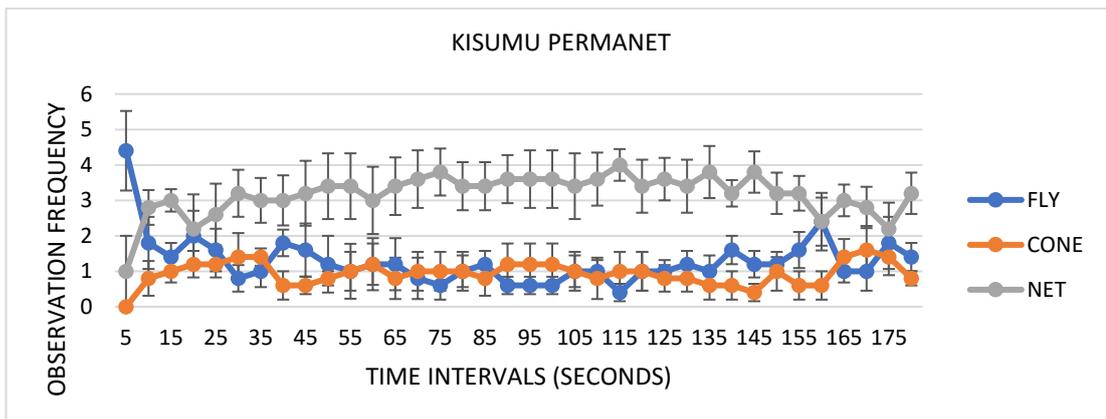


Figure 3-5 Line graphs of experiment 3 behaviour assay (untreated) showing mean observation frequency of activity on the net (grey line), the cone (orange line) and in flight (blue line) of insecticide susceptible *An. gambiae* KISUMU (top) and NGOUSSO (middle) strains and *An. funestus* FANG strain (bottom) after exposure to untreated netting using the video WHO standard cone bioassay method (n = 25, 5 mosquito x 5 net samples). Error bars show SEM.

3.5.2.3 Responses of *An. gambiae* during LLIN exposure

3.5.2.3.1 PermaNet/Duranet

Responses of susceptible mosquitoes to PermaNet (and Duranet – see Appendix 3) were similar to those observed with an untreated net, *i.e.* mosquitoes rested on the LLIN throughout suggesting no evidence for repellency or contact irritancy occurring within the 3 min (Figure 3-6). Insecticide resistant strains behaved similarly within the exposure period (Appendix 3).



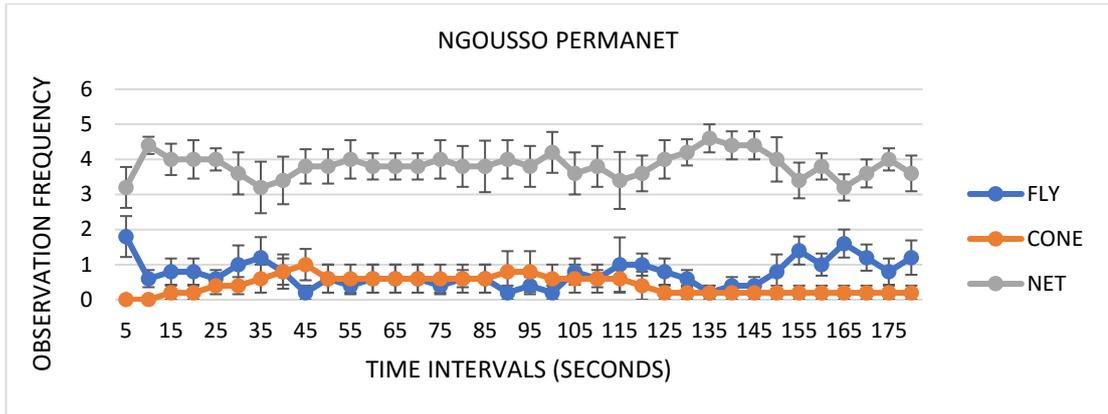


Figure 3-6 Line graphs of experiment 3 behaviour assay (PermaNet) showing mean observation frequency of activity on the net (grey line), the cone (orange line) and in flight (blue line) of insecticide susceptible *An. gambiae* KISUMU(top) and NGOUSSO (bottom) strains after exposure to PermaNet using the video WHO standard cone bioassay method (n = 25, 5 mosquito x 5 net samples). Error bars show SEM.

3.5.2.3.2 Olyset

Susceptible mosquitoes exposed to Olyset (Figure 3-7) behaved differently compared to both untreated nets and PermaNet (Figures 3-5, 3-6). Both Kisumu and Nguosso spent more time in flight (equally likely to be in flight or resting on net throughout). Increased flight activity results in reduced time in contact with the net.

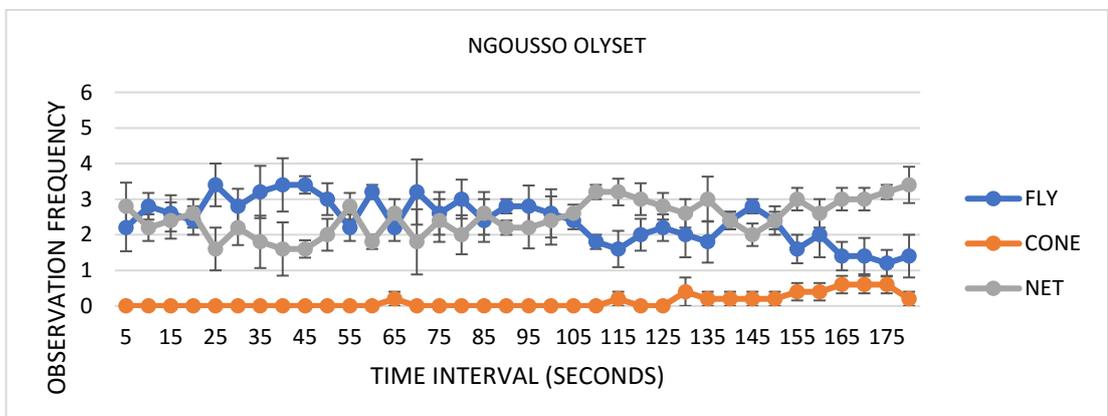
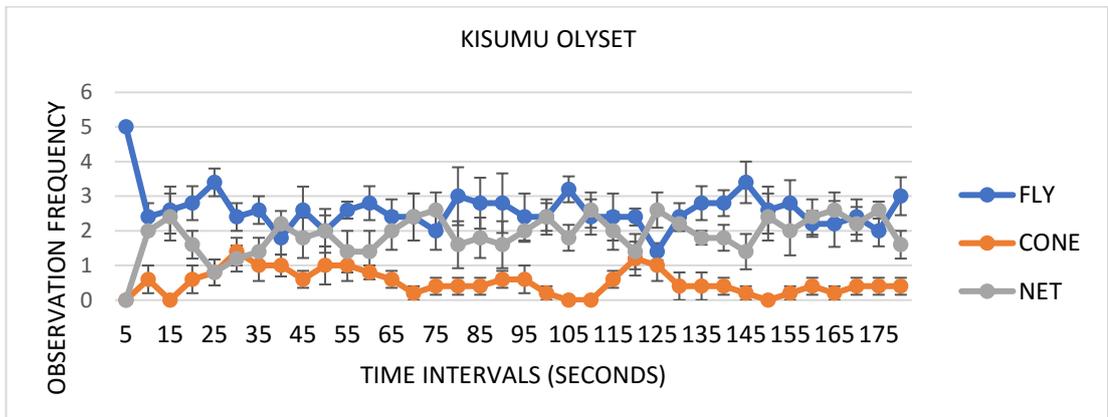


Figure 3-7 Line graphs of experiment 3 behaviour assay (Olyset) showing mean observation frequency of activity on the net (grey line), the cone (orange line) and in flight (blue line) of insecticide susceptible *An. gambiae* KISUMU (top) and NGOUSSO (bottom) strains after exposure to Olyset netting using the video WHO standard cone bioassay method (n = 25, 5 mosquito x 5 net samples). Error bars show SEM.

The increased flight activity upon Olyset exposure was not observed in the resistant strains (Figure 3-8). After a brief initial ‘unsettled’ period mosquitoes rested on the LLIN in a similar response as observed for both untreated and PermaNet. Both Tiassalé and VK7 spend most of the time in contact with the Olyset LLIN, with little disengagement from the net to fly or rest on the cone.

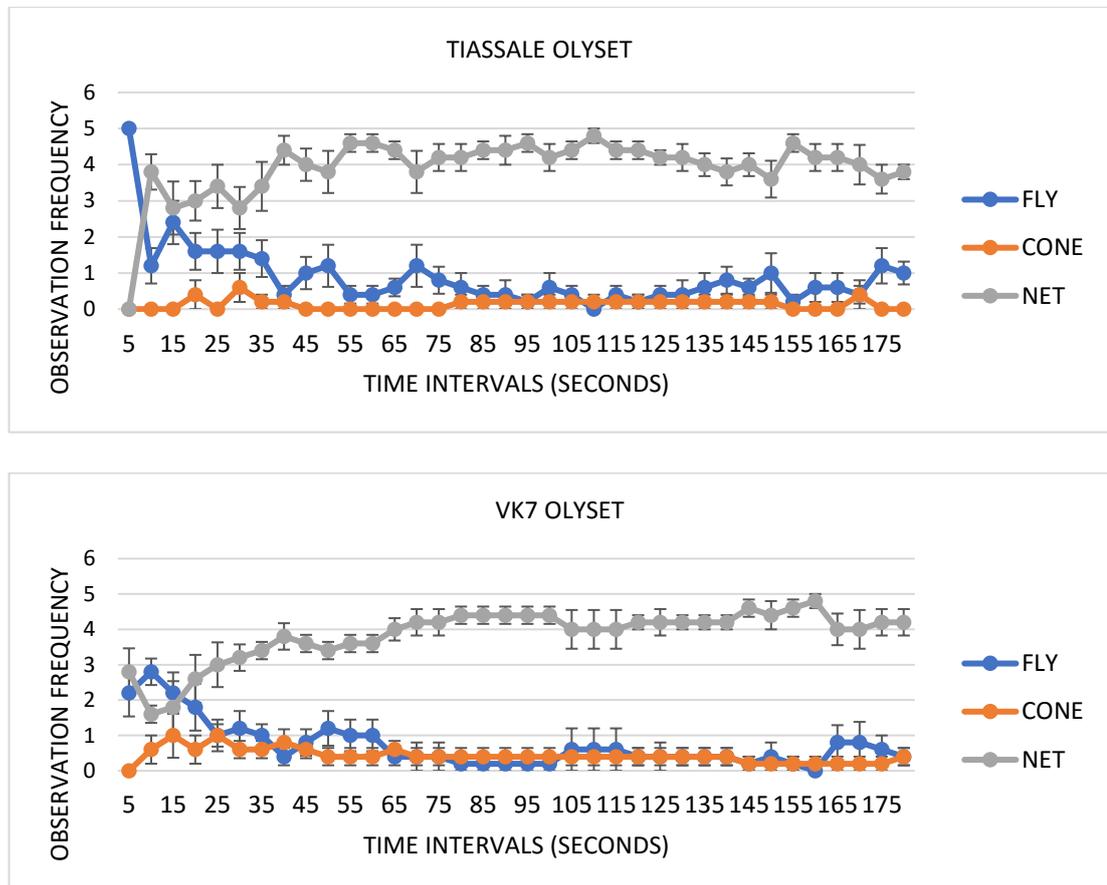


Figure 3-8 Line graphs of experiment 3 behaviour assay (Olyset) showing mean observation frequency of activity on the net (grey line), the cone (orange line) and in flight (blue line) of insecticide resistant *An. gambiae* TIASSALÉ (top) and VK7 (bottom) strains after exposure to Olyset netting using the video WHO standard cone bioassay method (n = 25, 5 mosquito x 5 net samples). Error bars show SEM.

3.5.2.3.3 Olyset Duo

Exposure to Olyset Duo, results in increased net contact in the susceptible Kisumu strain, yet not the Ngousso strain, and the resistant strains mosquitoes show increased flight and cone activity which is not observed with Olyset net alone. (see Appendix 3)

3.5.2.4 Summary – direct contact bioassays

Direct contact assays were performed to establish baseline behavioural responses of mosquitoes to insecticide treated net and determine levels of contact irritancy responses at the LLIN surface. There is clear evidence for a different entomological mode of action (how mosquitoes respond to an insecticide and how the insecticide acts on the mosquito) between type I (permethrin) and type II (deltamethrin) pyrethroid treated LLINs with susceptible mosquitoes. Only Olyset (type I) induces contact irritancy in susceptible mosquitoes and subsequently alters mosquito behaviour.

Box and whisker plots showing the ranges of median time spent either in contact with the net or in flight at untreated nets, Olyset and PermaNet, are summarised in Figure 3-9. After exposure to untreated net or PermaNet, both susceptible *An. gambiae* strains (Kisumu/Ngousso) and resistant *An. gambiae* strains (Tiassalé/VK7) spent the majority of time resting on the net. The length of time spent either in contact with the net compared with in flight was significantly different for all four strains against untreated and PermaNet ($p < 0.0001$).

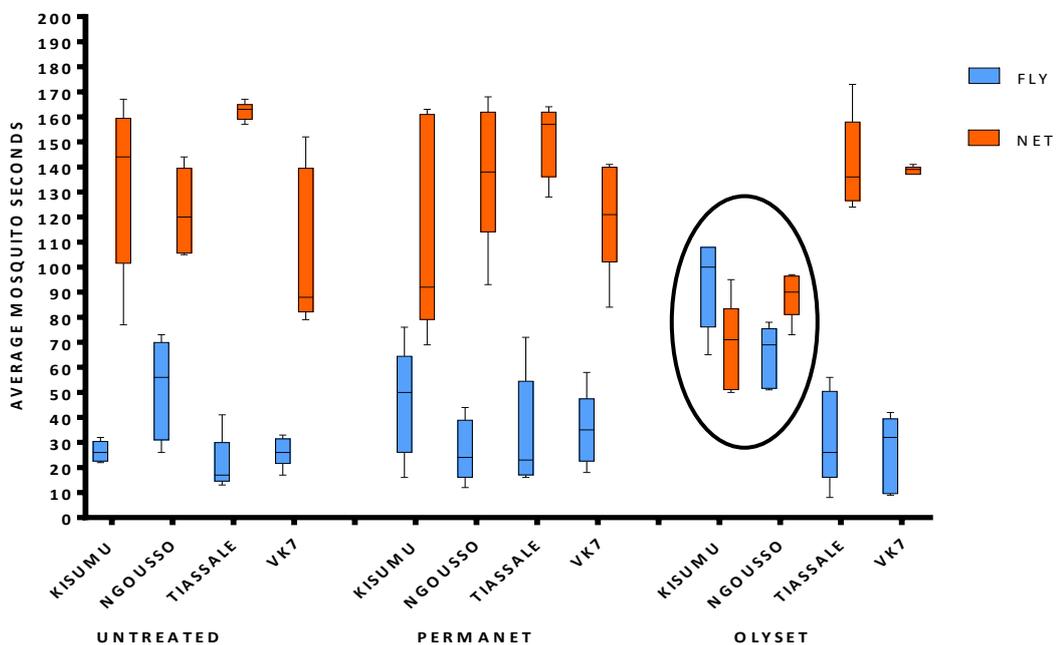


Figure 3-9 Box and whisker plots of experiment 3 (direct contact assays) showing comparisons of range of time spent in flight (blue bar) and in contact with net (orange bar) of insecticide susceptible (KISUMU, NGOUSSO) and insecticide resistant (TIASSALÉ, VK7) strains of *An. gambiae*, after exposure UNTREATED, PERMANET and OLYSET LLINs using the video WHO standard cone method. Box extends from the 25th to 75th percentiles. Median shown as line within each box. Whiskers range from minimum to maximum value. Circle highlights response of susceptible strains Kisumu and Ngousso against Olyset.

GEE analysis (Appendix 6) shows the effect of the treatment on contact with the net varies with type of strain as the interaction between strain and net is highly significant ($P < 0.0001$).

Notably different responses from those seen against untreated and PermaNet, were observed with both Kisumu and Ngousso after exposure to Olyset (highlighted by circle in Figure 3-9). Pairwise comparisons show there is a significant difference in time Kisumu spends on the Olyset net compared to PermaNet ($p=0.010$)/Untreated ($p<0.0001$). Interestingly, although Ngousso behaves similarly to Kisumu, away from the net, time spent on the Olyset net is statistically different from Kisumu against Olyset ($p=0.01$), and not statistically significantly different against PermaNet ($p=0.166$). Both susceptible strains spend significantly different amount of time on the net compared to IR strains. There is an absence of evidence for contact irritancy in resistant mosquitoes suggesting the impact of irritant properties of permethrin is ameliorated by IR.

To demonstrate this altered behaviour between Olyset and PermaNet, composite still images of Kisumu mosquitoes during a cone test video, after contact with Olyset and PermaNet, in a single replicate of the direct contact assay, were created (Figure 3-10). The top row shows aggregate superimposed positions taken every 5 seconds and the bottom row, every frame. Heightened flight/cone activity, as seen by increased mosquito movement density, is evident in the Olyset cones (left side) compared to the PermaNet (right side), where the mosquitoes prefer to remain on the netting.

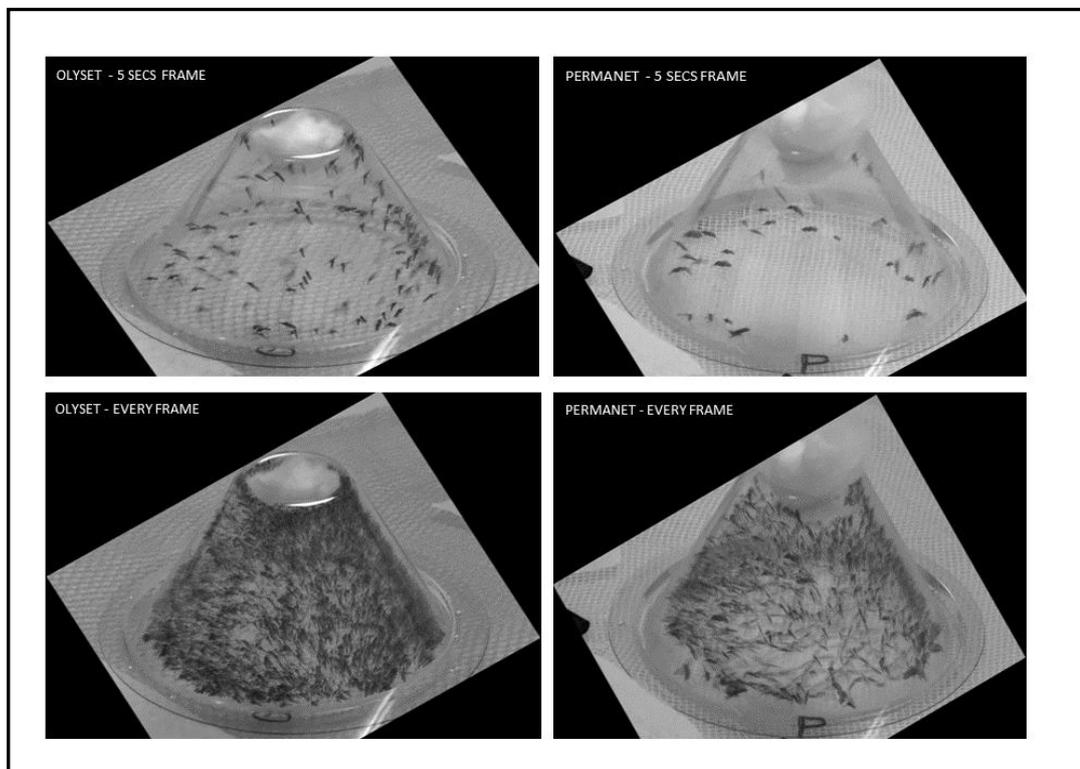


Figure 3-10 Composite video still images of insecticide susceptible *An. gambiae* (Kisumu) mosquito activity during a single replicate cone test in response to Olyset net (left side) and PermaNet net (right side). Composite images created by finding the minimum brightness value of every 5 seconds frame (top row) or all frames (bottom

row) at all locations in each frame. Some mosquitoes appear ‘chubby’ in the PermaNet cone (top right) revealing individuals remain static for much of the 3-min assay, whereas increase activity is evident in the Olyset cone (top left). Images taken every frame show increased mosquito movement density against Olyset (bottom left) compared to PermaNet (bottom right). Images created thanks to Dr Jeff Jones (LSTM) using ImageJ (Rueden et al. 2017).

Responses of all strains to the combination net, Olyset Duo (Figure 3-11), appear more random, differing from the patterns of activity following contact against mono-treated LLIN nets. Less time is spent in contact with the net and increased time in flight, but this is not statistically significant for the susceptible Ngousso strain, or either resistant strain. Yet, Kisumu spent more time in contact with the Olyset Duo net than seen with Olyset alone ($p < 0.0001$).

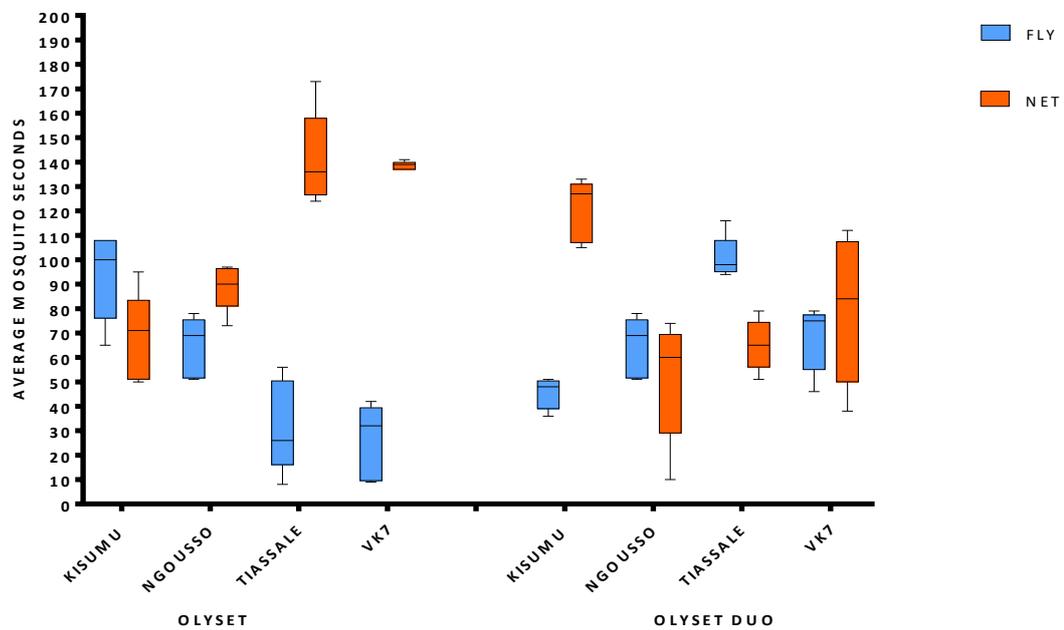


Figure 3-11 Box and whisker plots of experiment 3 (direct contact – Olyset and Olyset Duo) showing comparisons of range of time spent in flight (blue bar) and in contact with net (orange bar) of insecticide susceptible (KISUMU, NGOUSSO) and insecticide resistant (TIASSALÉ, VK7) strains of *An. gambiae*, after exposure to OLYSET and OLYSET DUO LLINs using the video WHO standard cone method. Box extends from the 25th to 75th percentiles. Median shown as line within each box. Whiskers range from minimum to maximum value.

3.5.2.5 Summary – direct contact bioassays (host present)

To determine if the levels of an LLIN's contact irritancy, measured in the absence of a host, were retained when competing with the range of attractants emitted by a host, the cone bioassays were repeated with a human arm placed behind the net (see Section 3.4.3.2). When a host was present, all strains spent significantly more time on the net than in flight ($p < 0.0001$) (Figure 3-12). The key finding from this experiment was that in the presence of a host, susceptible mosquitoes spent increased time in contact with the Olyset net than observed in the absence of a host, highlighted by the circle in figure 3-12. GEE analysis (Appendix 6) shows the effect of the treatment on contact with the net varies with type of strain as the interaction between strain and net is highly significant ($P < 0.0001$).

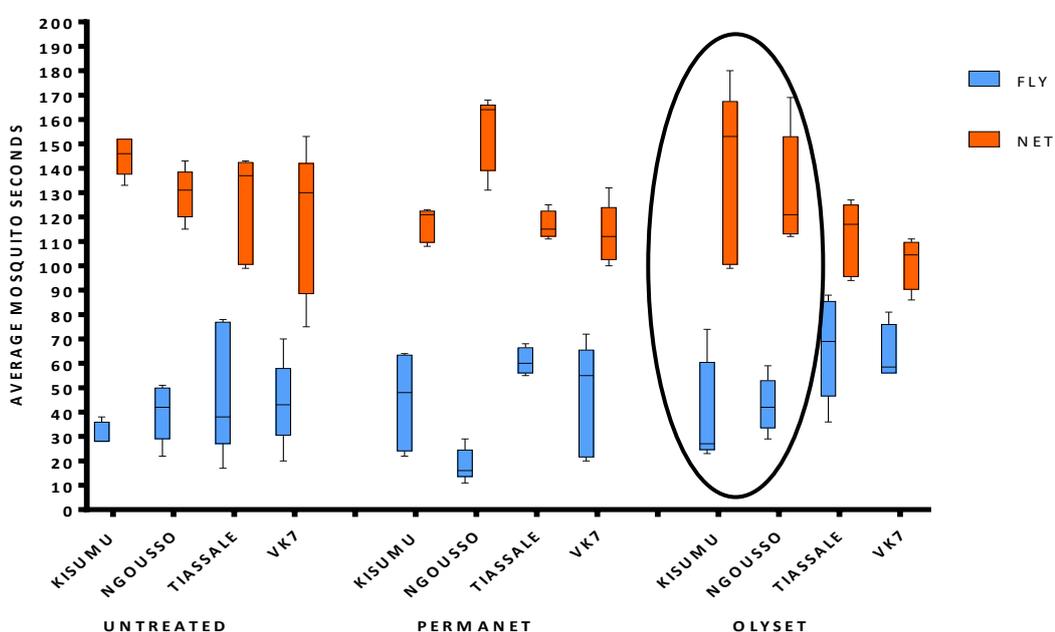


Figure 3-12 Box and whisker plots of experiment 3 (direct contact plus host assays) showing comparisons of range of time spent in flight (blue bar) and in contact with net (orange bar) of insecticide susceptible (KISUMU, NGOUSSO) and insecticide resistant (TIASSALÉ, VK7) strains of *An.gambiae*, after exposure UNTREATED, PERMANET and OLYSET LLINs using the video WHO standard cone method. Box extends from the 25th to 75th percentiles. Median shown as line within each box. Whiskers range from minimum to maximum value. Circle highlights response of susceptible strains Kisumu and Ngoussou against Olyset.

3.5.2.6 Comparisons of KD, mortality and net contact against Olyset with and without a host

To determine whether increased time spent on the Olyset net in the presence of a host had an impact on KD and mortality, these outcomes were compared with results from the standard cone bioassay experiments performed in the absence of a host (Section 3.5.2.1). As these assays were carried out at different times with different mosquito batches, the p-value must be viewed with caution. The KD and mortality determined in the presence and absence of a host against Olyset is shown in Figure 3-13.

Results show a significant increase ($p < 0.0001$) in KD for Ngouso ($20\% \pm 6.3$ SEM to $96\% \pm 4$ SEM), and Tiassalé (0% to $59\% \pm 4.6$ SEM) after exposure to Olyset LLINs in the presence of a host compared to no host. No significant difference was observed for Kisumu ($p = 0.38$) and there was no KD in the VK7 strain. Mortality significantly increased in the Ngouso strain in the presence of a host ($p = 0.0025$). There was no mortality recorded for either resistant strain.

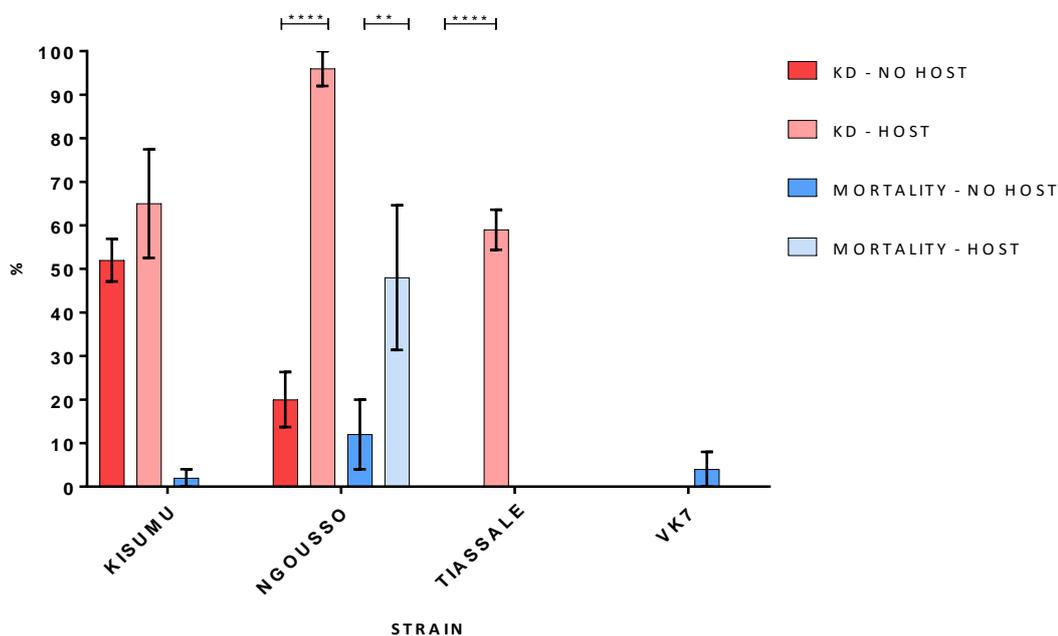


Figure 3-13 Bar chart of experiment 3 (non-host versus host assays) showing comparisons between percentage knock down (KD) and 24 h mortality of insecticide susceptible (KISUMU, NGOUSSO) and insecticide resistant (TIASSALÉ, VK7) strains of *An. gambiae* after exposure to Olyset net using WHO standard cone bioassay method, in CONTACT only and CONTACT + HOST experiments. Error bars show SEM. **** $p < 0.0001$. ** $p < 0.01$

GEE results (Appendix 6) show that there are significant differences in net contact responses in the absence and the presence of a host. After contact with Olyset in the presence of a host, susceptible strains Kisumu ($p < 0.0001$) and Ngouso ($p < 0.0001$), spend significantly

longer on the net than in the absence of a host. Similarly, this effect is also seen in the IR strains, Tiassale ($p=0.003$) and VK7 (<0.0001).

3.5.2.7 Summary - non-contact bioassays

To distinguish between true spatial repellency or irritancy following net contact, direct contact with the LLIN was prevented via the inclusion of an untreated net barrier (see Section 3.4.3.3). Full results are presented in Appendix 3. Generally, mosquitoes spent more resting time on the untreated barrier net than in flight (Figure 3-14) but the amount of time on the net was reduced relative to the contact assays (Figure 3-9). For example, there was no significant difference in the time that Kisumu spent against the barrier or in flight for the Olyset assays whilst for the second susceptible strain, Ngousso, significantly more time was spent on more time on the net than in flight ($p<0.0001$). Both susceptible strains spend less time in flight when contact was precluded, suggesting that the Olyset may act as contact irritant, rather than a repellent. Neither KD nor mortality was observed in any of the non-contact experiments indicating no airborne toxicity with any net type.

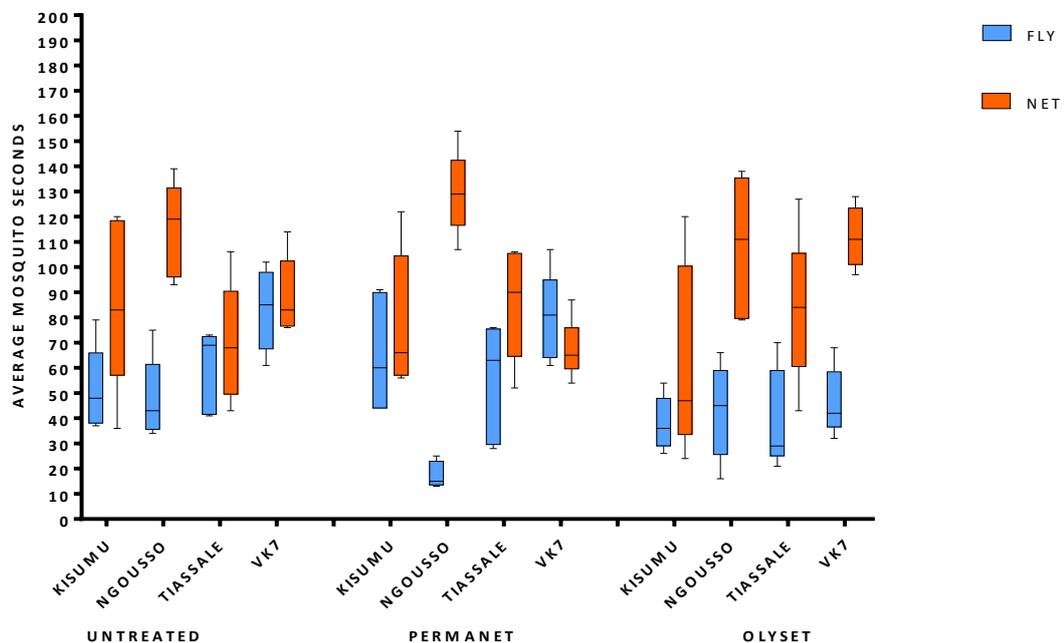


Figure 3-14 Box and whisker plots of experiment 3 (non-contact assays) showing comparisons of range of time spent in flight (blue bar) and in contact with net (orange bar) of insecticide susceptible (KISUMU, NGOUSSO) and insecticide resistant (TIASSALÉ, VK7) strains of *An. gambiae*, after exposure UNTREATED, PERMANET and OLYSET LLINs using the video WHO standard cone method. Box extends from the 25th to 75th percentiles. Median shown as line within each box. Whiskers range from minimum to maximum value.

3.5.2.8 Summary - responses of *An. funestus* during LLIN exposure

These experiments were carried out to investigate whether an alternative response to direct insecticide exposure was present in a susceptible and resistant strain of *An. funestus* compared to *An. gambiae* strains. Unlike resistant *An. gambiae* strains, resistant *An. funestus* strains do not possess the *kdr* mutation. Summary Figure 3-15 shows both insecticide susceptible Fang and resistant Fumoz spent significantly more time in contact with the net than in flight after exposure to untreated, Duranet, PermaNet and Olyset ($p < 0.001$). Susceptible and resistant strains of *An. funestus* follow the same pattern of behaviour as susceptible and resistant strains of *An. gambiae* against untreated, Duranet and PermaNet nets (see Appendix 2). Interestingly, the reduced net contact time observed with susceptible strains of *An. gambiae* exposed to Olyset nets was not observed in the susceptible *An. funestus* strain (see Appendix 2).

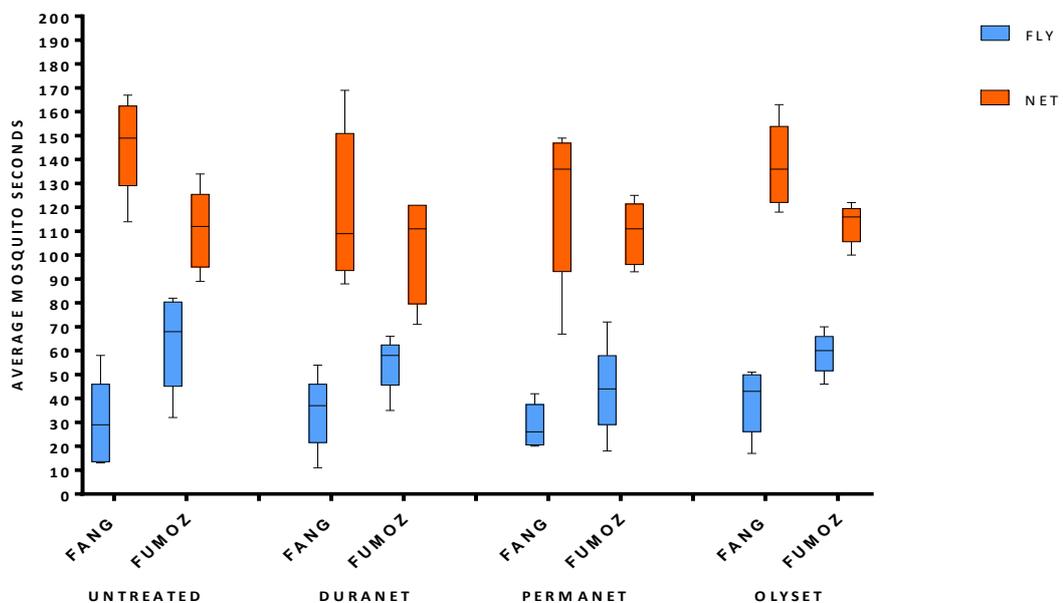


Figure 3-15 Box and whisker plots of experiment 3 (direct contact assays - *An. funestus*) showing comparisons of range of time spent in flight (blue bar) and in contact with net (orange bar) of insecticide susceptible FANG and insecticide resistant FUMOZ strains of *An. funestus*, after exposure UNTREATED, DURANET, PERMANET and OLYSET LLINs using the video WHO standard cone method. Box extends from the 25th to 75th percentiles. Median times shown as line within each box. Whiskers range from minimum to maximum value.

3.5.3 Experiment 2 - Knockdown (KD) and mortality

3.5.3.1 Performance of different LLINs in standard cone bioassays.

The performance of four LLINs against six *Anopheles* strains were compared using standard cone bioassay methodology (Figure 3-16). Two strains of *An. funestus* were tested against all treatments except Olyset Duo. Knock down at the end of the 3 min exposure was zero for all nets (not shown) and hereafter, references to KD data are to 60 min post exposure only. KD and 24 h mortality was zero in the insecticide susceptible strains of *An. gambiae* and resistant strain of *An. funestus*, after exposure to untreated netting. *An. gambiae* insecticide resistant strains (Tiassalé and VK7) showed no KD and 4% (± 4 SEM) mortality after exposure to untreated netting. Results for the Fang strain of *An. funestus* results were excluded as the assay showed high KD/mortality against untreated netting.

Against both susceptible strains, PermaNet and Olyset Duo met WHO targets of >95% knockdown and > 80% mortality. Exposure to Duranet resulted in 100% KD and 76% (± 14.7 SEM) mortality in Kisumu and 80% (± 12.6 SEM) KD and 60% (± 16.7 SEM) mortality in Ngousso strains, apart from Kisumu KD, these results are below the WHO threshold. Percentage rates against Olyset were all below the WHO threshold, where Kisumu showed 52% (± 4.9 SEM) and 2% (± 2 SEM) and Ngousso showed 22% (± 4.9 SEM) and 12% (± 8 SEM) KD and mortality respectively.

For insecticide resistant strains, knockdown rates were below WHO thresholds for all LLINs tested apart from Olyset Duo which showed 100% KD with Tiassalé. The VK7 strain achieved less than 4% (± 4 SEM) KD and mortality after exposure to all four LLINs. Similarly, mortality in the Tiassalé strain was below 8% for all LLINs except Olyset Duo which was 52% (± 13.6 SEM). Fumoz proved to be highly resistant showing zero KD and mortality against all nets tested. Against PermaNet, Tiassalé showed 52% (± 4.9 SEM) KD and only 4% (± 4 SEM) mortality, while VK7 and Fumoz showed no KD or mortality.

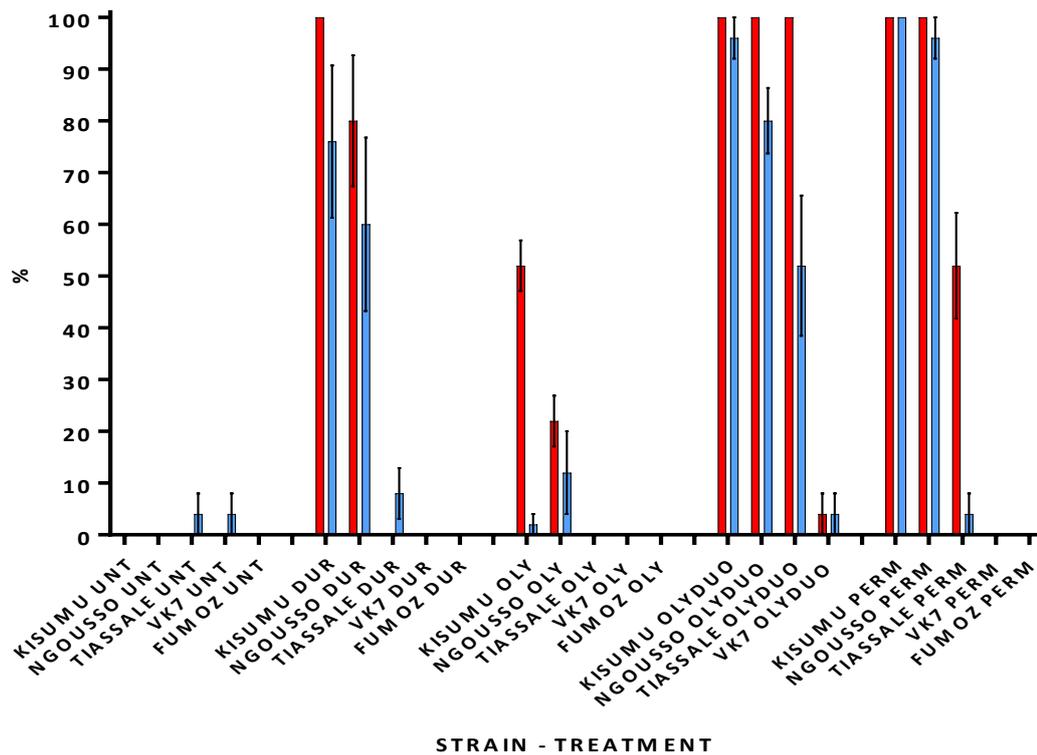


Figure 3-16 Bar chart of experiment 1 - WHO standard cone bioassay results showing mean percentage knock down (red bars) and 24 h mortality (blue bars) data for insecticide susceptible strains (KISUMU, NGOUSSO) and insecticide resistant strains (TIASSALÉ, VK7) of *An. gambiae* and insecticide resistant strain (FUMOZ) of *An. funestus*, after exposure to UNT (untreated), DUR (Duranet), OLY (Olyset), OLYDUO (Olyset Duo) and PERM (PermaNet) LLINs using the WHO standard cone bioassay method (n=25, 5 mosquito x 5 net samples). Error bars show SEM. 3 min KD results are not shown. Insecticide susceptible *An. funestus* FANG strain results are not shown.

3.5.3.2 Impact of number of mosquitoes per assay on cone bioassay

results

Results from bioassays using five individuals were compared to bioassays using ten individuals. For each strain and treatment, there was no significant difference in KD and 24 h mortality between x5 and x10 test individuals (Figures 3-17 and 3-18). Kisumu and Tiassalé KD and mortality data obtained in the WHO standard cone bioassay experiment using five individuals (Section 3.4.1.1) was used to compare with ten individuals in this experiment. As these assays were carried out at the different times with different mosquito batches, results must be viewed with caution.

KD and mortality were zero in all strains exposed to untreated netting. After exposure to PermaNet, five and ten individuals of both susceptible strains showed consistent KD (Figure 3-4) and mortality (Figure 3-5) of between 98% (± 2 SEM) - 100%. After exposure to Olyset, with both five and ten individuals of Kisumu, KD did not exceed 58% (± 4.9 SEM) and mortality 10% (± 4.5 SEM). Similarly, for Ngoussou, KD did not exceed 20% (± 6.3 SEM) and mortality 12%

(± 8 SEM). For the two resistant strains, although KD increased in Tiassalé against PermaNet, neither PermaNet nor Olyset induced mortality greater than 10% (± 4.5 SEM). Five individuals were used in each replicate in subsequent experiments in line with WHO recommendations.

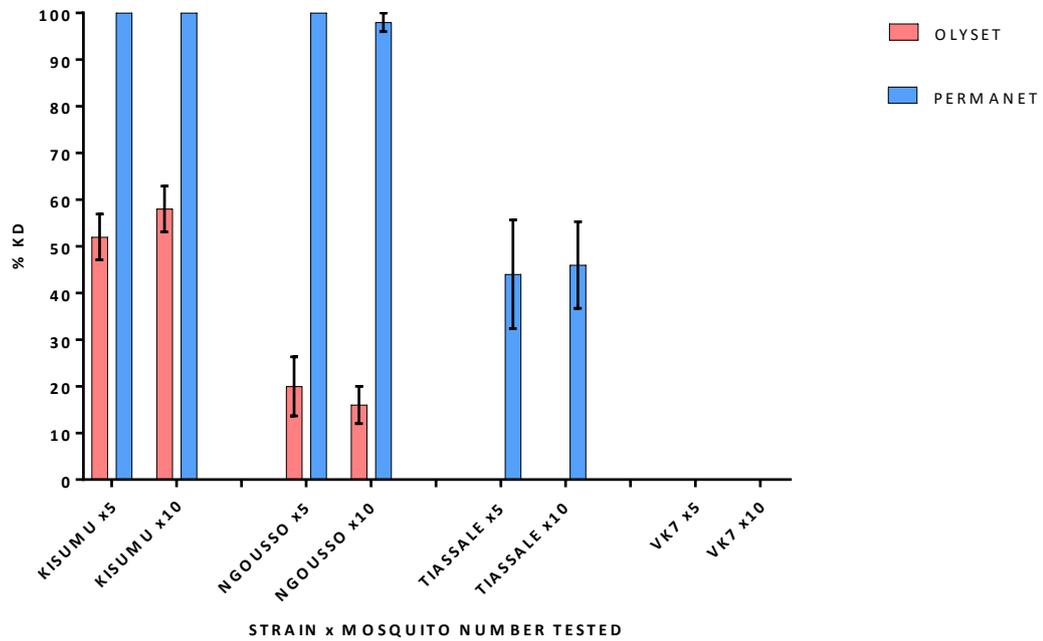


Figure 3-17 Bar chart of results for experiment 1 (5 versus 10 assay) - Knockdown (KD) showing percentage knock down for tests with 5 individuals (n=25, 5 mosquito x 5 net samples) and 10 individuals (n=50, 10 mosquito x 5 net samples) with insecticide susceptible (KISUMU and NGOUSSO) and insecticide resistant (TIASSALÉ and VK7) strains of *An. gambiae* after exposure to OLYSET (red bar) and PERMANET (blue bar) using the WHO standard cone assay method. Error bars show SEM. Untreated netting results are not shown.

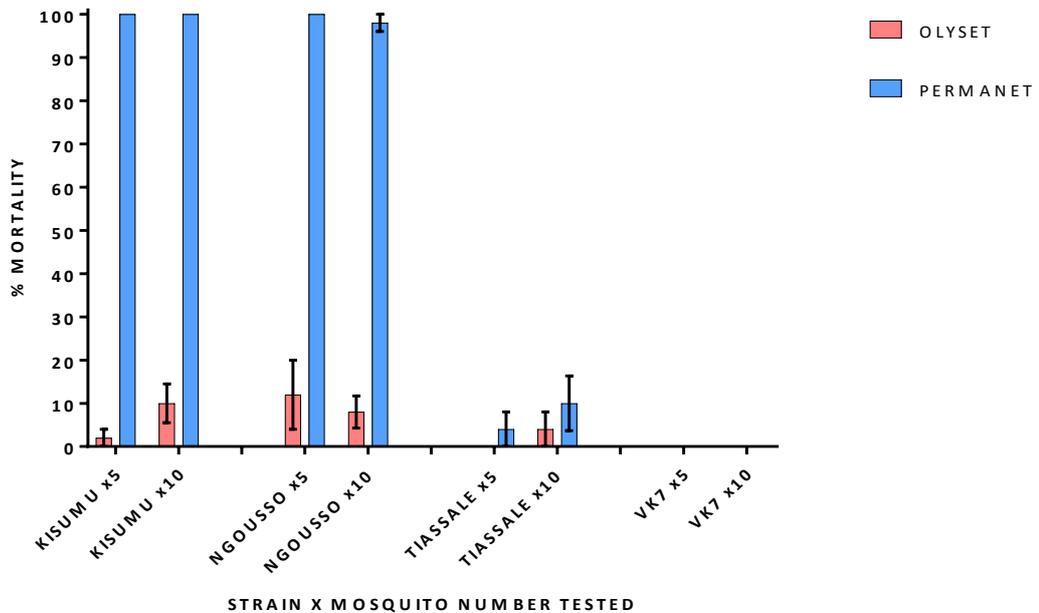


Figure 3-18 Bar chart of experiment 1 (5 versus 10 assay) - Mortality showing percentage 24 h mortality with 5 test individuals (n=25, 5 mosquito x 5 net samples) and 10 test individuals (n=50, 10 mosquito x 5 net samples) using insecticide susceptible (KISUMU and NGOUSSO) and insecticide resistant (TIASSALÉ and VK7) strains of *An. gambiae* after exposure to OLYSET (red bar) and PERMANET (blue bar) LLINs using the WHO standard cone bioassay method. Error bars show SEM. Untreated netting results are not shown.

3.5.3.3 Modified Wire - Ball assays

The wire-ball method yielded significantly improved knockdown and mortality results compared with the standard cone assay (Figure 3-19). Standard cone assays were carried out at the different times (Section 3.4.1.1) with different mosquito batches, therefore the P value must be viewed with caution. Both KD and 24 h mortality was zero after exposure to untreated netting.

In the susceptible Kisumu strain, both KD and mortality against Olyset, using the ball assay, were significantly increased compared with the cone bioassay ($p < 0.0001$) where KD increased from 52% (± 4.9 SEM) to 100% and mortality increased from 4% (± 2.5 SEM) to 93% (± 4.4 SEM). Against PermaNet, there was already 100% KD and mortality with both assays.

With the resistant Tiassalé strain, there was no significant difference in either KD or mortality against Olyset, between ball and cone methods. Yet there was a significant increase in KD and mortality of Tiassalé against PermaNet ($p < 0.0001$), where KD increased from 52% (± 4.9 SEM) to 100%, and mortality from 4% (± 2.5 SEM) to 74% (± 11.6 SEM). These results show the ball method, which aims to ensure constant LLIN contact, is substantially more effective at knocking down and killing both susceptible and resistant mosquitoes than the cone method.

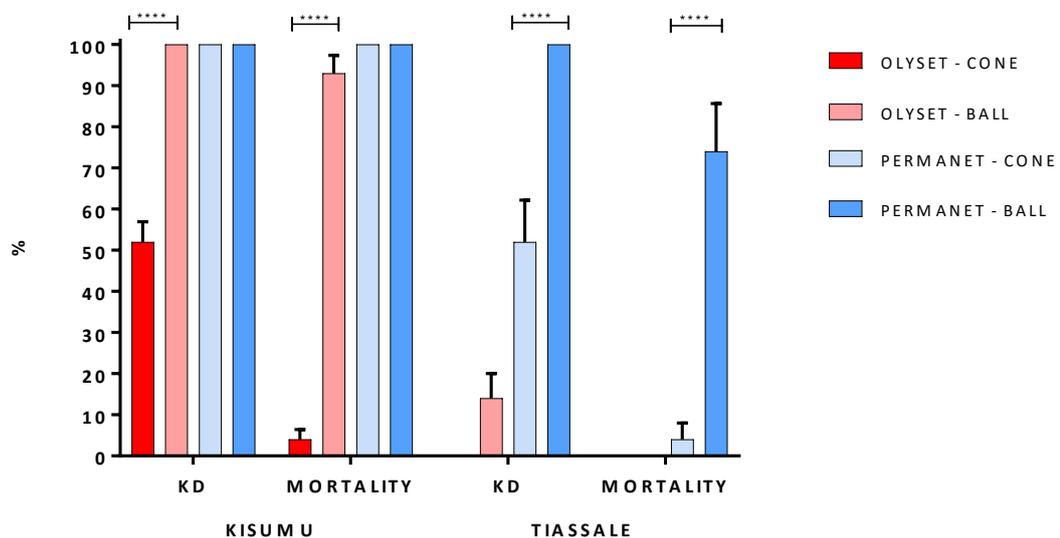


Figure 3-19 Bar chart of experiment 1 (adapted ball assay) showing mean percentage knock down (KD) and 24 h mortality for insecticide susceptible (KISUMU) and insecticide resistant (TIASSALÉ) strains of *An. gambiae* after exposure to OLYSET and PERMANET LLINs using the adapted wire ball method and WHO standard cone method (n = 25, 5 mosquito x 5 net samples). Error bars show SEM. **** $p < 0.0001$

3.5.4 Experiment 3 - LLIN contact time required for knock down

3.5.4.1 Timed bioassay experiment - Olyset Duo

In the first experiment, 3 min knock-down was not observed after exposure of mosquito strains to all LLINs using the WHO standard cone assay (Section 3.4.1.1). However, it was noted that after exposure of Kisumu to Olyset Duo, mosquitoes were immediately knocked down after transfer to holding cups at the end of the assay. This was not recorded as a 3 min knockdown as the mosquitoes appeared to be active within the cone at 3 min.

Hence, additional timed bioassay experiments were carried out to investigate when, within the 3 min exposure period, KD occurred. Using the standard WHO cone assay, five Kisumu mosquitoes were exposed to Olyset Duo for different durations and KD time recorded. The initial exposure was 30s, with subsequent exposure times increasing by increments of 30s to a total of 180s. Figure 3-20 shows the percentage knocked down immediately after transfer to cups following each exposure period. KD increases with increasing exposure time until 100% of individuals knocked down after 150s.

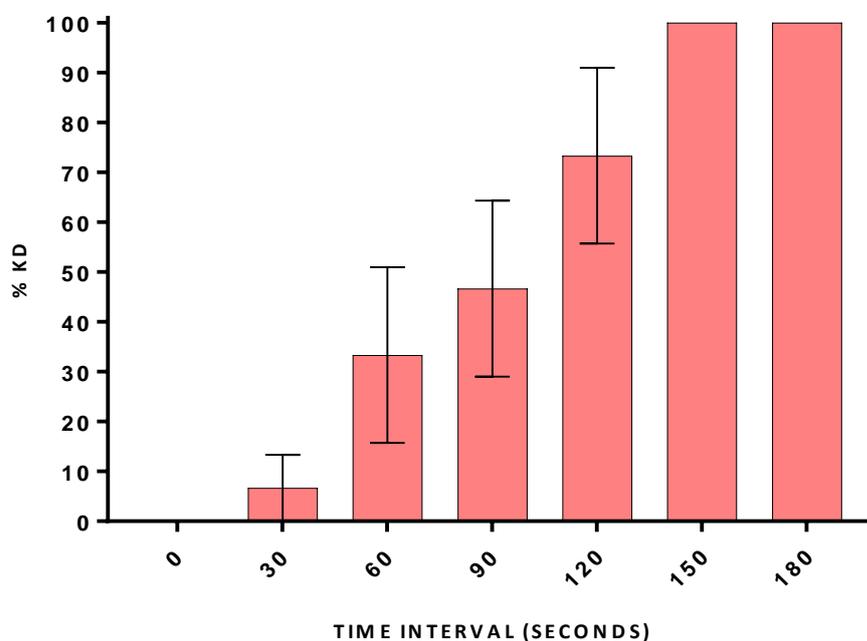


Figure 3-20 Bar chart of experiment 2 (timed exposure assay (Olyset Duo)) showing mean percentage knock-down (KD) of insecticide susceptible (Kisumu) strain of *An. gambiae* after exposure to Olyset Duo using the WHO standard cone bioassay. Total individuals knocked down after each timed exposure was recorded up to 180 seconds (n=90, 5 mosquito x 6 exposures x 3 net samples). Error bars show SEM.

3.5.4.2 Timed bioassay experiment - all LLINS

Further timed bioassay experiments were carried out to investigate KD and mortality results for Kisumu against the other LLINs. Unlike Olyset Duo which resulted in 100% KD after the 3 min assay, KD was zero after 3 min exposure to all other LLINs. Therefore, KD due to increasing exposure times, was recorded after 60 min instead (Figure 3-21).

There was zero KD against untreated netting. Against Duranet and Olyset Duo, within the first 30 seconds of an assay, mosquitoes have enough contact with insecticide to cause 100% knockdown 60 min post exposure. Similarly, PermaNet gave a KD of 93% (± 6.6 SEM). Olyset gave only 60% (± 11.5 SEM) knock down after 30 seconds. For all nets, a 3 min exposure results in over 90% knockdown after 60 min.

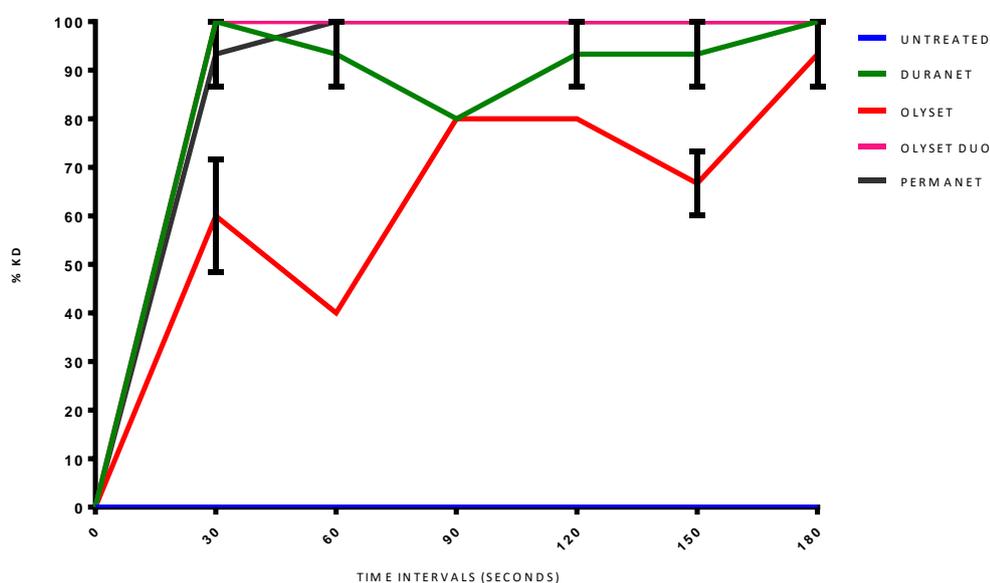


Figure 3-21 Line chart of experiment 2 (timed exposure assay (all nets)) – knockdown (KD) showing mean percentage knock down of insecticide susceptible (Kisumu) strain of *An. gambiae* 60 min after exposure to UNTREATED, DURANET, OLYSET, OLYSET DUO AND PERMANET using the WHO standard cone bioassay for increments of 30 seconds (n=90, 5 mosquito x 6 exposures x 3 net samples). Error bars show SEM.

Figure 3-22 shows that after exposing mosquitoes to Olyset Duo for 30s, 24 h mortality reached 100%. Similarly, PermaNet results in over 93% (± 6.6 SEM) mortality after 30s exposure. For Duranet, a 3 min exposure was necessary to induce 100% mortality and with Olyset, mortality only reached 20% (± 11.5 SEM) after the longest 180s exposure.

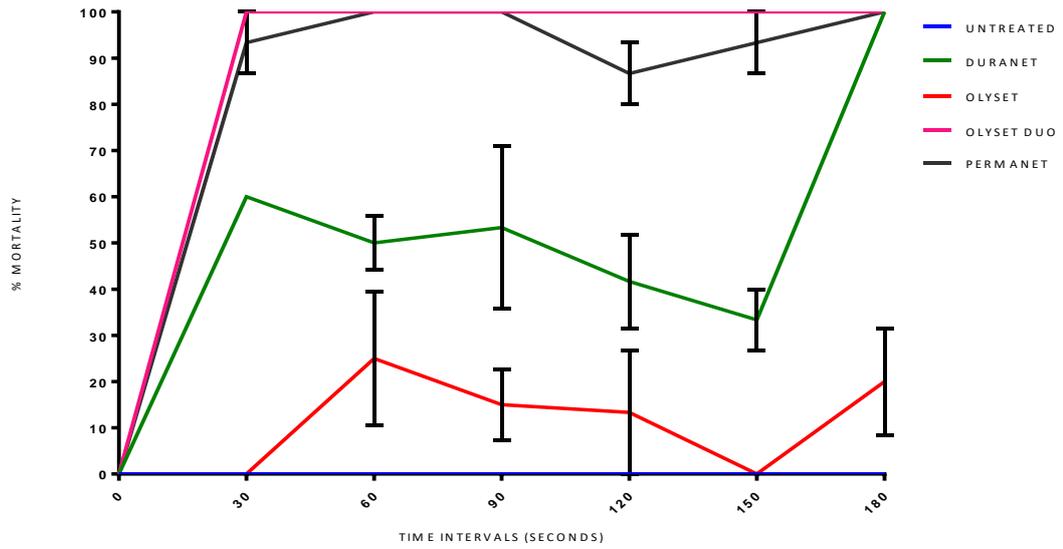


Figure 3-22 Line chart of **experiment 2 - timed exposure assay (all nets) – mortality** showing mean 24 h percentage mortality of insecticide susceptible (Kisumu) strain of *An. gambiae* after exposure to UNTREATED, DURANET, OLYSET, OLYSET DUO AND PERMANET using the WHO standard cone bioassay at 30s time intervals (n=90, 5 mosquito x 6 exposures x 3 net samples. Error bars show SEM.

3.6 Discussion

This aims of this chapter were to identify sources of variation in mosquito response to insecticide exposure in the WHO cone bioassay, among insecticide susceptible and resistant strains of *An. gambiae* and *An. funestus*, against various LLINs. Plus, the possibility of gathering additional data was explored using simple modifications to the standard WHO cone bioassay. The studies reported in this chapter evaluated existing WHO assay methods and explored adaptations to these standard methods, using both resistant and susceptible strains of *An. gambiae* and *An. funestus*. Some of the key implications of these results are discussed below.

3.6.1 Current WHO cone bioassay

The first objective was to evaluate the effectiveness of the current standard WHO cone bioassay method for the determination of performance of a selection of LLINs, based on their ability to knock down and/or kill insecticide susceptible and resistant populations.

Performances of WHO cone bioassays to measure the bioefficacy (the efficacy of the material in a biological environment) of LLINs were examined. Standard WHO methodologies were used to compare the response of a range of *Anopheles. spp.* to LLINs from different manufacturers (Section 3.5.2.1, Figure 3-3). Two of the four LLINs tested met WHO criteria for knockdown and/or mortality when tested against susceptible strains, these were PermaNet and Olyset Duo, while neither Duranet nor Olyset met the WHO criteria for the susceptible strains of *An. gambiae*. The reasons for the poor performance of Duranet in this study are unknown; the concentration of insecticide found in the Duranet was low, but within the acceptable range after HPLC analysis, however the net was old (removed from original packaging >1 year previous). KD and mortality results against Olyset were low for an unwashed, new net (removed from the original packaging 1 week prior to testing) containing the expected concentration of insecticide.

Numerous studies have reported on the effectiveness of Olyset nets in both laboratory and field experiments, and cone test results have been highly variable. While some studies showed Olyset nets (new and used) are highly effective at killing mosquitoes in field trials (Sharma et al. 2006; Ansari et al. 2006; Malima et al. 2008; Sharma et al. 2009; Dev et al. 2010; Soleimani-Ahmadi et al. 2012) and laboratory/experimental hut studies (Jeyalakshmi et al. 2006; Maxwell et al. 2006; Atieli et al. 2010; Sood et al. 2011); other studies show reduced mortality (<80%) in field trials of new and used nets against susceptible strains (Tami

et al. 2004; Lindblade et al. 2005; Itoh 2005; Dutta et al. 2014; Dennis J. Massue et al. 2016;; Boussougou-Sambe et al. 2017). Interestingly, studies where Olyset net was efficient at killing, occurred mainly on the Indian-subcontinent where *An. gambiae s.l* is not present, while studies revealing reduced mortality were carried out mainly in Africa, where *An. gambiae s.l.* mosquitoes predominate.

3.6.2 Current alternatives to the standard WHO cone

bioassay?

The second objective was to investigate alternative methods to the cone bioassay with a view to improve the knock-down and/or killing ability of LLINs, against susceptible and resistant mosquito strains.

Assays were performed with either five or ten mosquitoes per cone. Disturbance from other mosquitoes in the cone assay could potentially result in reduction in net contact and subsequent killing effect. It was shown that the number of mosquitoes tested did not have a significant impact on KD and mortality (Section 3.5.2.2, Figures 3-4 & 3-5). Next, a modified alternative to the WHO cone assay, the adapted wire-ball assay, was tested. Susceptible Kisumu mosquitoes were unable to escape contact with the treated surface, resulting in 100% mortality after 3 min exposure to Olyset net. Although this study found a marked improvement in KD and mortality using the ball method to test PermaNet against the insecticide resistant Tiassalé strain, mortality against Olyset in this strain was not improved using the modified ball method (Section 3.5.2.3, Figure 3-6).

An earlier study (Maxwell et al. 2006) reported similar results to this study, where both the cone and wire ball assay were used to test Olyset nets by bioassay. Both assays were carried out for 3 min, and the cone killed 92.6% of susceptible *An. stephensi* mosquitoes, while the ball killed 99.6%. The mortality seen in the cone differs greatly from results seen against *An. gambiae* strains in this current study, yet the difference between mortalities in the Maxwell study were deemed significant. The authors noted it took 449.7s until knock-down in the cone and 334.8s in the ball – a significant difference between the two methods ($p < 0.001$). In another study (Okumu et al. 2012), authors carried out both cone and wire-ball bioassay tests on the same nets monthly for six months, using susceptible *An. arabiensis* mosquitoes and noted all nets performed better on wire frames than on cone assays. Only Olyset induces contact irritancy

3.6.3 Modified video cone bioassay

The third and fourth objective of this chapter was to determine the effectiveness of the novel video cone bioassay as a tool for studying mosquito behaviour at the LLIN surface and assess the repellent and/or contact irritancy quality of an insecticide, and determine which variable (net, strain) has significant influence time spent on the net surface.

Video recordings of mosquito activity within the cone, with different treated material and mosquito populations, were made to describe the effects of insecticides on mosquito movement and behaviour. In general, on both untreated nets and LLINs, all mosquitoes (*i.e.* both susceptible and resistant strains) spent more time on the net than either in flight or resting on the cone, during the 3 min assay (Section 3.5.4, Figure 3-10). This pattern of behaviour was evident when exposing mosquitoes to PermaNet and Duranet. However, susceptible mosquitoes exposed to the Olyset net exhibited reduced net contact and increased flight activity. Using GEE analysis (Appendix 6), the effect of the treatment on contact with the net varies with type of strain as the interaction between strain and net is highly significant ($P < 0.0001$).

The mortality of susceptible mosquito strains was reduced after exposure to Olyset which may be a consequence of the reduced net contact observed (Section 3.5.2.1, Figure 3-3). Interestingly, as mentioned at the beginning of this discussion, reduced mortality in susceptible strains was also seen against Duranet. Video frequency observations of activity on the Duranet surface (see Appendix 3) were not noted to be different from the baseline activity seen against untreated netting or PermaNet, where mosquitoes spent more time in contact with the net. Rather than changes in mosquito behaviour explaining reduced mortality, this observation suggests that the actual Duranet used in these experiments may have contained sub-optimal concentrations of insecticide at the net surface.

Grieco et al. (2007), proposed a new terminological scheme that uses 'contact irritant' and 'spatial repellent' to refer to chemicals, that, by virtue of uptake by touch or odour, respectively, cause mosquitoes to sit apart from the source of stimulation. Their results are based on stimulus-dependent insecticide avoidance involved in contact irritancy rather than spatial repellency (Miller et al. 2009). Miller et al. propose new terms, engagement and disengagement, which apply to chemicals that, by their effects on locomotion, increase or decrease contact with the source of stimulation, respectively. Mosquitoes in the Miller study showed similar disengagement responses to Olyset net as those reported in this chapter (Section 3.5.4.3.2, Figure 3-14).

In the experiments reported here, susceptible mosquitoes exposed to Olyset nets showed increased flight activity, suggestive of either a spatial repellency or a contact irritant effect of permethrin. A spatial repellent would result in continued increased flight activity even when direct contact with Olyset was prevented. With Ngousso, time on the net increased in the non-contact experiments (Section 3.5.7, Figure 3-20) compared to the direct contact experiments (Section 3.5.5, Figure 3-16), yet with Kisumu, although more time was spent in contact with the net in the non-contact assay compared to direct contact, there was still no obvious preference for either flight or net (Section 3.5.7, Figure 3-20). However, this non-preference for either flight or net activity was also seen against PermaNet and untreated net in both susceptible and resistant strains. It is essential to carry out non-contact experiments using known spatial repellent pyrethroids, such as Transfluthrin/DDT, to evaluate their effect on mosquito behaviour, compared with results seen in this study.

Varying modes of action of the insecticides incorporated into the LLINs tested in this study explain the differences in observed behaviour. Pyrethroid insecticides affect both the peripheral and central nervous systems of insects (Soderlund et al. 2002). Type I pyrethroids (permethrin) are generally good knockdown agents due to their ability to induce repetitive firing in axons, resulting in restlessness, un-coordination and hyperactivity followed by prostration and paralysis. Type II compounds (e.g. deltamethrin, alpha-cypermethrin) cause a pronounced convulsive phase that results in better kill. The differing physiological effects of these chemicals are explained by the fact that the duration of the modified sodium currents by Type I compounds lasts only milliseconds while those of type II compounds last for several seconds or longer.

This difference between type I permethrin (Olyset) and type II deltamethrin (PermaNet/Duranet) corresponds with the differences in behaviour observed in this study. The type I insecticide results in more hyperactivity than Type II, as shown by the response of exposure of susceptible mosquitoes to Olyset. This hyperactivity may lead to a sub-lethal dose of the insecticide, therefore lower mortality than expected. Indeed, a comparative study (Hougard et al. 2003) measured irritancy and mortality by cone bioassay of pyrethroid nets using pyrethroid-susceptible and pyrethroid-resistant strains of *An. gambiae* and *Cx. quinquefasciatus*. They suggest that within a WHO cone, a high irritant effect can considerably reduce tarsal contact with treated netting material, even with forced contact under WHO cones showing only 62% mortality in the case of permethrin. In contrast, the response to type II insecticides in this study resulted in prolonged periods of net contact and subsequent higher mortality.

Susceptible strains in this current study were not irritated by type II pyrethroids, and instead, spent time on the net probably because they were intoxicated. Previous research (Grieco et al. 2007) proposed that if mosquitoes are intoxicated at concentrations lower than that required for a behavioural response, then toxicity supersedes other actions since the insect might be overcome before being stimulated through mechanisms of contact irritancy. Type II pyrethroids exhibit this toxic effect. Likewise, if a contact irritant response occurred more quickly than a toxic response, the contact irritant mechanism could function to preclude toxicity by causing the insect to move away from the chemical prior to acquiring a lethal dose, as with type I pyrethroids. The results reported in this study suggest that the irritant effect of Olyset took effect before the toxic effect could incapacitate the susceptible *An. gambiae* strains. Conversely, the differing response of the susceptible *An. funestus* strain to Olyset where mosquitoes remained in contact with the net, suggests this fragile strain was overcome by toxic effects before the irritant behavioural effect could take place (Section 3.5.8, Figure 3-21).

Similar results emerged from a study (Siegert et al. 2009) which showed susceptible mosquitoes exposed to Olyset nets had reduced landing attempts and elevated frequency of flight within 3 min compared to PermaNet. They also showed that their resistant strain landed more frequently on the Olyset net, like the findings seen in this study. Their results showed no evidence of pre-contact repellency; all effects emerged after fabric contact. The authors suggest that when contact-induced changes in mosquito behaviour were relatively delayed, the mosquito is typically knocked down or acquires a lethal dose, *i.e.* after contact with PermaNet. Yet, when contact-induced changes in behaviour occur early, the mosquito disengages from the fabric and thus lethality is lost, as seen with Olyset.

WHO recommends using cone assays to measure 'time to first take off' (TTFTO) to detect irritant or excito-repellent properties (WHO 2006). The method involves releasing single mosquitoes and recording the number of take-offs from the net surface and the cumulative flying time within the 3 min assay and has been used in various studies (Hodjati & Curtis 1997; Chandre et al. 2000; Hodjati et al. 2003; Hougard et al. 2003). A recent study (Kawada et al. 2014) demonstrated TTFTO revealed significant differences in the number of take offs observed in the *An. gambiae*, *An. arabiensis*, and *An. funestus* strains after exposure to Olyset and Permethrin papers compared to control tests. With the number of take-offs in these colonies significantly increased after exposure to permethrin 0.75% paper and an Olyset Net, the authors concluded that permethrin was repellent, but their methodology could not distinguish between repellency and irritancy.

In the current study, the Olyset Duo combination net showed higher KD and mortality than observed in the Olyset nets. A previous study (Koffi et al. 2015) exposed both susceptible *An. gambiae* mosquitoes to an Olyset net vs Olyset Duo net in cone bioassays resulting in 22% and 100% mortality respectively. Another study in Benin (Ngufor et al. 2014), showed improved efficacy of Olyset Duo versus Olyset, with overall mortality increasing from 27% to 50% for resistant strains of *An. gambiae* in experimental hut trials. Although both Olyset and Olyset Duo nets have the same concentration of permethrin, the bleed rate (rate of release from the net fibres to the surface) of the insecticide is reportedly higher in Olyset Duo *i.e.* the amount of chemicals on the yarn surface are greater in Olyset Duo, and thus, the effect of blood-feeding inhibition and the lethal effect induced by permethrin is greater than those in Olyset Nets (Ngufor et al. 2014; Koffi et al. 2015). A study (Djenontin et al. 2015) noted increased killing effect of permethrin when it was used in combination with pyriproxyfen on Olyset Duo relative to the Olyset Net for pyrethroid resistant *An.gambiae*. The authors suggest two hypotheses which might explain this pattern: either there is a difference in the bleed rate of permethrin in the combination LN relative to the Olyset Net or an additive/synergistic interaction between permethrin and pyriproxyfen. They suggest it is also possible that the presence of PPF on Olyset Duo interacts with the irritancy and/or repellence property of permethrin, thereby allowing mosquitoes to rest for longer thus picking up higher doses than Olyset Net alone. Despite this, in the current study, 24 h mortalities with Olyset Duo were still below WHO thresholds for resistant strains (Section 3.5.2.1, Figure 3-3).

3.6.4 Effect of the presence of a host

The fifth objective of this chapter was to examine variations in observed mosquito activity between susceptible and resistant individuals, after direct and non-direct contact with the LLIN surface, in the absence and presence of a host. GEE analysis (Appendix 6) shows a significant statistical difference in the time spent on the Olyset net in the presence of a host compared to the absence of a host, in both susceptible strains ($p < 0.0001$). While the presence of a host, did not influence time spent on the Olyset net with IR strains, Tiassale ($p = 0.533$) and VK7 ($p = 0.588$).

With the susceptible Ngousso strain, in the presence of a host, there was an increase in contact time on the Olyset net compared to contact time without the host. This increase in contact time resulted in a significant increase in both KD and mortality (section 3.5.6.1). An early study (Miller & Gibson 1994) looking at the behavioural response of insecticide

susceptible host seeking *An. gambiae* to permethrin impregnated netting, using baited tunnel assays, found that permethrin irritated mosquitoes, causing them to spend significantly more time away from the netting and relatively more time walking than at rest when they were on the netting. The authors concluded that mosquitoes are so strongly attracted to a host protected by netting, they will tolerate relatively high dose of irritating insecticides long enough to pick up lethal doses. The results of this study corroborate their findings where KD and mortality increased in the susceptible Ngousso strain in the presence of a host. Surprisingly, although Kisumu experienced increased contact time with the net in the presence of a host, this did not result in increased mortality, suggesting that the lower mortality observed with Olyset, is not solely a result of lower contact time with the net in this particular strain.

The reduction in flight duration when mosquitoes were exposed to Olyset net in the presence of a human host, observed in the current study, are opposite to the effects seen in a study by Siegert et al. (2009), where experiments with a nitrile-gloved hand or a bare human hand showed mosquitoes were very sensitive to the Olyset net in the presence of a bare human hand. The authors state that contact with the Olyset net reduced the frequency of mosquitoes landing at both host cue strengths (bare human hand and gloved human hand) compared with mosquitoes exposed to untreated net. Their explanation for increased disengagement/flight was that either mosquitoes temporarily stopped responding to the host, due to inhibition of chemosensory reception and loss of response to cues as a sublethal neurotoxic effect, or they were irritated after contact with the insecticide. They did not test mosquito behavioural response to Olyset in the absence of a host. The authors favour the loss of responsiveness to host cues mechanism because of the elevation of normal flight activity in the absence of a host. In the present study, attraction to a host appears to outweigh the effects of contact irritancy previously observed in the susceptible strains, in the absence of a host.

3.6.5 Absence of evidence for contact irritancy in IR mosquitoes

One of the key findings from the present study was that resistant strains of *An. gambiae* did not exhibit contact irritancy after exposure to Olyset nets seen in the susceptible strains (Section 3.5.4, Figure 3-14). This reduction in response to permethrin in resistant mosquitoes may be due to the presence of *kdr* mutations, as has been previously proposed (Chandre et al. 2000; Kawada et al. 2014). Insensitivity to the spatial repellent Transfluthrin has been

linked to reduced insecticide susceptibility and increased *kdr* allele frequency in *Ae. aegypti* and the authors suggested that *kdr* might also decrease excito-repellency behaviours (Wagman et al. 2015). In the results reported here, increased 'repellent' activity (defined as increased flight compared to net contact) was seen in individuals lacking *kdr* (*i.e.* the susceptible strains of *An. gambiae*). Therefore, as neither insecticide susceptible nor resistant *An. funestus* possess *kdr* (Irving & Wondji 2017), these strains would be expected to behave like insecticide susceptible *An. gambiae* strains and spend little time on Olyset nets. Instead they behaved like insecticide resistant *An. gambiae*, spending most of their time on the net. The susceptible Fumoz strain possibly became intoxicated before they could escape net contact, while the survival of the resistant Fang mosquitoes illustrates the efficiency of metabolic mechanisms to process insecticides.

Reduced irritability to permethrin has also been observed in pyrethroid resistant *An. stephensi* (Hodjati & Curtis 1997; Hodjati & Curtis 1999; Hodjati et al. 2003). In this species, even though the resistant strain spent longer on the net, it did not accumulate a sufficient dose of insecticide to kill it, even when offered a simultaneous blood meal (which increased contact time). Resistant strains spent between 1.7 to 2.2 times longer resting on the net than the susceptible strain, showing evidence for less irritability with the resistant strains. During long periods standing on the treated netting, the resistant mosquitoes fed as much, or more than the susceptible mosquitoes, and eventual mortality was less with the resistant strain (Hodjati et al. 2003).

3.6.6 How much exposure to an LLIN is required to kill a mosquito?

Timed cone bioassay experiments were performed to determine the point at which, during the 3 min assay, susceptible mosquitoes become intoxicated by insecticide (Section 3.5.3). Cone bioassays with shortened exposure times showed that it took less than a 1 min for LLINs to intoxicate susceptible mosquitoes (Section 3.5.3.2, Figures 3-8 & 3-9). After only 30s, contact with Olyset Duo and PermaNet gave 100% KD and mortalities of 100% and 93% (± 6.6 SEM) respectively. KD was slower against Olyset than all other LLINs, showing only 60% KD after 30s, compared to over 90% for the other LLINs, increasing to 93% (± 6.6 SEM) after 180s exposure, yet mortality to Olyset was only 20% (± 11.5 SEM). These findings correspond with the reduced mortality ($2\% \pm 2$ SEM) seen in Kisumu against Olyset already discussed in previous experiments (Section 3.5.2.1, Figure 3-3).

3.6.7 Can the cone bioassay be improved?

The cone bioassay is a tool used for investigating the bioefficacy of an LLIN in the laboratory. It is quick, easy, requires low numbers of mosquitoes and allows for tight control around experimental conditions and mosquito movements. The presence of a host influences knock-down, mortality and behaviour, as shown in this study. LLINs are designed to protect a host so a host is always present. Results in this study show improved performance of nets with host present. The method is straightforward and could, therefore, be modified to include a human in the standard WHO cone bioassay to evaluate insecticide efficacy. If the assay is used to look at behaviour of mosquitoes and their interaction with a host baited LLINs in the field, then the use of a host would be critical to provide more realistic outputs.

The cone assay may not act as a suitable proxy for effectiveness of all LLINs in the field. This is particularly true for insecticides that have an excito-repellent effect, such as permethrin. It could be argued that the test is useful against non-repellent insecticides, but it is not practical to actively exclude certain insecticides based on their repellent qualities, especially with the need to assay the effectiveness of new active ingredients coming into the vector control arena.

This study has shown the behaviour of mosquitoes within the cone affects net contact time, resulting in variable knock-down and mortality results. Modifications of the cone assay, such as the ball assay, ensures greater net contact and would, therefore, produce more reliable data. This assay is however cumbersome, it requires larger samples of netting and a wire cage structure which is not widely available. The standard WHO plastic cone is, however, widely available and cheap.

WHO guidelines for testing mosquito adulticides (WHO 2006) feature an alternative method for testing LLIN efficacy; WHO test tubes (cylinders) to test net samples. The netting material is attached to a piece of paper of the same size as a WHO test paper (12 x 15 cm), before insertion into the tube. Mosquitoes are introduced from the holding chamber and exposed for 3 min before being blown back into the holding chamber. Further work comparing this tube method with the recommended cone method would be very useful, especially to investigate effects of mortality and behaviour against Olyset. The confines of the tube may result in less opportunity to avoid net contact apart from the wire mesh ends of the tubes, which will act as an escape from the treated net sample.

In WHO procedures, it is standard for LLINs that do not meet the threshold criteria in cone bioassays to be evaluated using an alternative assay; the tunnel test (WHO 2013c). The main drawback for the tunnel test is the need to use an animal as the host. This has practical and ethical issues. The assay is carried out over a 12 h period which is uncomfortable for the animal. In this assay, the mosquitoes have access to a blood meal and this may impact mortality. The test does however investigate effects of the LLIN on blood feeding inhibition, rather than simple mortality, and thus provides additional information on net performance. It is, therefore, important to ensure the cone test remains a viable option as an effective, useful bench-top assay.

3.6.8 Limitations

The WHO cone bioassay is a standardised method to evaluate LLIN performance, therefore for the first aim of this chapter, it would have been preferable to follow exactly WHO procedures for sampling LLINs where 50 mosquitoes are exposed to one large piece of LLIN from four different nets (WHO 2013c). However, in this study, only a single new net of each type was available for all experiments, with 25 mosquitoes tested per net, using one technical replicate on each of the five net sections. Another improvement would be to ensure that treatments and controls were always conducted simultaneously for all experiments; statistical comparisons were not possible for all experiments as different sources of mosquitoes were used. It is important to note, however, the addition of the video component to the assay changed the aim of experiments from a test of net efficacy to an exploratory, feasibility study, where standard operating procedures had yet to be established.

This current study has shown that in the presence of a host, permethrin loses some of its 'repellency' action, and the Olyset net is ineffective at knocking down and killing both insecticide susceptible and resistant strains alike. Physical qualities of the Olyset net may account for assay effectiveness, *i.e.*, the large hole size of Olyset nets created problems, especially in the host assay, mosquitoes passing through the holes and escaped the assay. This was also seen in Chapter 2 with the repeated exposure/feeding assays. Other authors have noted the large pore size and the problems it causes in the laboratory (Ansari et al. 2006; Maxwell et al. 2006). For the cone bioassays this could be solved by ensuring the single layer of netting is very loosely attached to the plastic sheet, so the net is not stretched or including a fine inert mesh in the apparatus set up, behind the Olyset net, to prevent escape,

it is important however to ensure the mesh does not have an impact on mosquito behaviour, or by having 2 layers of netting to make it problematic for the mosquitoes to pass through.

The manufacturers of Olyset state that even though Olyset has a larger than average mesh size to improve ventilation, field data has shown that penetration of the net by mosquitoes is rare (Olyset Technical Brochure, Sumitomo Chemical Co, Ltd, 2013). Firstly, they state that Olyset Net contains the insecticide permethrin, which is highly repellent to insects. As mosquitoes reach the net, they detect the permethrin and are repelled. Secondly, they suggest it is impossible for mosquitoes to pass through the mesh without touching the net (Figure 3-23), in doing so, they receive a lethal dose of insecticide which prevents biting and leads to rapid knockdown and kill.

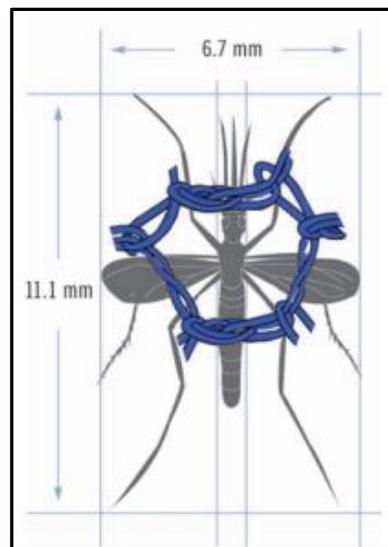


Figure 3-23 A diagram to illustrate the size of Olyset net compared with an *Anopheles* mosquito. Manufacturers state it is impossible for mosquitoes to pass through the mesh without touching the net (Image modified from Olyset technical brochure 2013, Sumitomo, Japan).

The inability of the experimental design to continuously track movement of individual mosquitoes was another limitation. However, reducing the time intervals to <5 seconds may not improve results as our study showed mosquito behaviour was generally static, and it was unlikely activity would change significantly if observed at higher frequencies. It would be useful to document the frequency of ‘mosquito to mosquito’ contact, as it was observed in the video footage in this current study, that movement in one mosquito may be initiated by another mosquito. It is possible the movement ‘frenzy’ within the cone may have a strong impact on collective behaviour, especially in the presence of an attractive host.

Using an alternative, more comprehensive event-logging software package (e.g. BORIS Behavioral Observation Research Interactive Software) would be useful to save time on processing and presentation of results. Finally, when measuring time in contact with the net

it was not possible to distinguish whether the mosquitoes were resting because they were intoxicated or because they were unaffected by the presence of insecticide; however, this could only be inferred from the subsequent mortality results.

3.7 Conclusion

The objective of the WHO cone test is to force mosquitoes into contact with the netting to assess whether they absorb sufficient quantities of insecticide from the net within the specified exposure period to meet the knockdown/mortality thresholds. The test assumes that the period of contact with the net is constant for all net types and mosquito populations. Results from this chapter demonstrate that this assumption does not always hold true.

Video analysis was able to determine a difference in behaviour of phenotypically different mosquitoes towards different insecticide treated netting. Importantly the results provided clear evidence for a different entomological mode of action of Olyset and PermaNet LLINs and showed that the excito/repellent properties of permethrin can be ameliorated by IR and/or the presence of a host.

This current study highlights the potential of the cone bioassay to provide further information on the behavioural responses following net exposure. The final experimental study detailed in the next chapter will take an even closer look at the 'micro behaviour' of mosquitoes at the LLIN interface whilst taking a blood meal and, investigate possible variation in behaviour as a consequence of the IR trait.

4 Investigating feeding behaviour of susceptible and resistant strains of *Anopheles gambiae* at the LLIN interface

4.1 Introduction

In chapter 3, responses to insecticide after direct contact with treated netting were shown to be significantly impacted by IR phenotype, LLIN net type, and the presence of a host. In this chapter, the mosquito - host interaction in the presence of an LLIN is scrutinised, by further observing mosquito behaviour during a blood feed at the net interface.

To achieve this, a novel behavioural assay which is small, portable and simple, has been developed. The 'Thumb test' is a bench-top test devised to examine detailed events during approach, landing, blood feeding, departure and associated behaviours at the bed net surface, using video tracking. In addition, with infra-red lighting - which is invisible to the mosquito (Gibson 1995) - we can observe behaviour under complete darkness, mimicking mosquito host-seeking and feeding conditions in the wild.

The Thumb test records behaviour within a 10x10x10cm cube of transparent Perspex chamber using a simple video camera and macro lens. The events are clearly defined and the start and endpoints rigorously demarcated. This simple video system uses a human hand as bait and can be adjusted to allow test mosquitoes to blood feed or not, with the host present as an attractant in all cases. The test can be run by a single operator that also functions as bait. These bioassay systems facilitate rapid evaluation and quantitative characterisation of vector control treatments for insecticidal and/or repellent properties.

Mosquitoes use a variety of senses to locate potential sources of a blood meal and host-location behaviour is divided into 3 phases, which include long-range, middle-range, and short-range (Gibson & Torr 1999; Day 2005). In this chapter, short-range cues are under investigation. Once the mosquito is close enough to touch the host, these short-range cues include vision, heat, sound, and olfaction (Khan & Maibach 1966; Takken & Knols 1999; Zweibel L.J. and W. Takken 2004). Host temperature and tactile chemical cues on the host's skin (Takken & Knols 1999; Verhulst et al. 2010) help the mosquito to identify the host, initiate probing, and start blood feeding (Bowen 1991; de Jong & Knols 1995). A recent study (Won Jung et al. 2015) showed mosquitoes target an optimal site on the host, in order to

penetrate the skin and blood vessels without alerting the host animal, by use of olfactory receptor neurons located in sensory hairs contained in the piercing structure of the mosquito mouthpart (Service 2008).

In the study reported here, the behaviour of insecticide susceptible (Kisumu) and resistant (Tiassalé) strains of *An. gambiae* were investigated during exposure to a pyrethroid treated net (PermaNet 2.0) and untreated control. In addition, behaviour of young, 3-5 d old, insecticide resistant mosquitoes was compared with older (17-19 d old) individuals of the same strain, as aging insecticide resistant mosquitoes have been shown to lose their IR status (Rajatileka et al. 2011; Chouaibou et al. 2012; Christopher M Jones et al. 2012).

4.2 Aims and Objectives

This study aimed to demonstrate host seeking behaviour of *An. gambiae* at an LLIN where infra-red video camera systems were used to observe the final sequence of behavioural events during landing and blood feeding on human blood through an LLIN.

The specific objectives of this chapter were to:

- Evaluate the effectiveness of the novel thumb assay to provide a basic description of feeding behaviour of individual mosquitoes at untreated net interface.
- Compare behaviour at untreated netting with behaviour at an LLIN net interface.
- Determine difference in behaviour at the LLIN net interface between insecticide susceptible and insecticide resistant strains.

4.3 Materials and methods

4.3.1 Mosquito colonies

An insecticide susceptible (Kisumu) and insecticide resistant (Tiassalé) strain of *An. gambiae* were used in this experiment. All mosquitoes tested were female, non-blood fed and aged 3-5 d (both Kisumu and Tiassalé) or 17-19 (Tiassalé only) d post emergence. Mosquitoes were reared, maintained and tested in conditions previously described in chapter 2 (Section 2.3.1).

4.3.2 Long lasting insecticide treated nets

Untreated nets and PermaNet 2.0 were used in this experiment. Insecticide activity was determined by HPLC analysis as described in chapter 3 (Section 3.5.1). One piece (5cm x 5cm) of netting from each net was used for all tests.

4.4 Experimental design

Assay apparatus (Figure 4-1) consisted of the test chamber: measuring 10cm x 10cm x 10cm, it consists of 3 sides of opaque plastic, 2 facing sides of transparent plastic and one side untreated netting, thumb and entrance ports cut into opposite sides of the chamber. Once assembled, the chamber was held together with rubber bands and positioned so the camera can view mosquito activity through one side while illuminated from the other side. The thumb rested against either untreated or treated netting positioned in the front thumb port to provide a constant blood meal source for the assay duration (bottom left and right). A view from within the chamber is seen in the bottom right.

The chamber was placed centrally, supported by bricks and aligned with the camera and light source. The camera was positioned 30cm to the right side of the chamber while the acrylic diffuser was positioned 40cm to the left of the chamber. The LED light source was placed 40cm behind the diffuser (Figure 4-2).

Distinct behavioural events of individual mosquitoes were quantified during blood feeding on a human host through either untreated or treated netting. Experiments were carried out in an LSTM insectary and each mosquito was released into a test chamber (designed and constructed at LSTM) and allowed to fly freely with access to a blood meal from a human host. All experiments were carried out in complete darkness and recorded with a video camera under infra-red light. Assays were performed after the first hour of the scotophase.

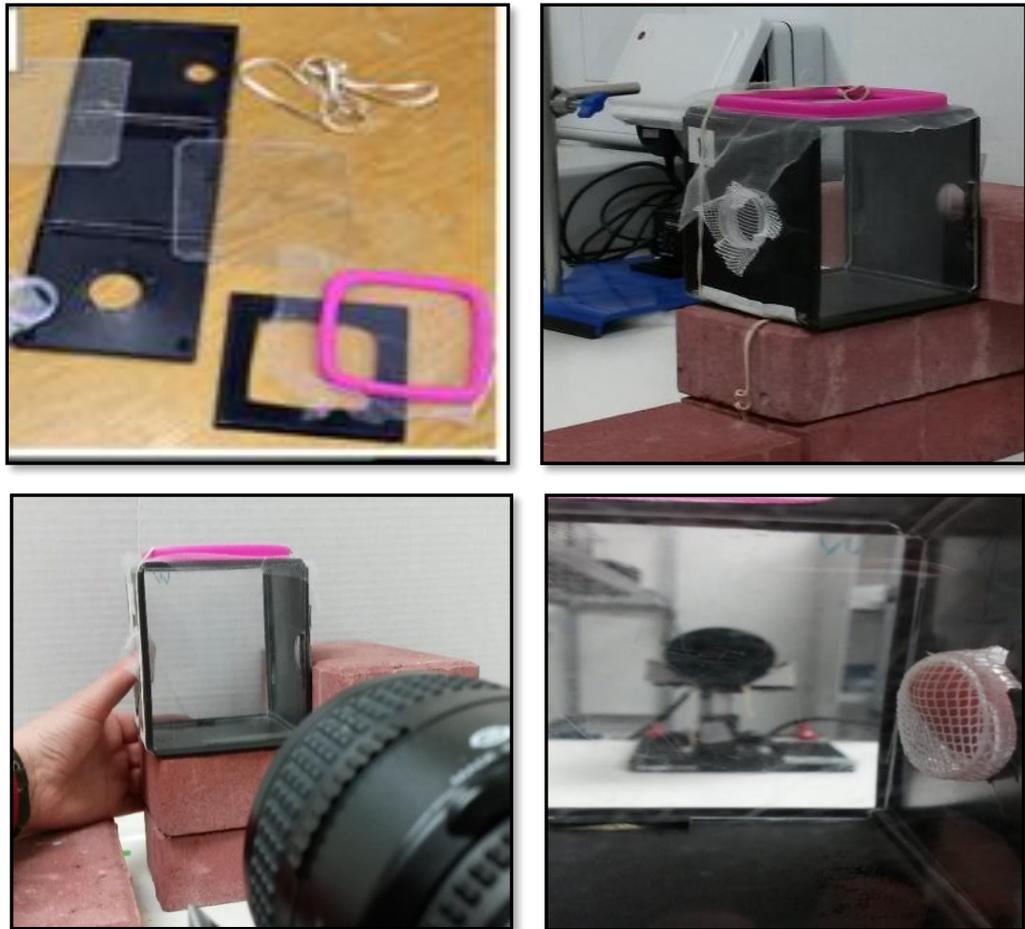


Figure 4-1 Photographs of thumb assay experimental components showing unassembled test chamber components (top left) and assembled chamber (top right) showing the thumb/net port (front) and mosquito entry port (rear). The thumb rests can be seen directly behind netting in the feeding port (bottom left and right). Transparent plastic facing sides of the chamber allow activity within the chamber to be illuminated using an LED light source (not shown) and video recorded via camera (bottom left and right).

The video recording camera equipment comprised a near infra-red enhanced camera (MQ013RG-E2, Lambda Photometrics Ltd, UK) with lens (Nikon AF Micro NIKKOR 60mm f/2.8D) and stand. The LED light source (M850L3, ThorLabs Ltd, UK) delivered light at a wavelength of 850nm. A 20cm x 20cm acrylic sheet (Amazon.co.uk) was used to diffuse the LED light. The camera was connected to a gaming laptop (Schenker XMG P722, Germany) and images were captured at 30 frames per second using commercial digital video recording software (StreamPix v.5, Norpix, Canada). All data were stored on a 3TB hard drive (Toshiba Canvio HDWC130EW3J1, Amazon.co.uk).

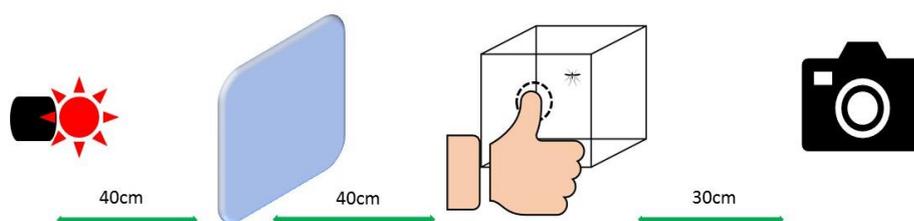


Figure 4-2 Photograph and diagram of thumb assay experimental set up showing alignment of experimental set up for the thumb feeding assay, with camera + lens (far right), testing chamber and supporting bricks (middle), acrylic diffuser sheet suspended using clamps (left) and LED Infra-red-light source suspended using clamps (far left).

4.4.1 Thumb test procedure

4.4.1.1 *Young mosquitoes – Kisumu and Tiassalé*

To investigate whether behavioural responses to an insecticide treated net varies with mosquito age, two ages of mosquitoes were tested. WHO tube bioassays (WHO 2016b) using Deltamethrin (0.05%) papers were carried out to investigate whether there was a difference in mortality seen between the two populations (Appendix 4).

Starved of sugar 3 h prior to testing, ten mosquitoes were removed from stock cages and transferred into 125ml cups covered with untreated net. At the start of testing, individual mosquitoes were removed from the cup by mouth aspiration into the test chamber. Video recording was started, and the thumb was placed behind the netting. The progress of the recording was viewed on the laptop which was shrouded in heavy duty black-out material – to prevent light contamination that might influence the mosquito’s behaviour.

Keeping their thumb motionless at the test net surface, the observer sat for up to 20 min or less if the mosquito had fed and departed from the thumb before then. Fed mosquitoes were gently removed and placed into a separate paper cup, with 10% sugar solution provided and after 24 h, mortality was recorded. This procedure continued until approximately 20 mosquitoes had successfully fed per treatment group. Individuals which did not appear in

the recorded field of view within 5 min (no show) or did appear but failed to feed (unfed) within 10 min (some of these mosquitoes landed and probed but did not ingest blood within 10 min) were classed as non-responders and were discarded.

4.4.1.2 Older mosquitoes – Tiassalé only

Only the resistant strain was tested to determine whether IR reduced with age. Following the same procedure as described in Section 3.1, a sample of older (17-19 d old) non-blood fed female mosquitoes (n = 10) of Tiassalé strain against both LLIN types, were tested.

4.5 Behavioural characteristics

Video recordings were processed manually in real-time playback and closely analysed using slow motion. Table 4.1 shows nine strictly defined and distinct behavioural events (Abe *et al*, in preparation) which were observed, and time of occurrence recorded. These times were converted from mm:ss format into total seconds which was converted into duration of each event. Figure 4-3 shows three photographic examples of event observations seen on the video footage; event no. 5 (from probing to insert); event no.6 (insert to start feeding visible) and event no. 7 (feeding before diuresis).

Table 4-1 Thumb assay behavioural events showing nine strictly defined behavioural events which were observed and recorded during each video recording of a successful feed through either untreated or treated netting in the thumb feeding assay. The events appear in order of occurrence based on expected/anticipated blood feeding behaviour of the mosquito. Events defined by Abe *et al* (in preparation).

No.	Event	Definition
1	Until appearance	Arrives in camera field of view but does not land on net
2	Appearance to contact	Touches net but does not land, wings do not stop
3	Contact to landing	Legs contact net, wings stop
4	Landing to start point of probing	Tilts forward pushing proboscis into net
5	From probing to insert	Insertion of proboscis into thumb, stops probing
6	Insert to start feeding visible	Abdomen starts expanding
7	Feeding before diuresis	Continued feeding before blood droplet appears at the tip of the abdomen
8	Feeding with diuresis until withdrawal	Appearance of blood droplet at the tip of the abdomen, tilts forward and pulls proboscis out of thumb, proboscis visible above netting
9	Resting on net before departure	Flies or walks off net, losing all contact and leaves field of view



Event no. 5) Probing to insert



Event no. 6) Insert to start feeding visible



Event no. 7) Feeding before diuresis

Figure 4-3 Photographs of mosquito silhouette images taken during thumb assay showing examples of three behavioural events: number 5) 'from probing to insert', number 6) 'insert to start feeding visible' and number 7) 'feeding before diuresis'. Widening of the mosquito abdomen is seen blood feeding progresses. Images stills taken from video footage during analysis.

4.6 Data analysis

Mean activity for all net types were determined by two-way ANOVA (Tukey's multiple comparisons test). Analyses were performed using GraphPad Prism Version 7.03 (GraphPad Software Inc.). Further analysis, using multivariate models, will be carried out for future publications on this assay (Hughes *et al* 2019 (Appendix 2) and Guy *et al* 2019: In Prep).

4.7 Results

Thumb assay behavioural responses are illustrated in stacked bar charts below. Grey shades show activity pre-net contact. Periods of feeding (while in contact with the net) are shown by orange through to dark red and periods of net contact (after feeding) are shown in green.

4.7.1 Baseline activity of *An. gambiae* mosquitoes blood feeding at an untreated net surface

Times spent approaching, landing and feeding for individual (n=20) insecticide susceptible (Kisumu) and insecticide resistant (Tiassalé) *An. gambiae* mosquitoes at the untreated net interface, are shown in Figures 4-4 and 4-5. After feeding, 50% (10/20) Kisumu and 65% (13/20) Tiassalé remained resting on the net until the maximum test time of 20 min.

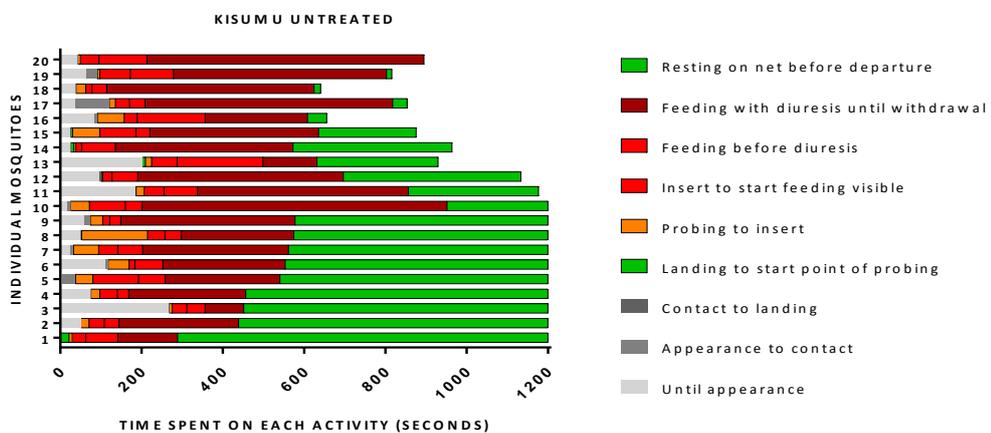


Figure 4-4 Stacked bar chart of thumb assay behavioural responses (Kisumu - untreated) showing time spent on each defined behavioural event (see colour key) after exposure of insecticide susceptible *An. gambiae* Kisumu strain individuals (1-20) to untreated netting up to a maximum of 20 min (1200s) using the thumb box test system. Each row, indicating an individual mosquito, are ranked based on resting time for ease of visualisation.

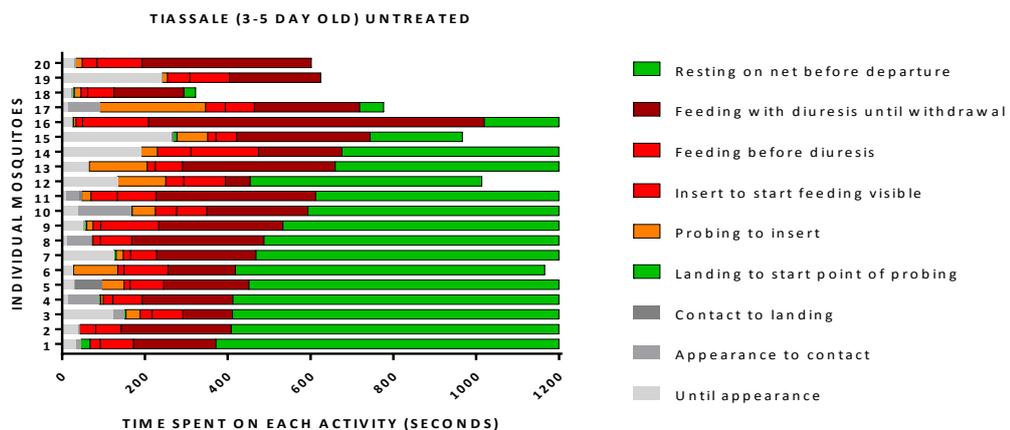


Figure 4-5 Stacked bar chart of thumb assay behavioural responses (Tiassalé- untreated) showing time spent on each defined behavioural event (see colour key) after exposure of young (3-5 d old) insecticide resistant *An. gambiae* Tiassalé strain individuals (1-20) to untreated netting up to a maximum of 20 min (1200s) using the thumb box test system. Each row, indicating an individual mosquito, are ranked based on resting time for ease of visualisation.

4.7.2 Activity of *An. gambiae* mosquitoes blood feeding at PermaNet LLIN surface

Times spent approaching, landing and feeding for individual (n=20) insecticide susceptible (Kisumu) and insecticide resistant (Tiassalé) *An. gambiae* mosquitoes at the PermaNet interface are shown in Figure 4-6 and 4-7. With access to the host, mosquitoes readily fed through an LLIN, although the duration of blood feeding and resting time on the net was markedly reduced compared to untreated nets. In contrast to the untreated net, only 5% (1/20) individuals from each strain remained on the net for the 20-min assay time.

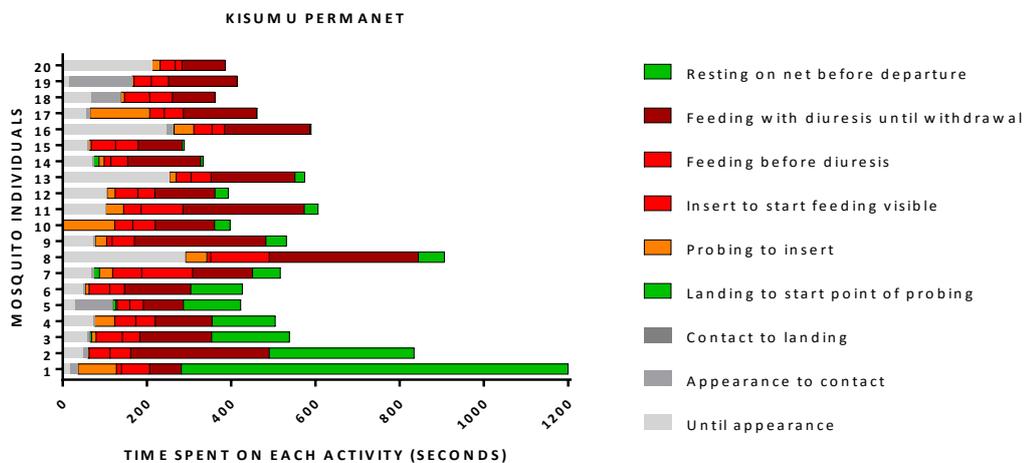


Figure 4-6 Stacked bar chart of thumb assay behavioural responses (Kisumu - PermaNet) showing time spent on each defined behavioural event (see colour key) after exposure of insecticide susceptible *An. gambiae* Kisumu strain individuals (1-20) to PermaNet netting up to a maximum of 20 min (1200s) using the thumb box test system. Each row, indicating an individual mosquito, are ranked based on resting time for ease of visualisation.

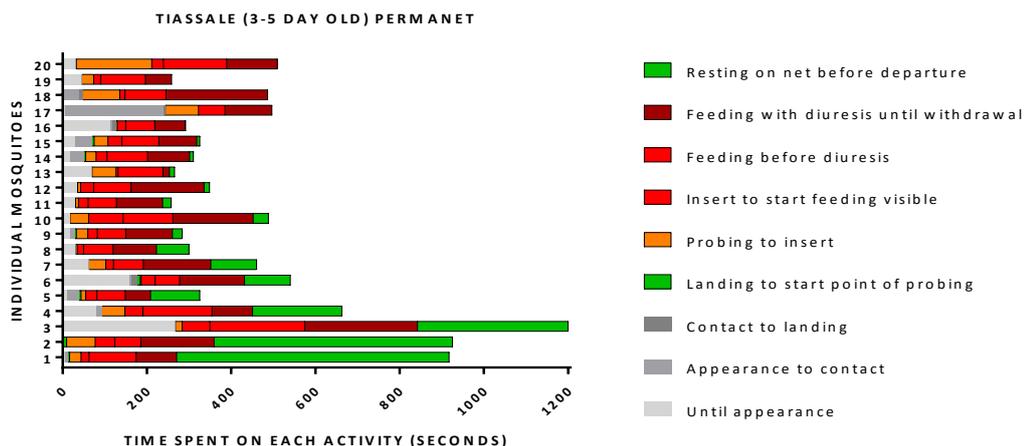


Figure 4-7 Stacked bar chart of thumb assay behavioural responses (Tiassalé - PermaNet) showing time spent on each defined behavioural event (see colour key) after exposure of young (3-5 d old) insecticide resistant *An. gambiae* Tiassalé strain individuals (1-20) to PermaNet netting up to a maximum of 20 min (1200s) using the thumb box test system. Each row, indicating an individual mosquito, are ranked based on resting time for ease of visualisation.

4.7.3 Activity of older *An. gambiae* at untreated and treated net surfaces

To verify predicted reduction in insecticide susceptibility due to increased age, WHO tube bioassays performed on the two different aged cohorts of the Tiassalé strain (Appendix 4) showed 18% (± 2.63 SEM) mortality in younger mosquitoes compared to 55% (± 5 SEM) in older individuals, revealing a significant increase (T test $p < 0.0017$) in susceptibility as mosquitoes aged.

Times spent approaching, landing and feeding for individual young ($n=10$) and old ($n=10$) insecticide resistant (Tiassalé) *An. gambiae* mosquitoes at the both untreated net and PermaNet interface are shown in Figure 4-8. Up to 40% of older insecticide resistant individuals remained on untreated netting (lower Section) for the maximum assay time, while none remained on PermaNet (upper Section) over the same period. Again, reduced time spent blood feeding was observed when individuals successfully fed through the PermaNet.

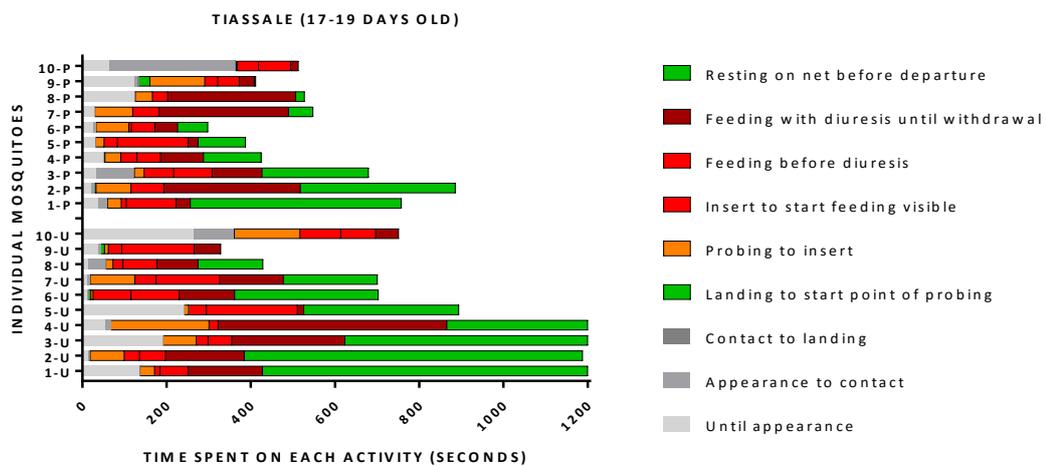


Figure 4-8 **Stacked bar chart of thumb assay behavioural responses (Tiassalé - PermaNet)** showing time spent on each defined behavioural event (see colour key) after exposure of older (17-19 d old) insecticide resistant *An. gambiae* Tiassalé strain individuals to untreated (U1-10) and PermaNet (P1-10) netting up to a maximum of 20 min (1200s) using the thumb box test system. Each row, indicating an individual mosquito, are ranked based on resting time for ease of visualisation.

4.7.4 Mean activity for all net types and mosquito strains

Results reveal no difference in times of feeding behaviour between insecticide susceptible and resistant strains, only between treatments (Figure 4-9 and Appendix 5). Insecticide susceptible (Kisumu) individuals spent a similar amount of time on each behavioural activity as resistant (Tiassalé) individuals when exposed to untreated netting or PermaNet. Young (3-5 d) and old (17-19 d) insecticide resistant individuals spent a similar amount of time on each activity when exposed to PermaNet.

When comparing differences in feeding behaviour between treatments, insecticide susceptible (Kisumu) individuals and (young) insecticide resistant (Tiassalé) individuals spent significantly less time ‘feeding with diuresis until withdrawal’ ($p < 0.0001$) and ‘Resting on net before departure’ ($p < 0.0001$) through the untreated net than PermaNet. These differences in blood feeding and resting/net contact duration are examined in detail below.

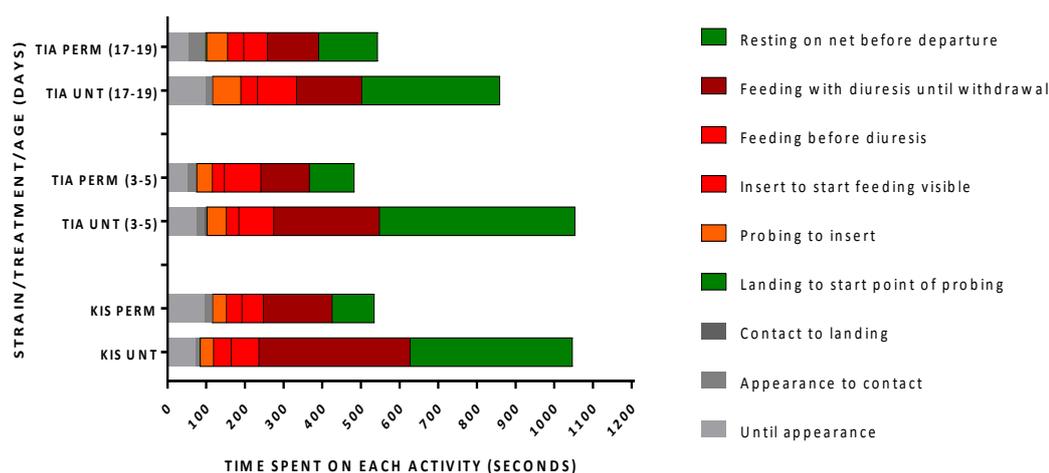


Figure 4-9 Stacked bar charts showing mean time of each defined behavioural event (see colour key) after exposure of insecticide susceptible *An. gambiae* Kisumu strain individuals (n=20), young (3-5 d) (n=20) and older (17-19 d) (n=10) insecticide resistant *An.gambiae* Tiassalé strain individuals to untreated and PermaNet netting up to a maximum of 20 min (1200s) using the thumb box test system.

4.7.4.1 Blood feed duration

Total time spent blood feeding (combining three-blood feeding behavioural events "insert to start feeding visible", ‘feeding before defecation’ and ‘feeding with defecation until withdrawal’) are illustrated in Figure 4-10. There was a statistically significant decrease in total time spent blood feeding after exposure to untreated net versus PermaNet, in both insecticide susceptible ($p < 0.0001$) and resistant ($p = 0.013$) individuals. There was however no such difference between treatment observed with the older Tiassalé mosquitoes. There is no difference in blood feeding duration between strains when Kisumu and Tiassalé (both ages) feed through untreated net or PermaNet.

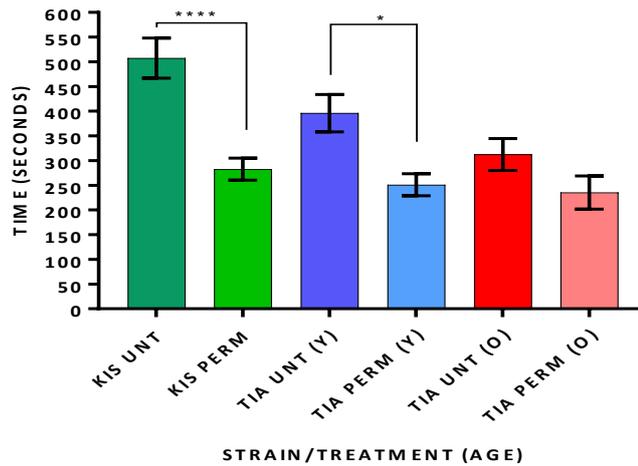


Figure 4-10 Bar chart showing mean time spent blood feeding (combined behavioural events: insert to start feeding visible; feeding before defaecation; feeding with defaecation until withdrawal) in insecticide susceptible *An. gambiae* Kisumu strain individuals (n=20), young (3-5 d, n=20) and older (17-19 d, n=10) insecticide resistant *An. gambiae* Tiassalé strain individuals to untreated (UNT) and PermaNet (PERM) netting using the thumb box test system. ****p<0.0001, *p<0.05.

4.7.4.2 Net contact duration

Total time from all behavioural events involving net contact (excluding ‘until appearance’ and ‘appearance to contact’) were combined to investigate observed differences in total time spent in contact with the net, depending on treatment (Figure 4-11). After exposure to untreated net versus PermaNet, there is a significant difference between total time spent in contact with the net for insecticide susceptible and both aged resistant individuals (p < 0.0001 (young), 0.03 (old), with mosquitoes spending less time in contact with treated nets.

Insecticide susceptible *An. gambiae* spend 0.45x less time in contact with PermaNet (430±52) than untreated netting (965±49s), while insecticide resistant *An. gambiae* spend 0.43x times less in contact with PermaNet (411±57s) compared to untreated netting (958±62s). Older mosquitoes spent 0.56x less time in contact with the LLIN compared to untreated netting (447±68s vs 800± 128s respectively).

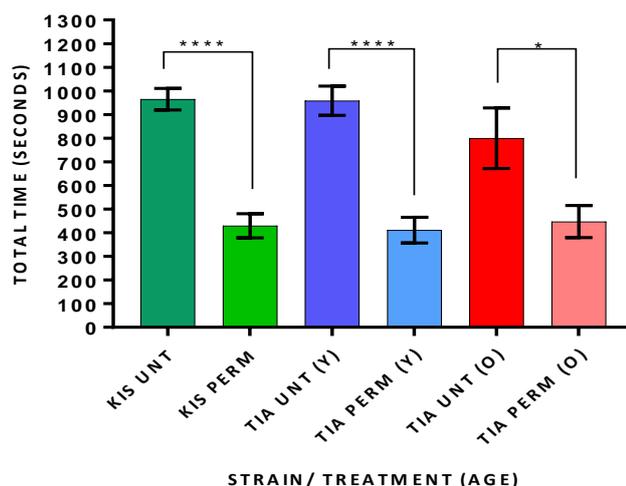


Figure 4-11 Bar chart showing mean time spent in contact with the net (combined behavioural events excluding until appearance and appearance to contact) in insecticide susceptible *An. gambiae* Kisumu strain individuals (n=20), young (3-5 d, n=20) and older (17-19 d, n=10) insecticide resistant *An. gambiae* Tiassalé strain individuals to untreated (UNT) and PermaNet (PERM) netting using the thumb box test system. ****p<0.0001, *p<0.05

4.7.5 Responders and survivors per experiment

The number of no-shows (mosquitoes which did not appear in the video recording field) within 5 min or those individuals which probed but remained unfed within 10 min, against both treatments are shown in Table 4-2. A total of 77% of susceptible Kisumu strain, responded against untreated net and 60% against PermaNet. Against untreated and PermaNet respectively, young Tiassalé gave a 67% and 60% response rate, compared to older Tiassalé of 83% and 53%. Remarkably, just over half Kisumu survived 24 h later (49% mortality) after feeding through PermaNet. There was zero mortality against untreated netting for both strains and Tiassalé against PermaNet.

Table 4-2 Responders and survivors in the thumb feeding behaviour experiment of insecticide susceptible (Kisumu) and young and old insecticide resistant (Tiassalé) *An. gambiae* individuals after exposure to untreated and PermaNet, in the presence of a host using the thumb box test system. 'No shows' refers to mosquitoes which did not appear in the video frame within 5 min. 'Unfed' refers to those individuals which were visible but did not successfully feed within 10 min. 'Fed' refers to those individuals which successfully fed during and up to 20 min. Percentages of total mosquitoes tested are shown. Young are 3-5 d, old are 17-19 d.

train	Kisumu		Tiassalé (Young)		Tiassalé (Old)	
	Unt n (%)	Perm n (%)	Unt n (%)	Perm n (%)	Unt n (%)	Perm n (%)
No show (≤ 5 min)	2 (8)	7 (21)	4 (13)	3 (9)	0 (0)	5 (26)
Unfed (≤ 10 min)	4 (15)	6 (18)	6 (20)	10 (30)	2 (17)	4 (21)
Fed (≥ 20 min)	20 (77)	20 (61)	20 (67)	20 (61)	10 (83)	10 (53)
Total (n)	26	33	30	33	12	19
% 24h Mortality	0	49	0	0	0	0

4.8 Discussion

This study aimed to demonstrate host seeking behaviour of *An. gambiae* at an LLIN where infra-red video camera systems were used to observe the final sequence of behavioural events during landing and blood feeding on human blood through an LLIN. Using a simple bench top video assay, in darkness under infra-red lighting, the approach, feeding and resting behaviour of individual *An. gambiae* mosquitoes to human bait, the effectiveness of the novel thumb assay to provide a basic description of feeding behaviour of individual mosquitoes at untreated net interface was evaluated.

By observing specific events at the LLIN interface compared to untreated net, the evidence shows that susceptible and resistant mosquitoes readily feed through an LLIN, though duration of blood feeding and resting was reduced in both strain types when feeding through an LLIN compared to an untreated net (Section 4.6.4, Figure 4-9). Significant differences in the behaviour between susceptible or resistant populations were not detected. Yet, responses of each strain significantly varied between net type. Some of the key implications of these results are discussed below.

4.8.1 LLINs reduce net contact and feeding times compared to untreated netting

In the presence of a human host, mosquitoes spent significantly more time in contact (feeding and resting upon the net before alighting) with untreated netting compared to PermaNet. Contact with treated nets cuts feeding time by half (Section 4.6.4.2., Figure 4-11). A behavioural study (Siegert et al. 2009) shows susceptible *An. gambiae* mosquitoes interaction with LLINs fitted over a human hand resulted in reduced number of visits to the host, compared to untreated netting. The authors showed reduced frequency of contact with a LLIN (PermaNet) over time, compared with mosquitoes interacting with an untreated net. Their observations of mosquitoes interacting with LLINs established that both disengagement (due to temporary loss of host response) and lethality cause reductions in visits to the host. Interestingly, they showed mosquito recovery to full-host seeking response within 24 h, meaning mosquitoes are available for biting the next day. Another study (Cooperband & Allan 2009) looked at the landing durations of different species of mosquito, using video recordings of behaviour within a glass chamber, in which a general trend of greater landing duration and total contact times on untreated surfaces compared with treated surfaces for all species and insecticides (Bifenthrin, Deltamethrin and Lambda-cyhalothrin) in agreement with our observations of reduced resting times on LLINs.

4.8.2 Susceptible and resistant mosquitoes successfully feed and rest in a similar way

Insecticide resistant mosquitoes behave in the same way as insecticide susceptible mosquitoes in their quest for a blood meal. Our study shows no significant difference in patterns of approach, probing, feeding or resting behaviour in Kisumu and Tiassalé mosquitoes after exposure to treated netting, compared to untreated (Section 4.6.4, Figure 4-9). Both strains spent similar time in contact with the net with both treatments and there was no significant difference in response rate. Nearly 50% of Kisumu survived 24 h after feed through PermaNet, compared to 100% in Tiassalé.

These results suggest that although they are likely to die after contact with insecticide, the susceptible mosquito will feed regardless of the danger of contact with the LLIN. An early study (Hossain & Curtis 1989) allowed insecticide susceptible *An. gambiae* mosquitoes to feed on a human arm through pieces of impregnated nylon net and those mosquitoes which fed through the treated net, many survived for 24 h. When feeding through a net with a similar concentration to a commercial LLIN, *i.e.* permethrin dose of 0.8g/m^2 , the authors showed that 40% of *An. gambiae* mosquitoes fed and survived. Olyset netting used in this current study contained 0.95g/m^2 permethrin and although Olyset was not used in the thumb experiments, further thumb experiments carried out using Olyset have shown a 44% mortality after feeding (Amy Guy, unpublished data). A dose of 2.5g/m^2 was required to completely prevent feeding through permethrin-impregnated nylon netting on a human arm (Hossain & Curtis 1989).

Comparing the response of *An. stephensi* to three pyrethroids (Hodjati et al. 2003) showed the resistant strain, when in contact with treated fabric covering a human arm, spent a longer time in contact with the fabric than the susceptible strain. However, they did not find a significant difference in the percent of mosquitoes which fed through the treated net between resistant and susceptible strains, corresponding with our findings.

The experiments in this chapter measured the responses of one susceptible and one resistant strain and it is possible that different responses might be observed between resistant strains with different mechanisms. The Tiassalé strain possesses *kdr* and metabolic IR mechanisms, 1014F *kdr* allele and *ace-1*, Elevated P450s: CYP6M2, CYP6P3, CYP6Z2/3 (Edi et al. 2014). Observations seen in the thumb assay may vary with differing IR alleles and further experiments on different resistant strains is warranted.

4.8.3 Older resistant mosquitoes do not behave differently from younger mosquitoes.

It was shown by WHO tube bioassay that there was an increase in mortality associated with age in our Tiassalé population (Section 4.6.3). However, immediate (24 h) mortality and pattern of individual mosquito behaviour in older mosquitoes was similar to behaviour in younger mosquitoes (Section 4.6.5). Older mosquitoes have been shown to lose resistance to insecticides as they age (Lines & Nassor 1991; Mourya et al. 1993; Rajatileka et al. 2011; Chouaibou et al. 2012; Jones et al. 2012). In this study, as no difference in feeding behaviour between resistant and susceptible individuals was seen, it is perhaps not unexpected that there was no difference in behaviour in older mosquitoes from the resistant strain compared to their younger counterparts.

4.8.4 Limitations

The thumb experiment was used to directly observe, video record and illustrate the feeding behaviour of mosquitoes, at the net interface, under laboratory conditions. How this translates into behaviour in the wild is unknown. An early study (Hossain & Curtis 1989) showed that when a human arm was pressed against a whole permethrin impregnated net with within a room, *An. gambiae* released mosquitoes failed to feed through the net. They suggested the free-flying foraging behaviour takes longer than that seen in the laboratory, with multiple net encounters, leading to uncoordinated and unsuccessful blood feeding. Mosquitoes in this current study were able to orientate towards the thumb with relative ease, both strains showing a 61% feeding rate.

Sublethal exposure to neurotoxic compounds can negatively affect sensory organs and reduce efficiency of host location in different mosquito species (Lee W. Cohnstaedt & Allan 2011). Flight tracks of host-seeking female *Cx. quinquefasciatus*, *An. albimanus*, and *Ae. aegypti* in a wind tunnel were video-recorded to compare activation of host-seeking and patterns of flight orientation to host odours. During host-seeking flights, all three-mosquito species differed significantly in-flight duration, velocity, turn angle, and angular velocity. Mosquitoes were then exposed to sublethal levels of pyrethroid insecticides to evaluate the effects of the neurotoxicants 24 h post-exposure. Significant reductions in time of activation to flight and flight direction were observed in mosquitoes exposed to deltamethrin and permethrin. Additionally, pesticide-treated *Cx. quinquefasciatus* mosquitoes flew

significantly slower, spent more time in flight, and turned more frequently than untreated controls.

This study looked at the micro-behaviour of mosquitoes at the net interface from within the confines of a small box and cannot be directly compared with results in wind tunnels/room assays. Close-range experiments (Siegert et al. 2009) allowed insecticide susceptible and resistant *An. gambiae* mosquitoes to voluntarily visit a PermaNet and Olyset mitten-covered hand, and found mosquito behaviour was modified such that the frequency of landings diminished, flying increased, and knockdown and lethality accordingly were reduced compared with mosquitoes interacting with untreated fabric. Mosquitoes landed sooner and more frequently in the presence of strong host cues (*i.e.*, bare hand versus nitrile-gloved hand). Disengagement with the fabric-covered hand occurred more abruptly and earlier with Olyset Net than with PermaNet, where the decline in response to the host was more gradual. The authors propose one mechanism underlying the observed disengagement is temporary loss of the ability to sense host cues. The results presented here suggest this is not always the case, as shown by increased disengagement from the Olyset net in the cone experiments, excluding a host, in Chapter three.

Previously, video mosquito landing observations have used wind tunnel tests (Healy & Copland 1995; Healy et al. 2002; Lacey & Cardé 2011; Spitzen et al. 2013; Spitzen et al. 2014; Lee W Cohnstaedt & Allan 2011) and glass chamber assays (Cooperband & Allan 2009), but there have been very few studies which successfully focused on the behaviour and responses, of insecticide susceptible and/or resistant mosquitoes, whilst feeding through treated nets (Hossain & Curtis 1989; Hodjati et al. 2003; Agramonte et al. 2017).

To visualise the movements of mosquitoes around a whole bed net, recent studies (Parker et al. 2015; Angarita-Jaimes et al. 2016; Parker et al. 2017) use a novel tracking system to observe free-flying *An. gambiae* insecticide susceptible mosquitoes in response to human-occupied nets. They showed total flight and net contact times were lower at LLINs than untreated net, but the essential behaviour of the response remained unaltered. These findings corroborate with our direct observations at the net surface

The thumb test needs to be carried out on additional strains of susceptible and resistant mosquitoes to improve the data set. It is important to carry out more replicates on each strain. Further investigations to quantify a range of delayed or sub-lethal effects on those mosquitoes (including insecticide resistant strains) that survive beyond the 48h period immediately after exposure are currently underway using simple benchtop assays. This will

enable investigators to measure and quantify impacts of insecticide exposure on those mosquitoes that survive beyond the 48h period immediately after exposure that currently is the limit of investigation. Many traits that are key determinants of vectorial capacity, such as adult lifespan and willingness to re-feed, can be influenced by insecticide exposure but these are not recorded in current evaluations insecticide products.

4.9 Conclusion

Precisely how mosquitoes interact with insecticides and how insecticide treatments influence the responses of mosquitoes have never been fully quantified. Moreover, many basic details of the interactions between mosquitoes and insecticides are missing. This current study utilises new technological systems to investigate novel behavioural parameters previously undescribed.

The thumb assay enables the characterisation of responses to insecticide exposure in individual mosquitoes, whilst blood feeding from a host. The assay allows for the comparison of vector control chemistries, the assessment of the impact of IR on the performance of existing products, and the observation any behavioural differences caused by IR traits.

5 General discussion

5.1 Overview

The overall aim of this study was to look beyond the parameters and boundaries of these standard assays, to address gaps in current knowledge on how insecticide exposure and IR might alter behaviour and/or affect lifelong fitness of malaria mosquitoes. The evidence used to test the null hypothesis that the impact of insecticide exposure on mosquito populations is confined to acute toxicity and there are minimal long-term consequences to fitness or variation in behaviour for mosquitoes surviving this exposure, is presented below.

Current approaches for determining the IR status of mosquito populations and the effectiveness of vector control interventions targeting them, include two simple bench top bioassays, the WHO Cone test and Tube test. However, these bioassays do not capture the lifetime impact of IR, nor do they shed any light on behavioural IR. An important novel element that was applied to existing assays in all three experiments, was the incorporation of a human host to more realistically test responses to a human-occupied LLIN.

Firstly, WHO cone bioassays were used to demonstrate that longevity was shortened in resistant mosquitoes exposed to LLINs, and to quantify the magnitude of this previously unreported, delayed mortality effects in resistant mosquito strains. Secondly, analyses of video recorded mosquito behaviour during cone bioassays, showed that mosquito-net interactions and subsequent changes in mosquito behaviour were dependent on LLIN type, mosquito IR status and the presence or absence of a host. Thirdly, a novel benchtop video assay, the Thumb Test, was used to characterise the nocturnal feeding behaviour of mosquitoes at the LLIN interface. Results showed that both insecticide resistant and insecticide susceptible mosquitoes would readily feed, apparently to repletion, through an LLIN. However, the duration of acquiring a blood meal, and therefore time spent in direct contact with the net, was significantly reduced in resistant and insecticide susceptible strains when feeding through treated netting.

Together, the results from all three experimental chapters revealed that IR does influence or alter a mosquito's behavioural responses to an LLIN, with potentially significant costs, the most important of which is a significantly reduced lifespan (delayed mortality effect) following insecticide exposure. Some of the key implications of these results are discussed below.

5.2 Delayed mortality effect

The first objective of this current study was to determine the long-term (beyond first 24 h as adults) effects of IR on mosquito longevity. Therefore, the aims of the experiments in Chapter 2 were to look beyond the first 24 h of the standard WHO cone bioassay to investigate the lifelong impact of insecticide exposure on daily survival, i.e how survivorship after exposure to insecticides change throughout the lifetime of IR mosquitoes as compared to susceptible mosquitoes, and the malaria transmission potential of moderately and highly resistant laboratory populations of the major African malaria vector *An. gambiae*.

Substantial delayed mortality effects were observed after exposure to PermaNet. Typically, under optimal conditions, *P. falciparum* requires a minimum of 9 d and typically ~10-16 d to develop to the infective sporozoite stage in sub-Saharan Africa (Beier 1998; Vaughan 2007). If the reduction in survival recorded in the present study is applied, then moderate and highly resistant mosquitoes exposed to the PermaNet LLIN would not survive long enough to become sporozoite positive mosquitoes capable of transmitting malaria. The exception was the extremely resistant VK7 strain for which no reduction in survival occurred following exposure to PermaNet. Survival of most strains tested was reduced following repeated insecticide exposure, but the magnitude of this shortened longevity was dependent on the exposure regime and the strength of IR trait present in each strain (Section 2.6.3, Table 2-4).

The magnitude of delayed mortality effects has been predicted to diminish as the degree of IR increases (Viana *et al*, 2016), a hypothesis that was reinforced recently by field studies in Burkina Faso (Natalie Lissenden, unpublished data). In those experiments, wild *An. coluzzii* mosquitoes proved to be highly resistant to pyrethroid insecticides, and results of both cone and experimental hut field trials suggested that exposure to LLINs had no significant impact on post-exposure longevity.

In the current study, all resistant female mosquitoes were provided with a sugar source, and in some tests, were blood fed. Mosquitoes that blood fed through untreated nets lived longer than those that did not receive a blood meal during exposure to untreated nets. *i.e.* after exposure to untreated net every 4/5 d, the median survival of the blood fed highly resistant Tiassalé strain was twice that of those mosquitoes fed on sugar alone (30 d compared to 15 d, respectively). Similarly, the median survival of the resistant Tororo strain was 17 d after blood feeding through untreated net, compared to 13 d after feeding on sugar alone.

As well as using protein from blood meals to develop eggs, female hematophagous insects use blood as an energy source for survival and flight (Nayar & Sauerman Jr 1975a). The authors state that blood-fed female mosquitoes survive and performed energy functions, for 3-8 d more than unfed females, depending on the species. Continuous feeding on sucrose solution also provides continuous availability of energy reserves for normal functions such as flight, respiration and metabolism (Nayar & Sauerman Jr 1975b). Previous reports show that when female *An.gambiae* (Gary & Foster 2001) or *Ae. aegypti* (Styer et al. 2007) feed on sugar and blood together, they survive longer than on sugar alone. Blood feeding, and multiple or repeat bloodmeals particularly, has previously been shown to increase longevity in insecticide resistant females (Oliver & Brooke, 2014).

As shown in the current study, when resistant mosquitoes blood fed through LLINs, compared to those exposed to LLINs and sugar fed only, the median survival decreased post PermaNet exposure but increased post Olyset exposure, *i.e.* after exposure to PermaNet, median survival of blood fed Tiassalé mosquitoes decreased from 12 d (sugar fed) to 9.5 d, and median survival of blood fed Tororo reduced from 9 d (sugar fed) to 7 d. Yet, remarkably, after feeding through Olyset net, mean survival of Tiassalé mosquitoes increased from 18 d (sugar only) to 27 d, while blood fed Tororo mosquitoes survived a median 16 d compared to 13 d (sugar only). The reduced LLIN effectiveness seen with Olyset has been demonstrated in both Chapter 2 and Chapter 3 and is discussed below.

5.3 Behaviour near the LLIN surface

The second pair of objectives of this study were to determine the long-term (beyond first 24 h as adults) effects of IR on behavioural responses to contact with LLIN netting, and behavioural avoidance of contact with LLIN netting. During the 3 min bioassay period, susceptible and resistant strains spent significantly more time in contact with PermaNet and untreated net than time in flight ($p < 0.001$). Resistant mosquitoes are less likely to avoid the LLIN compared to susceptible mosquitoes.

However, after exposure to with Olyset, Kisumu and Ngousso strains spent equal amounts of time in contact with the net and in flight, therefore the mosquitoes potentially obtained a reduced dose of insecticide. This behavioural response where mosquitoes show a preference not to rest on the insecticide treated net surface has been previously described (Siegert et al. 2009; Kawada et al. 2014) and suggests a contact irritant quality of the permethrin incorporated in the LLIN.

In this study, mosquitoes that were susceptible to pyrethroids according to WHO tube bioassays were not always killed by a 3-min exposure to an Olyset net. In contrast, these same strains typically exhibited close to 100% mortality 24 h after exposure to PermaNet. The stark difference in immediate and delayed mortality assay outcomes between these two LLINs, led to the experiments described in the second experimental chapter, where video recording revealed a probable explanation why Olyset performed badly in the cone bioassay.

This reduced exposure to Olyset, potentially resulting in a sub-lethal dose of insecticide, may contribute to hormetic effects in the resistant mosquito (Cutler 2013; Guedes et al. 2017). Hormesis is the stimulatory effect associated with low (sublethal) doses of compounds that are toxic at higher doses (Guedes & Cutler 2014). The direct relevance of hormesis to IR is its occurrence in insecticide resistant populations, as demonstrated by deltamethrin resistant weevils (Guedes et al. 2010). The authors show that upon failure of the insecticide to suppress the insecticide resistant populations, sub-lethal field exposure to insecticides not only leads to control failure but boosts the growth of the resistant population. Exposure to sublethal doses can induce production of enzymes that are important in detoxification processes, and induction of esterases (hydrolases), which are important in insecticide metabolism (Guedes & Cutler 2014). Further work looking at this phenomenon in insecticide resistant mosquitoes is warranted.

Interestingly, both resistant strains did not exhibit this same 'irritated' behaviour after exposure to Olyset, as they remained in contact with the netting for much of the assay (Section 3.5.5, Figure 3-15). Typically, during the 3 min exposure period to Olyset net, susceptible strains spend an average of 44% of time in contact with net, compared to 77% in resistant strains. After exposure to PermaNet, susceptible strains spent on average 70% and resistant strains 75%, respectively, of the 3 min in contact with the net, suggesting no such irritation response occurred with this LLIN.

These results suggest the accuracy of the standard WHO cone bioassays will depend on the type of insecticide within the LLIN being tested, rendering it unreliable when used to compare products from different manufacturers. An alternative bioassay method – the modified ball assay – resulted in significantly improved KD and mortality for susceptible strains exposed to Olyset ($p < 0.0001$) (Section 3.5.2.3, Figure 3-6). The ball assay has been shown to be more effective than the cone bioassay method for testing Olyset (Maxwell et al. 2006) but, despite being a WHO recommended assay, is not widely adopted.

5.4 Impact of a host

The third objective of this study was to determine the long-term (beyond first 24 h as adults) effects of IR on behavioural responses to contact with LLIN netting with a host. When a host was present, the susceptible strains spent significantly more time in contact with Olyset net than in the absence of a host (Kisumu $p < 0.0001$, Ngousso $p = 0.034$) (Section 3.5.6, Figure 3-18). Kisumu spent twice the amount of time in contact with the net 137s (± 15.96 SEM) with a host compared to 68s (± 8.1 SEM) without a host. Ngousso spent 130s (± 16.55 SEM) in contact with the net with a host compared to 89s (± 4.3 SEM) without a host. Kisumu and Ngousso spent 76% and 72% of total assay time, respectively, in contact with the net with a host compared with 38% and 49% of assay time in the absence of a host. There was no significant difference in time spent on the PermaNet or untreated netting with/without a host, in both resistant and susceptible strains.

The addition of a host to the cone bioassay resulted in significantly increased knockdown rates for the Ngousso susceptible strain and resistant Tiassalé strain ($p < 0.0001$) (Section 3.5.6.1, Figure 3-19). KD in Ngousso increased from 20 to 96% and 24 h mortality increased from 20% to 96%, while KD in Tiassalé increased from 0% to 59%. The assay outcome for the VK7 strain remained unaffected by the presence of a host. This extremely resistant strain may be a low responder to host cues, however to date, there have been no investigations of the effects of IR on variations in olfactory sensitivity to host cues.

Knock down for Kisumu increased from 52%-65% though this was not significant, and surprisingly, despite a 2-fold increase in mean time spent in contact with the Olyset net with a host compared to without, there was no mortality observed with the Kisumu strain. Whereas, increased net contact in the ball-assay yielded significantly higher mortality to Olyset in the Kisumu strain, yet once again this was not seen in this current study using the WHO cone bioassay, even in the presence of a host.

The fourth objective of this study was to determine the long-term (beyond first 24 h as adults) effects of IR on blood-feeding behavioural response after contact with LLINs. The thumb assay enables quantification of the impact of IR on blood feeding behaviour of individual mosquitoes. Mosquitoes feeding through PermaNet spent less time in contact with the net and fed for a shorter duration than mosquitoes feeding through untreated nets, in both insecticide susceptible and resistant strains (Section 4.6.4.1 & 2, Figures 4-10 & 4-11). A surprising finding was that in the Thumb experiments, the susceptible Kisumu strain successfully fed through a PermaNet with an average feeding duration of 283s, moreover,

less than half were dead 24 h later (Section 4.6.5, Table 4-2). Compared with the timed cone experiments in the absence of a host, the susceptible Kisumu strain suffered 93% immediate (24 h) mortality (Section 3.5.3.2, Figure 3-9) after only 30s in contact with PermaNet, These results highlight the positive effect blood feeding has on insecticide tolerance (Spillings et al. 2008; Oliver & Brooke 2016). An early study (Halliday & Feyereisen 1987) showed the toxicity of topically applied DDT to adult female *Cx. pipiens* decreased very rapidly about 2-fold to a minimum at 24h after a blood meal, then increased within 72 h back to values typical on non-blood fed insects.

Recent research used video-tracking of multiple free-flying mosquitoes responding to human-occupied bed nets (untreated and PermaNet). In laboratory tests, a single *An. gambiae* accumulated 18–96s of PermaNet net contact in a 60 min test (Parker et al. 2015) whereas in the field tests with *An. arabiensis*, the range was 46–82s (Parker et al. 2017). However, mortality was not recorded as it was not possible to recapture all mosquitoes in the experimental hut following tests. In another flight tracking study (Spitzen et al. 2014), the mean time susceptible *An. gambiae* individuals spent in contact with the net (PermaNet) was 70.1s resulting in 1 h knock down and death within 24 h. The Authors showed a positive correlation between the time resting on the net and knockdown/mortality rate.

In both the cone assays and the thumb tests, the presence of a host dramatically affected the behaviour of mosquitoes and, in many cases, significantly altered the test outcomes. Mosquitoes have an innate ‘inner drive’ to find and blood-feed on humans (Dethier 1957; McCall & Kelly 2002) and female mosquitoes use body odour, CO₂, moisture, heat and visual cues to orient towards a host (Gibson & Torr 1999; Zweibel and Takken 2004; Spitzen et al. 2013; Hawkes & Gibson 2016). In some instances, blood feeding (Takken et al. 2001; Qiu et al. 2013), parasite infection (Cator et al. 2012; Smallegange et al. 2013; Gleave et al. 2016), temporal changes (Rund et al. 2016), activation of the mosquito immune system (Cator et al. 2013) and inherited differences in host preference (Zweibel L.J. and W. Takken 2004; Wang et al. 2010; McBride et al. 2014; McBride 2016) can affect olfactory sensitivity of the malaria mosquito to host cues.

5.5 Insecticide bioavailability

Where sufficient contact with treated surfaces was assured, as in the ball bioassays, LLINs killed higher proportions of susceptible and resistant mosquitoes than the cone bioassay (section 3.5.2.3, Figure 3-6). Analysis of video observations of the cone test reveal that mosquitoes can escape LLIN contact altogether by resting on the cone or flying, but within

the ball assay, there is no untreated surface on which the mosquito can rest. This constant contact with insecticide in the ball assay almost certainly accounts for the significantly increased mortality seen in Kisumu on Olyset netting.

Furthermore, in the cone assays, increased flight behaviour was seen among susceptible *An. gambiae* mosquitoes in response to Olyset compared to other LLINs; within the ball assay, this heightened activity may aid to ensure 'all over body' coverage with insecticide, leading to increased insecticide penetration into the mosquito. A study (Aldridge et al. 2016) investigating the impact of topical application site on the efficacy of Permethrin and Malathion against *Cx. quinquefasciatus* showed that insecticide exposure at the primary body, *i.e.*, head, thorax, and abdomen, return much higher mortality from insecticides compared with contact at the appendages, *e.g* legs and wings.

Highlighted in a recent study (Andriessen et al. 2015), is a novel method to test the bioavailability of insecticide particles on the LLIN surface. The authors exposed resistant Tiassalé mosquitoes to insecticide particles which had been bound to a PermaNet LLIN using electrostatic forces. Particle transfer to mosquitoes was visualized by applying fluorescent dust on the electrostatic netting and the quantity of transferred fluorescent particles served as a visual proxy for particle transfer/contamination. Results show that in a standard 3-min WHO cone exposure assay, mosquitoes obtained fluorescent particles across the entire body including tarsi, antennae, proboscis, thorax, and lower abdomen, demonstrating that a substantial dose was transferred from the electrostatic netting to the mosquito; Tiassalé mortality increased from 10% using the non-electrostatic net to 100% using the electrostatic net. The authors attribute the increased mortality with increased all over body contact with the deltamethrin due to the electrostatic coating. An aspect which the authors did not investigate was to contrast levels of contamination from a standard net compared to a charged net, where less fluorescent particle transfer onto the body would be expected.

Similarly, in this current study, constant exposure to PermaNet in the ball assay, compared to the cone assay, resulted in significantly increased KD and mortality in the insecticide resistant Tiassalé strain (section 3.5.2.3, Figure 3-6), showing that increased contact with the deltamethrin impregnated LLIN can affect the mortality of resistant strains. This did not occur against Olyset net, suggesting that increased contact with the permethrin impregnated LLIN is not sufficient to kill resistant mosquitoes.

5.6 Improvements towards a better benchtop assay

This current study has shown the cone bioassay is unreliable when assay outcome, *e.g.* knockdown and immediate mortality of susceptible mosquitoes, is influenced by the irritant/repellency qualities of the active ingredient within the LLIN. Permethrin contained within Olyset has been shown to be a contact irritant which agitates susceptible mosquitoes after initial contact, resulting in reduced contact with the net (Siegert et al. 2009; Kawada et al. 2014).

In WHO practices, it is standard for LLINs that do not meet the threshold criteria in cone bioassays ($\leq 95\%$ KD and $\leq 80\%$ mortality) to be evaluated using an alternative assay: the tunnel test (WHO 2013b). The main drawback for the tunnel test is the need to use an animal as the host which has ethical and practical issues. Moreover, tunnel test assays are carried out over a 12 h period which is both uncomfortable for the animal and slows the testing process, and the mosquitoes have access to a blood meal which, as shown here, can significantly impact mortality. It is therefore important to try to ensure the cone test remains a viable option as an effective, useful bench top assay.

However, the current study suggests a much simpler solution. The presence of a host, in the form of an arm placed behind the board, is sufficient to overcome the contact irritancy induced by permethrin exposure. This modification of the cone assay also provides a better approximation of how LLINs operate by 'attract and kill' (Lynd & McCall 2013; Sutcliffe & Yin 2014; Parker et al. 2015). The presence of a host in the cone bioassays resulted in increased mortality with Olyset. If studies wish to use the results of the cone test as a proxy for effectiveness in the field, the addition of a host to preliminary laboratory experiments should yield more reliable results. Further repeated experiments should be performed to determine the effect of a host on bioassay outcome.

5.7 Limitations of current WHOPES guidelines

Thus far, this study has shown that bioassay testing methods must be tailored to the characteristics and mode of action of each insecticide class, and that, therefore, WHO cone bioassays may not be suitable as a method to evaluate all new LLINs coming onto the market, especially those with hitherto undefined modes of action. However, taking this into account, WHO guidelines state that when testing efficacy of nets with insecticides other than pyrethroids, it may be necessary to modify certain test procedures in Phase I laboratory tests, depending on the mode of action of the new insecticide. For example, for LLINs with slow-acting insecticides, mortality may be recorded 24, 48 and 72 h after exposure (WHO 2013b).

One such product currently proving difficult to test in the cone bioassay is the combination LLIN Interceptor®G2 (alphacypermethrin and chlorfenapyr). Chlorfenapyr is a slow acting insecticide that requires the mosquito to be metabolically active for toxicity to occur (Oxborough et al. 2015). In addition, Chlorfenapyr appears to be more toxic to mosquitoes during exposure at night, when cellular respiration and metabolic activity are at their peak. In cone bioassays, carried out in daylight, the Interceptor G2 LLIN did not achieve the WHO cone test threshold of 80% mortality (N'Guessan et al. 2016b). Instead, tunnel tests performed at night were carried out to demonstrate the threshold of 80% mortality and 90% blood feeding inhibition required by WHO in their LLIN evaluation.

This illustrated the limitations of current WHOPES guidelines (Glunt et al. 2014; Oxborough et al. 2015) which were developed for pyrethroid LLINs (WHO 2006, WHO 2013b). The danger that insecticides that are highly effective against wild mosquitoes would be overlooked at the screening stage of evaluation through bioassay and never progress to field evaluation, has been raised (Oxborough et al. 2015). The authors stated that the current emphasis on Phase I test criteria and thresholds developed and tailored for pyrethroids will not serve for the new classes of insecticide and they recommend a revision of the WHOPES LLIN evaluation guidelines, putting emphasis on tunnel tests which can simulate or allow expression of night-phase, host-seeking behaviour on the net.

This recommendation would apply to active ingredients, such as chlorfenapyr, which are not suitable for testing using a WHO standard cone assay. However, for LLIN chemistries that are not slow-acting, this study has shown that the inclusion of a host in the standard WHO cone assay, may counter problems of poor assay outcome currently experienced in the standard assay when testing active ingredients known to have irritant qualities.

5.8 Current and future work

This thesis investigated aspects of IR trait fitness costs and mosquito behaviour not previously described, the results have identified several important questions meriting follow-up:

Longevity experiments identified the existence of delayed mortality effects at a magnitude that could have significant implications for malaria transmission.

These findings need further validation in wild insecticide resistant populations to assess their relevance to operational control. There are some constraints to testing this hypothesis in the field, namely, difficulties in aging and determining the history of insecticide exposure of wild

mosquitoes. Alternatively, this phenomenon could be investigated under semi-field conditions where wild mosquitoes could be exposed to LLINs under realistic but contained conditions.

Additional potential sub-lethal impacts of insecticide exposure require further investigation

Longevity, rates of re-feeding, egg development, fertilisation, oviposition, hatching rates and impacts on adult progeny after insecticide exposure are currently being measured in follow up studies in the department. In addition, further experiments into fitness costs of IR such as costs to energetic resources, sex ratio, eclosion rates, mating competitiveness, wing length/body size and flight ability could be undertaken.

The relationship between IR, insecticide exposure and ability to transmit the Plasmodium parasite is poorly understood

Laboratory infection experiments incorporating repeated insecticide exposure, would be an optimal way to investigate this question but this is very challenging to do in practice given the risks associated with handling Plasmodium infected mosquitoes. Insecticide exposure has been shown to affect parasite development and significantly reduce the prevalence of *P. falciparum* oocysts in resistant strains of *An.gambiae* (Alout et al. 2014; Kristan et al. 2016). In this current study, the increased survival of resistant mosquitoes due to blood feeding, does not necessarily reflect survival of resistant mosquitoes in the wild. Yet the increased longevity seen after blood feeding may have an impact on transmission (Oliver & Brooke 2014) and studies have shown increased susceptibility to *Plasmodium* in wild insecticide resistant mosquitoes (Alout et al. 2013; Ndiath et al. 2014).

Are cone assay alternatives effective at evaluating LLINs in phase 1 trials?

The wire-ball assay offers considerable benefits as an alternative to the WHO cone assay. It ensures that the mosquito cannot escape net contact, providing a more accurate and reliable response to the insecticides. This method needs to be evaluated and compared against the standard WHO cone assay, using multiple insecticide and resistant strains, against current and emerging LLIN products.

Future work?

The aim of this thesis was to explore procedures to investigate the impact of exposure to LLINs on mosquito survival and behaviour at the net interface. Current (cones) and new

(thumb) bioassay systems were used to investigate novel behavioral parameters that previously were not amenable to observation or quantification. The natural extension of the findings of this current study is to explore the potential to develop behavioural test protocols focusing on vector behavioural responses during interaction with, and sub-lethal or delayed impacts following exposure to LLINs. This work forms part of a BMGF-funded research project at LSTM looking at developing a suite of test protocols to strengthen phase I evaluations and inform optimal deployment of new vector control tools.

Conclusion

This study explored whether bench top assays could be used or adapted to answer key questions on how physiological IR may affect behaviour, and consequently vectorial capacity. The results revealed that mosquito behaviour can affect the outcome of current standardised bioassays, raising questions about their suitability as tools for assessing IR status and for measuring effectiveness of current and new active ingredients. However, this study has shown that both the video cone and the thumb are useful and simple additions to the bench top assay repertoire. The study findings also highlight the importance of investigating the impacts of IR beyond immediate mortality. The existence of previously ignored delayed mortality effects offers an explanation as to why, to date, there have been no reports where malaria has increased due to the impact of pyrethroid resistance on LLIN effectiveness (Strode et al. 2014; Bhatt et al. 2015; Kleinschmidt et al. 2018). Nonetheless, it is possible that delayed mortality effects, or other fitness costs from LLIN exposure, may be reducing the impact of IR on LLIN performance (Kristan et al. 2016; Viana et al. 2016; Alout, Labbé, et al. 2017). However, results seen in this study, using highly resistant populations from Burkina Faso, warn that as the degree of IR increases, the magnitude of these fitness costs may diminish and eventually disappear.

6 References

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7 Appendices

7.1 Appendix 1 - KM curves (chapter 2, experiment 4)

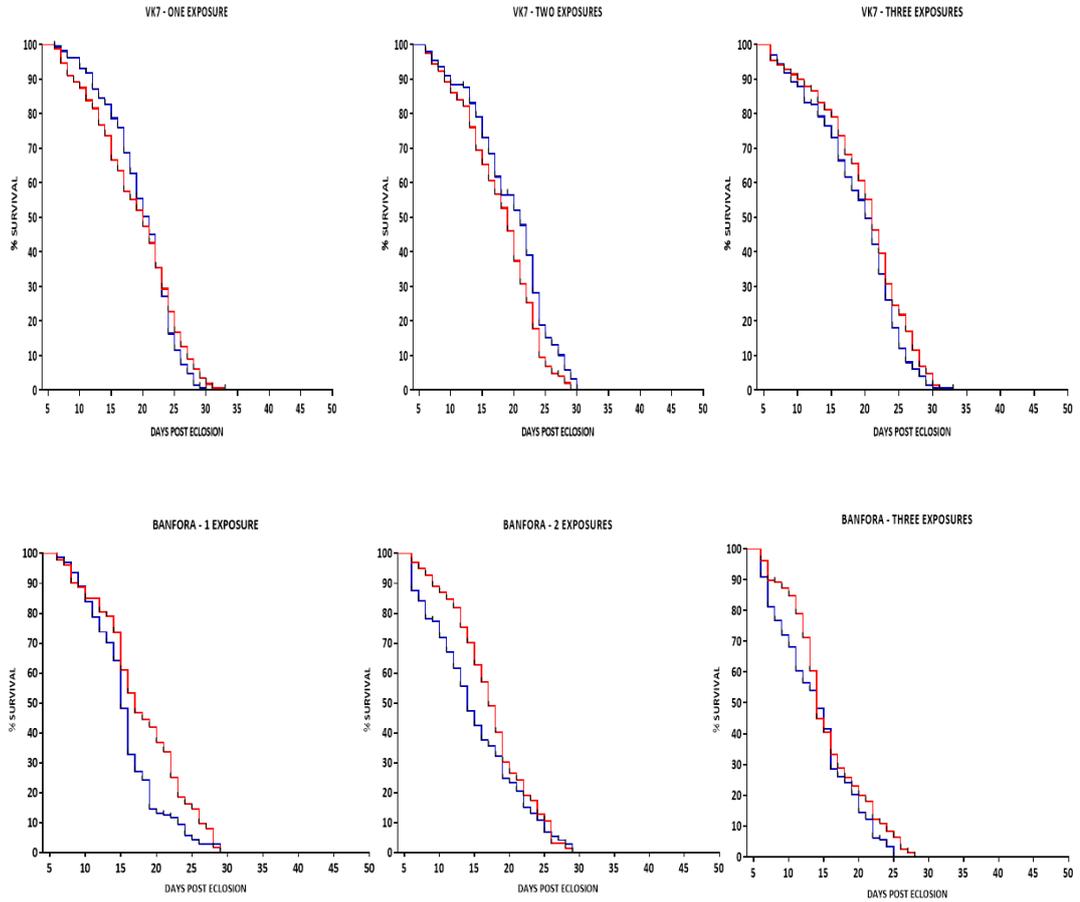


Figure 7-1 Kaplan Meier curves for **experiment 4** showing mean percentage survival for insecticide resistant VK7 and Banfora strains of *An. gambiae*, following all three episodes of exposure to PermaNet (blue line) and untreated net (red line) on days 4; 4,5; or 4,5,6 days post eclosion. Results for two experiments, each with 70 mosquitoes/net/strain were pooled for this analysis.

7.2 Appendix 2 – Published paper and paper in prep.

Viana, M., Hughes, A., Matthiopoulos, J., Ranson, H. and Ferguson, H.M., 2016. Delayed mortality effects cut the malaria transmission potential of insecticide-resistant mosquitoes. *Proceedings of the National Academy of Sciences*, 113(32), pp.8975-8980.

Angela Hughes, Geraldine M. Foster, Mayumi Abe, Agnes Matoke, Natalia Angarita-Jaimes, Amy Guy, Catherine E. Towers, David Towers & Philip J. McCall 2019 The ‘Thumb Test’: a laboratory benchtop test for quantifying mosquito behaviour at the insecticide-treated net interface (in prep)

7.3 Appendix 3 – Frequency charts (chapter 3)

7.3.1 Contact - Untreated

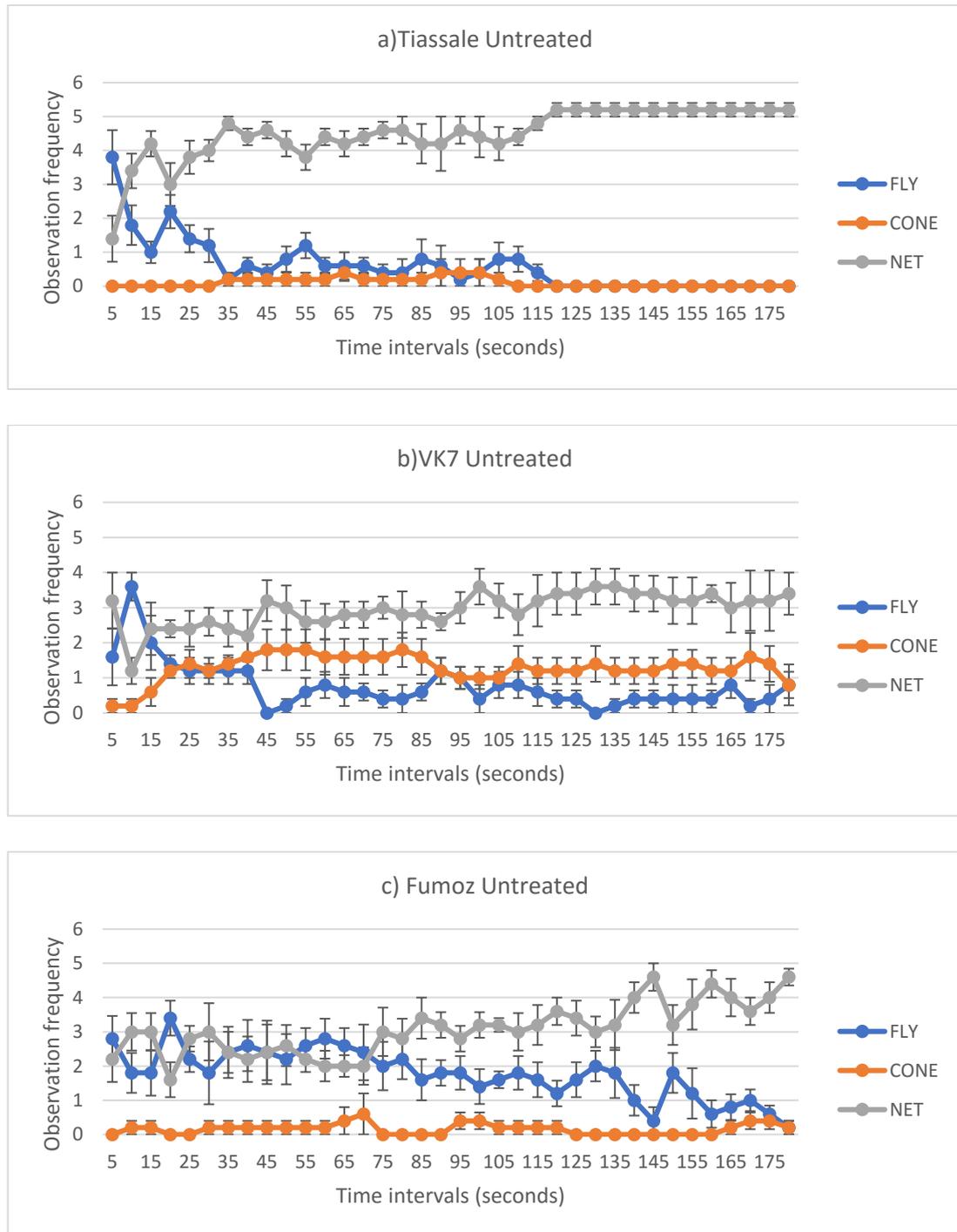
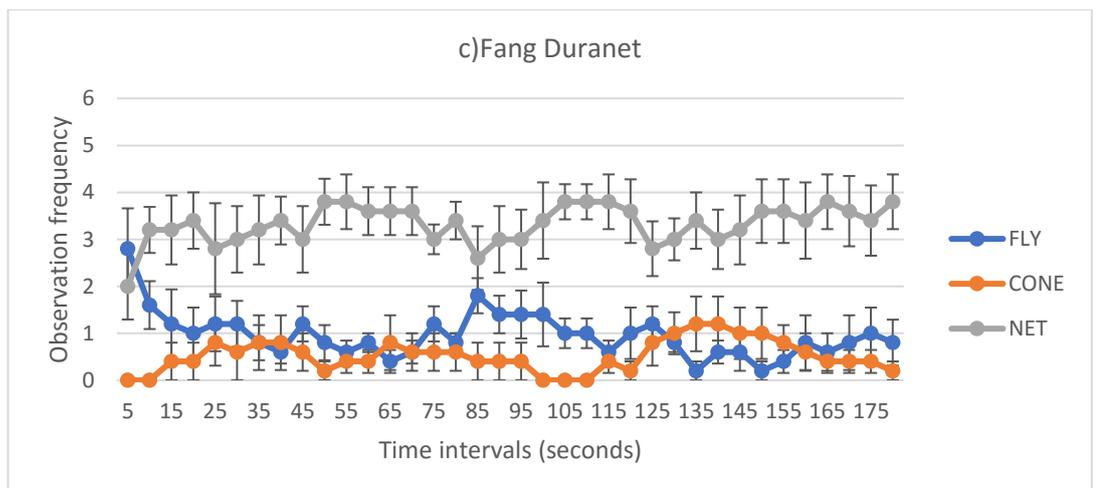
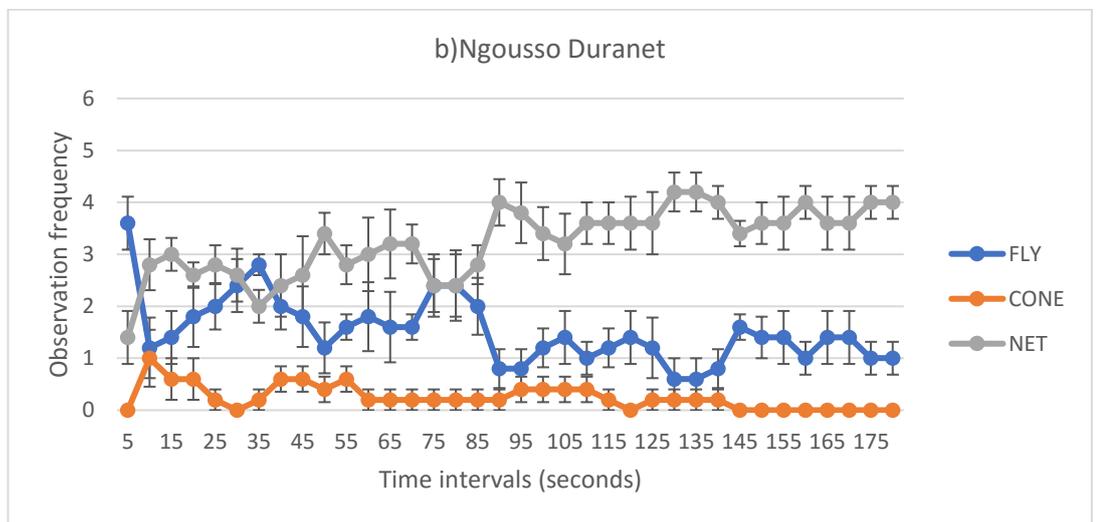
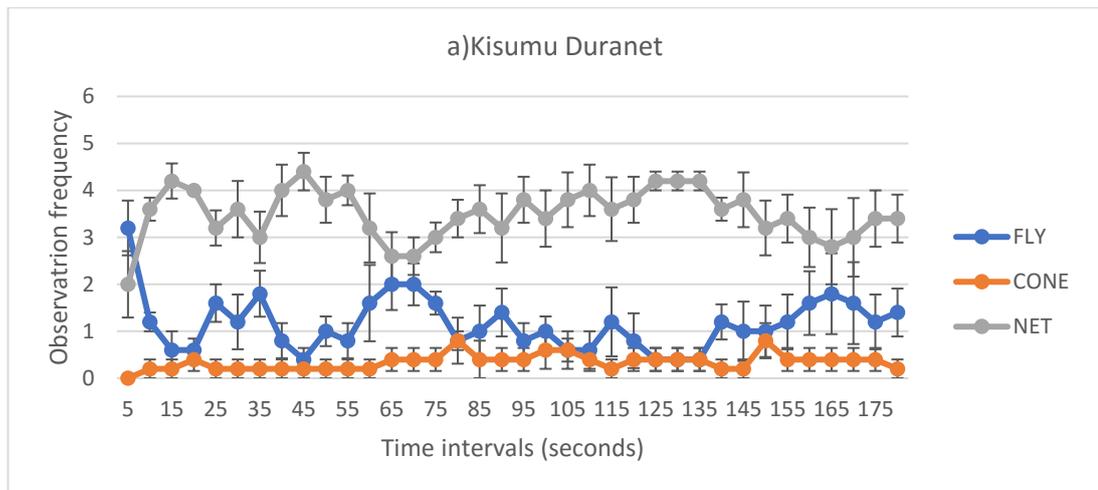


Figure 7-2 (a-c) Line graphs for untreated net (contact) - showing frequency patterns of activity across the 3-min (180 second) assay period after exposure in each resistant strain using WHO standard cone bioassay method in direct contact experiments. At each interval mosquitoes are counted either positioned on the net (grey line), positioned on the cone (orange line) and active in flight (blue line). Standard error bars show variation around the mean.

7.3.2 Contact - Duranet



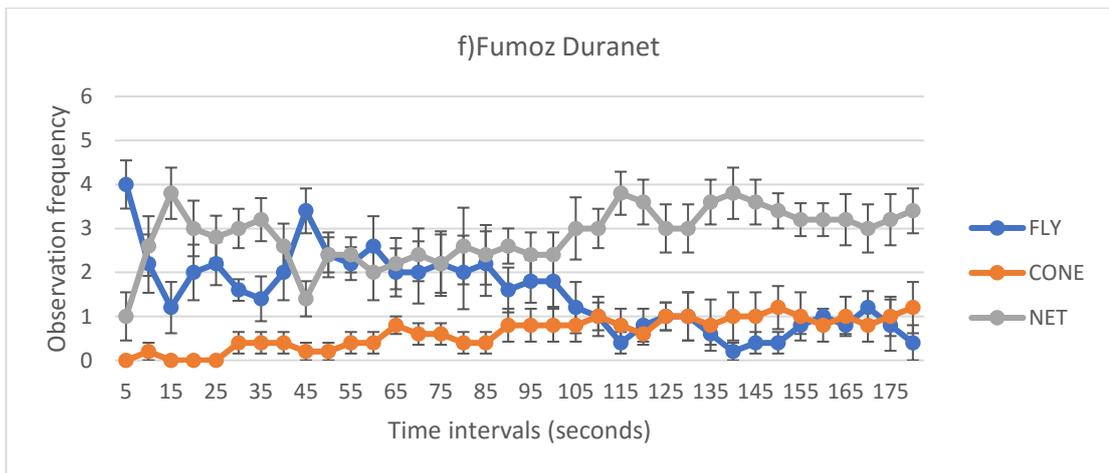
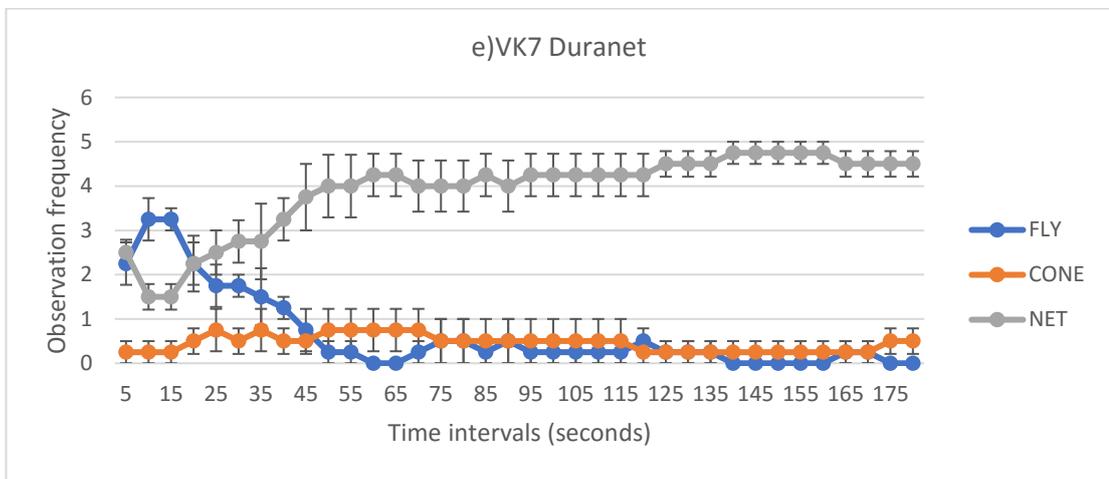
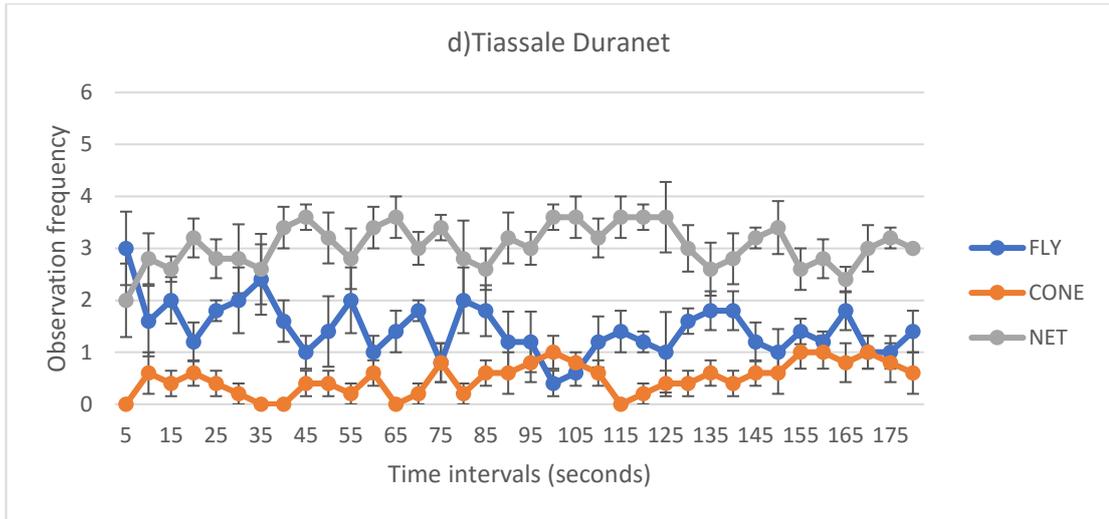
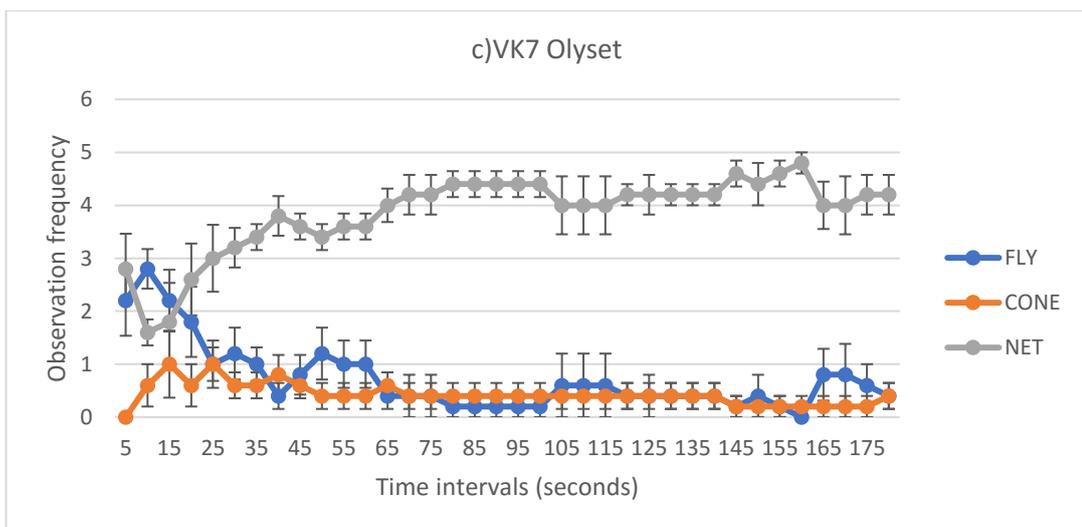
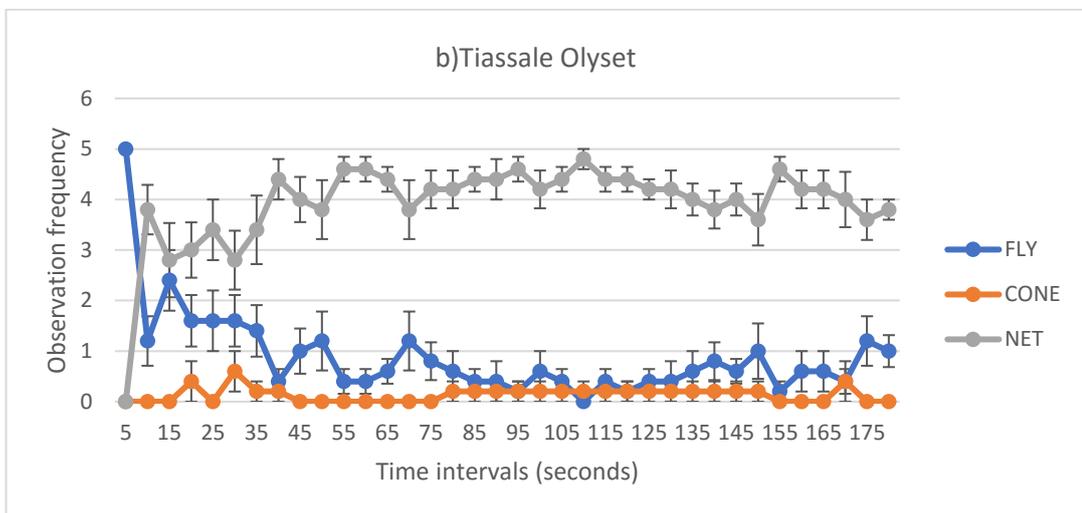
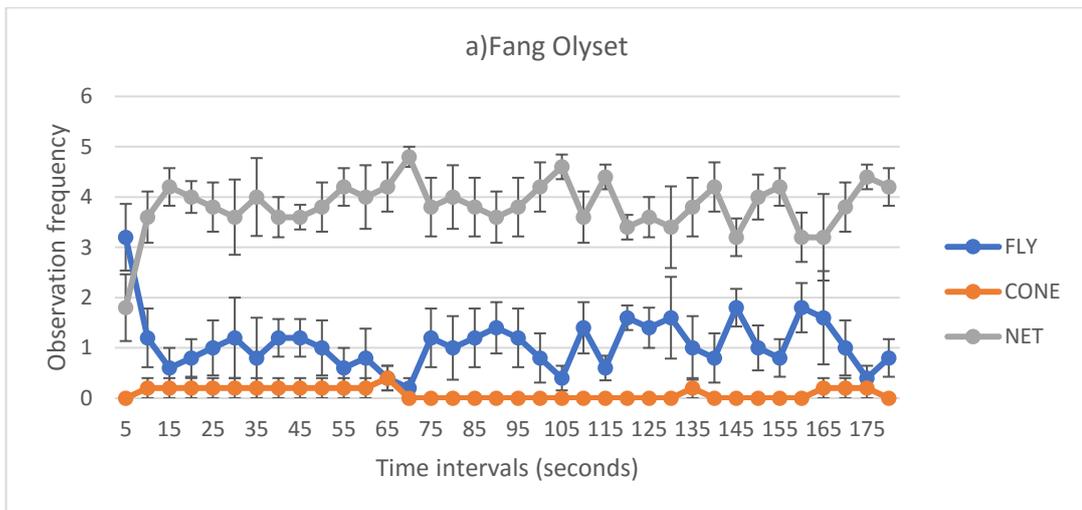


Figure 7-3 (a-f) Line graphs for Duranet net (contact) - showing frequency patterns of activity across the 3-min (180 second) assay period after exposure in each susceptible and resistant strain of *An. gambiae* and *An. funestus* using WHO standard cone bioassay method in direct contact experiments. At each interval mosquitoes are counted either positioned on the net (grey line), positioned on the cone (orange line) and active in flight (blue line). Standard error bars show variation around the mean.

7.3.3 Contact - Olyset



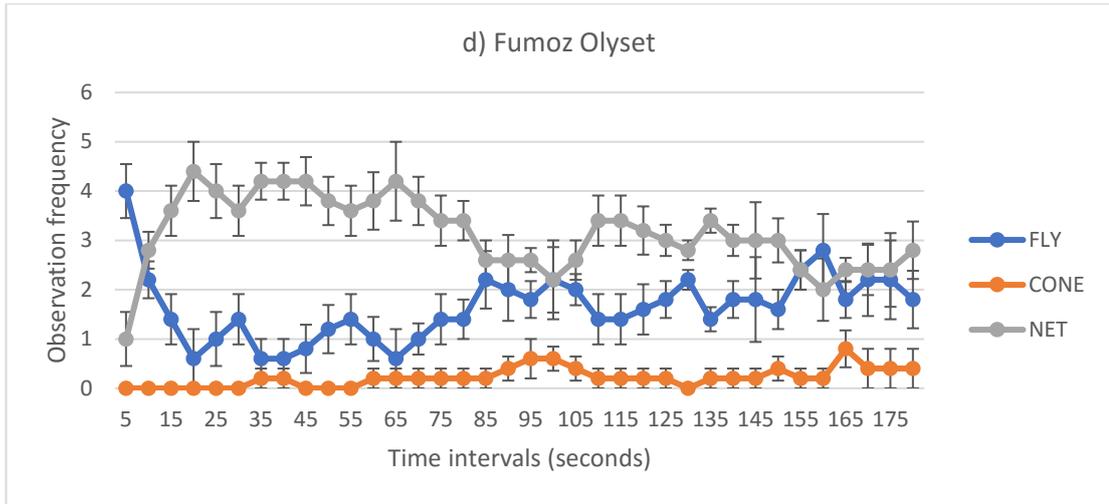
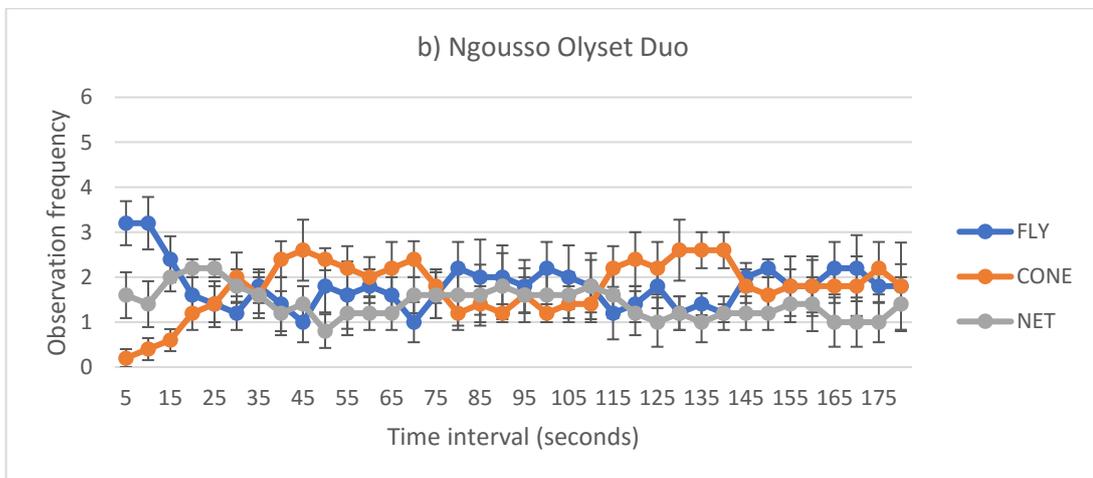
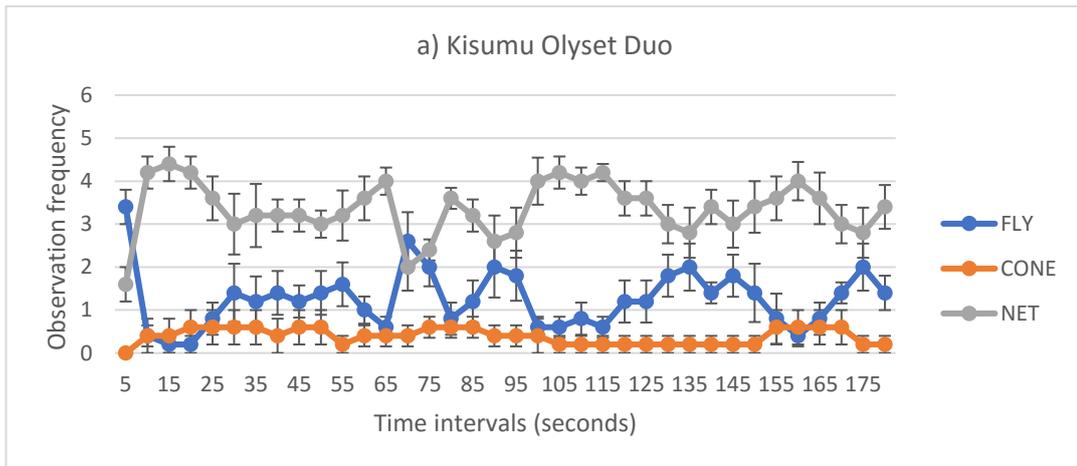


Figure 7-4 (a–d) Line graphs for Olyset net (contact) - showing frequency patterns of activity across the 3-min (180 second) assay period after exposure in susceptible *An. funestus* strain and all resistant strains of *An. gambiae* and *An. funestus* strains using WHO standard cone bioassay method in direct contact experiments. At each interval mosquitoes are counted either positioned on the net (grey line), positioned on the cone (orange line) and active in flight (blue line). Standard error bars show variation around the mean.

7.3.4 Contact - Olyset Duo



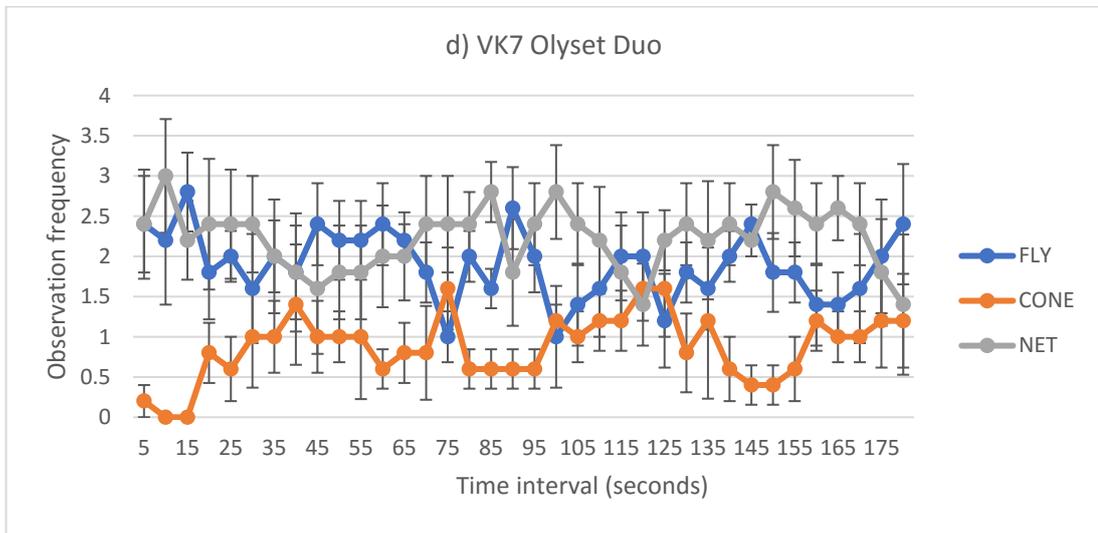
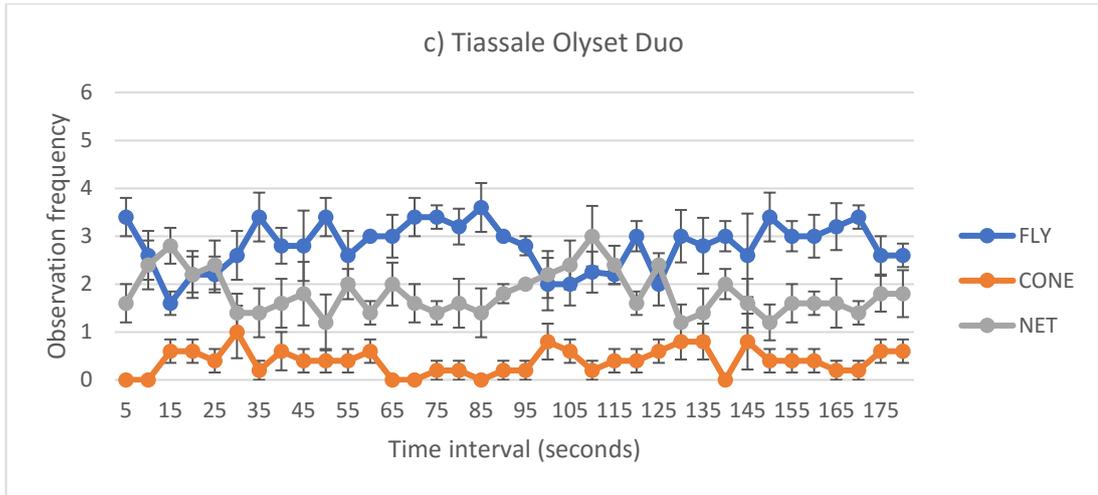
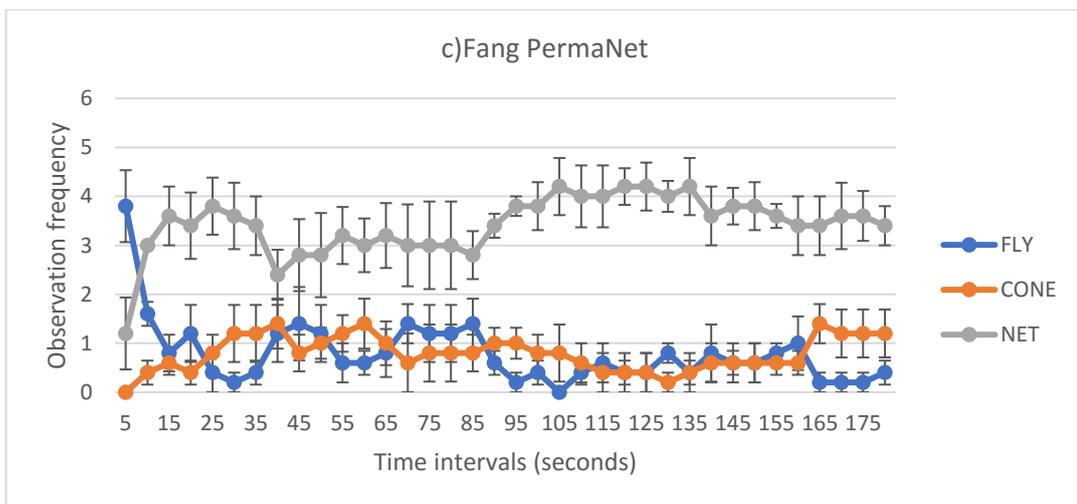
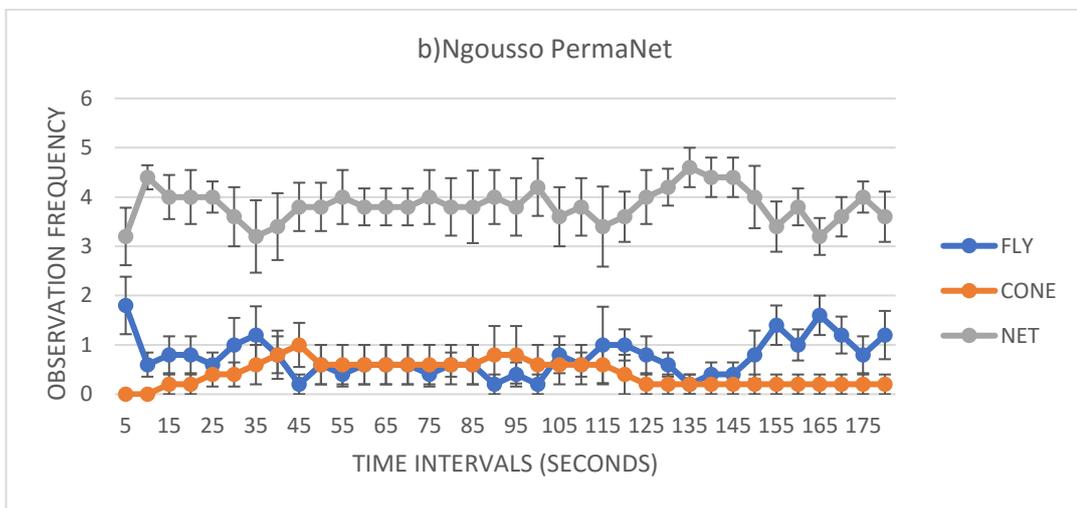
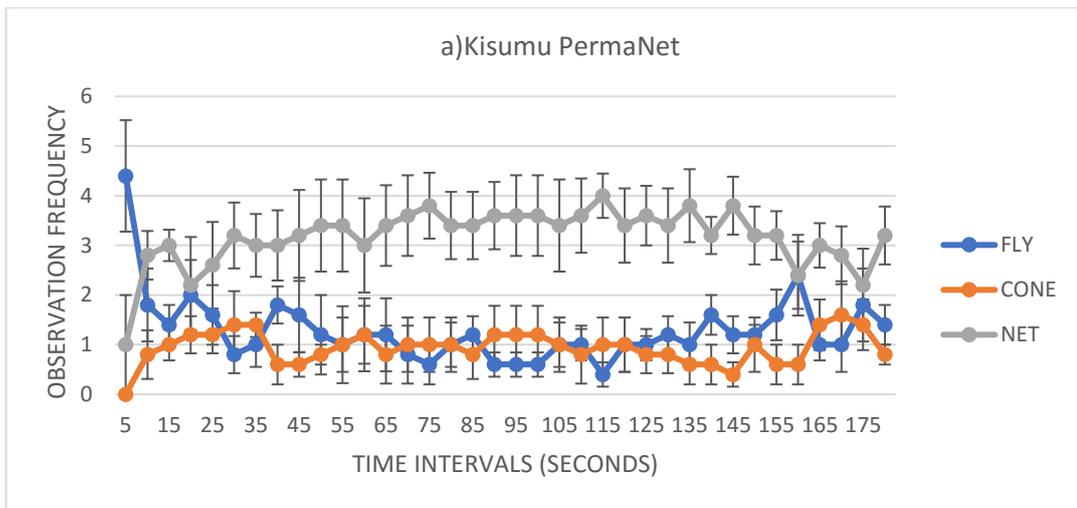


Figure 7-5 (a-d) Line graphs for Olyset Duo (contact) - showing frequency patterns of activity across the 3-min (180 second) assay period after exposure in each susceptible and resistant *An. gambiae* strain using WHO standard cone bioassay method in direct contact experiments. At each interval mosquitoes are counted either positioned on the net (grey line), positioned on the cone (orange line) and active in flight (blue line). Standard error bars show variation around the mean.

7.3.5 Contact - PermaNet



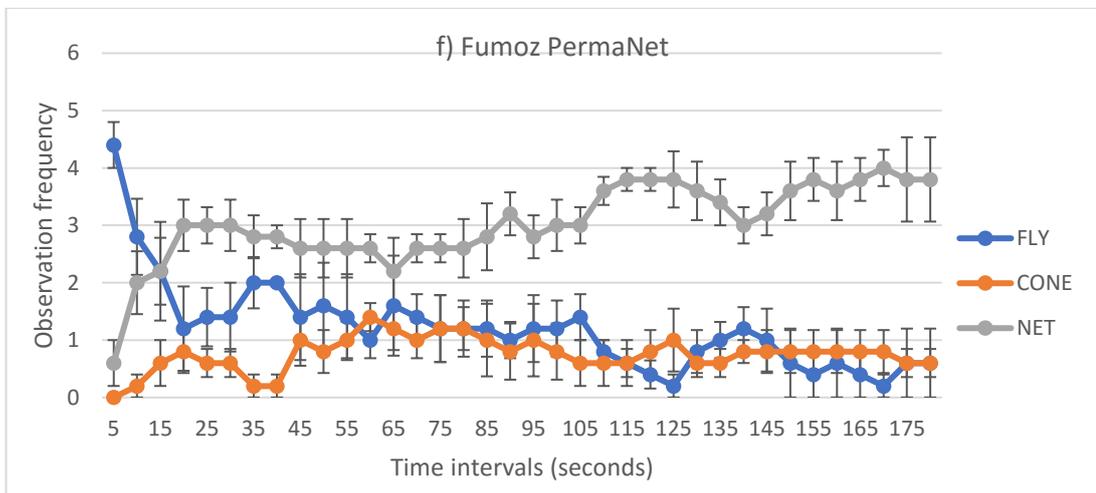
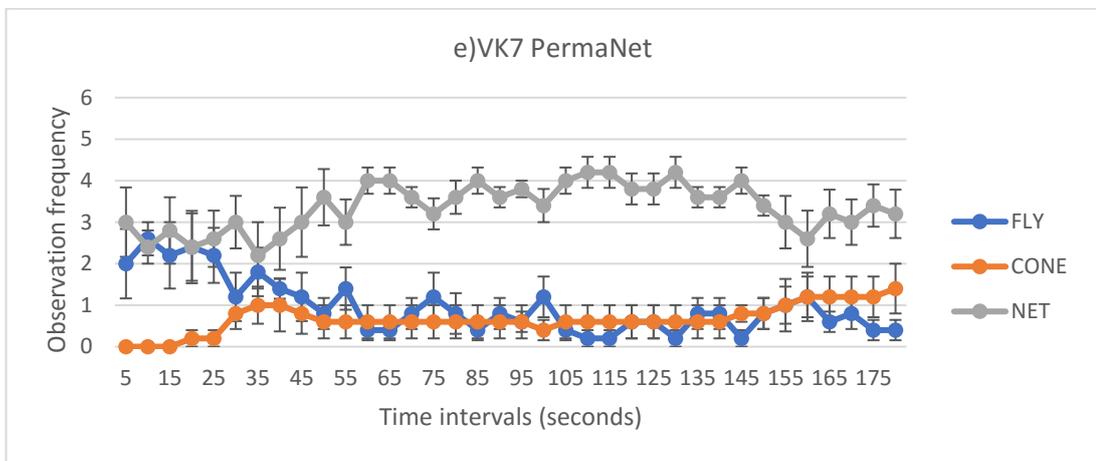
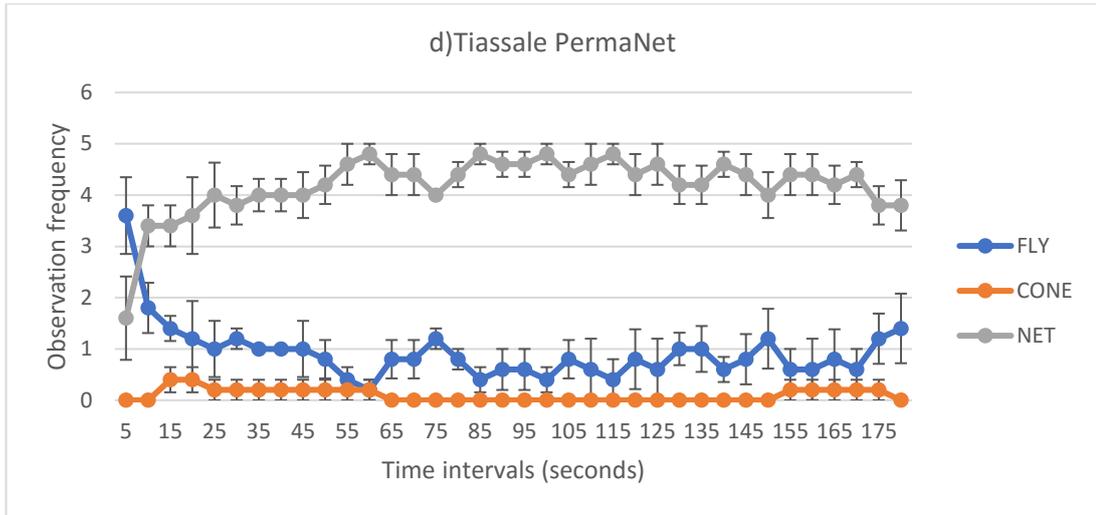
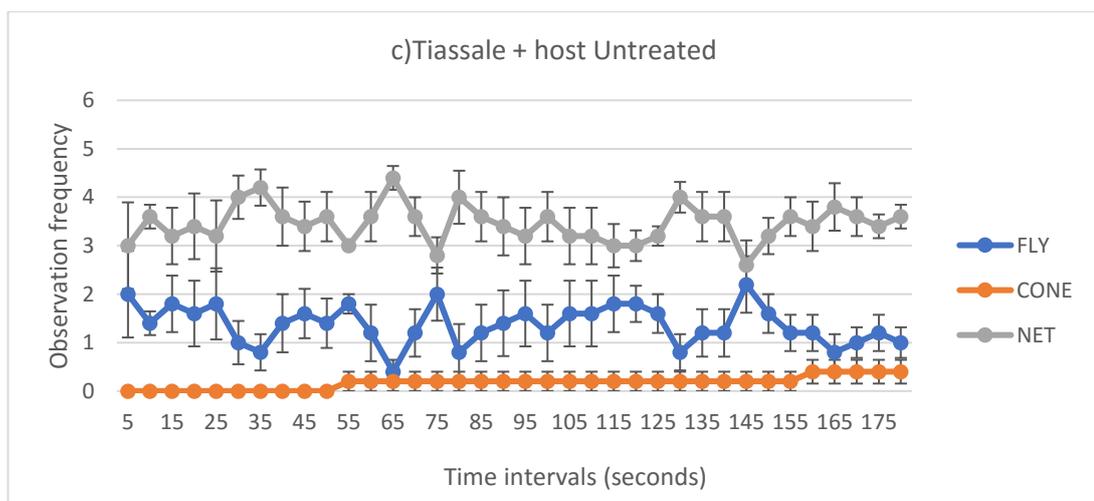
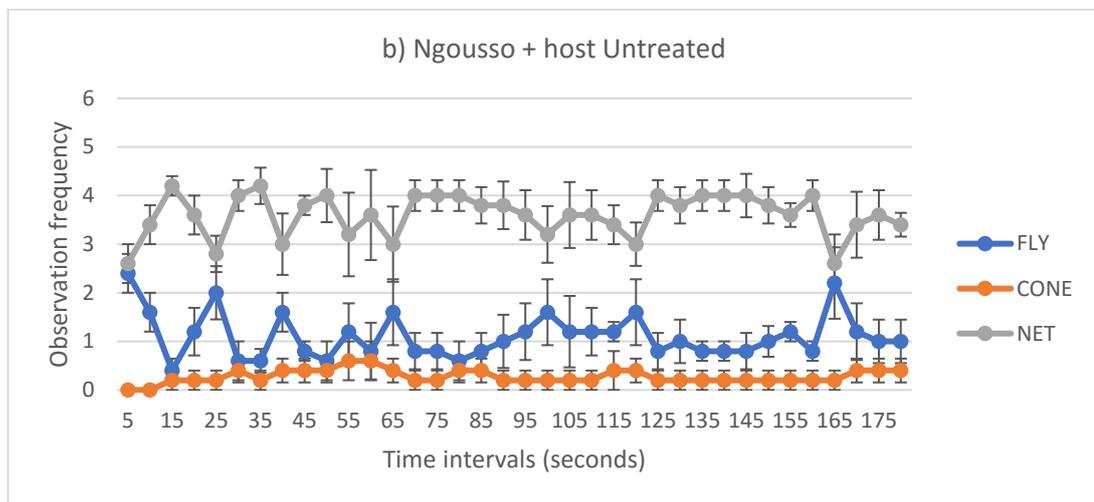
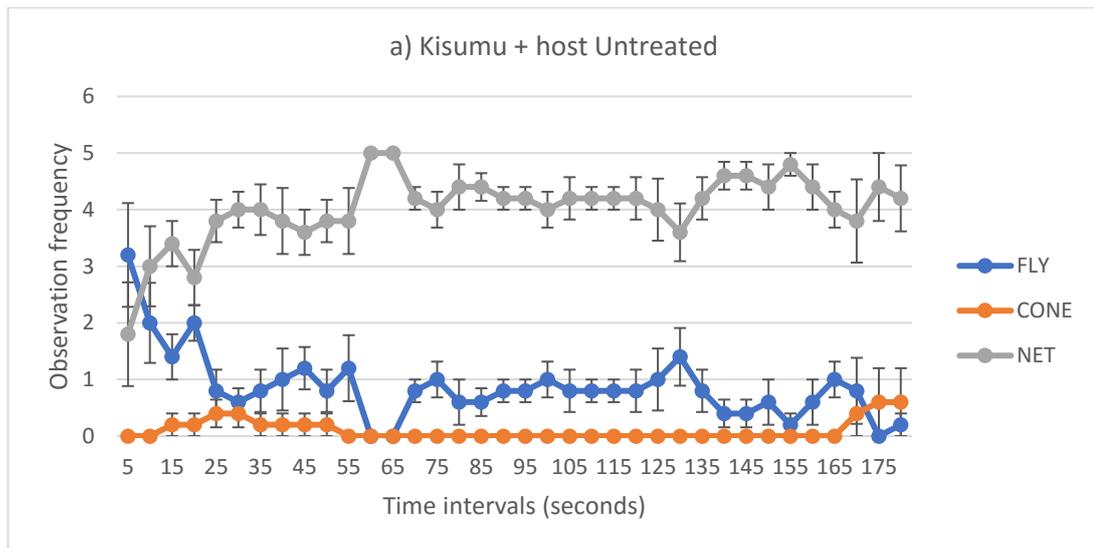


Figure 7-6 a) – f) Line graphs for PermaNet (contact) - showing mean frequency patterns of activity across the 3-min (180 second) assay period after exposure in all susceptible and resistant strains of *An. gambiae* and *An. funestus* using WHO standard cone bioassay method in direct contact experiments. At each interval mosquitoes are counted either positioned on the net (grey line), positioned on the cone (orange line) and active in flight (blue line). Standard error bars show variation around the mean.

7.3.6 Host – Untreated



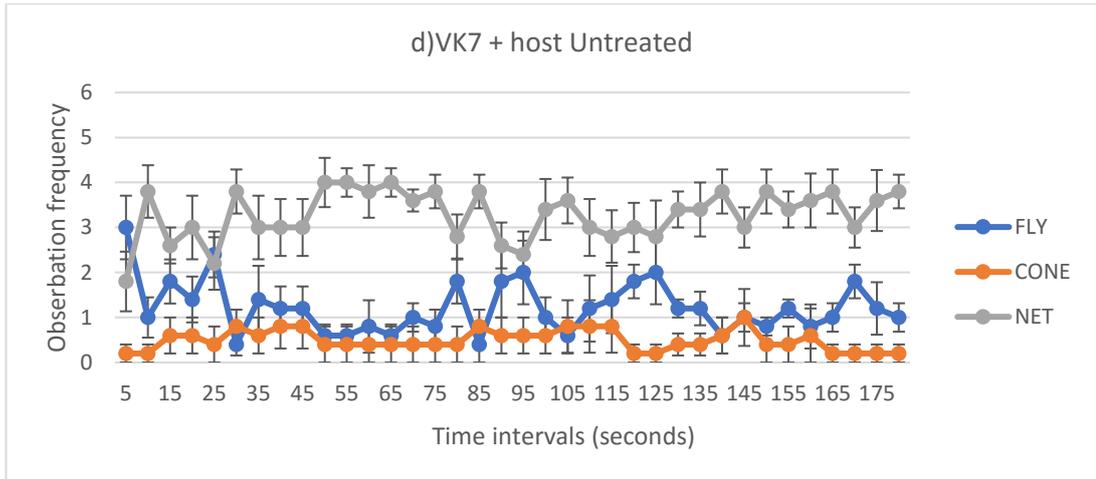
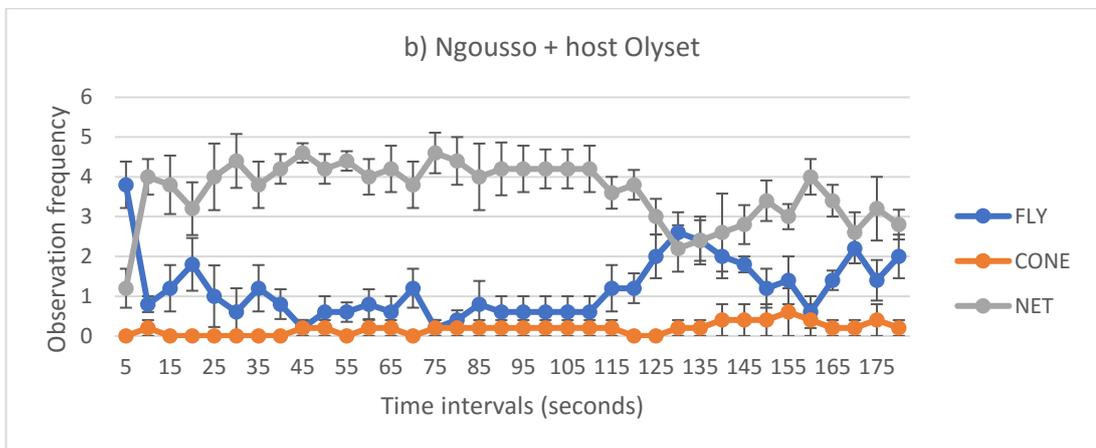
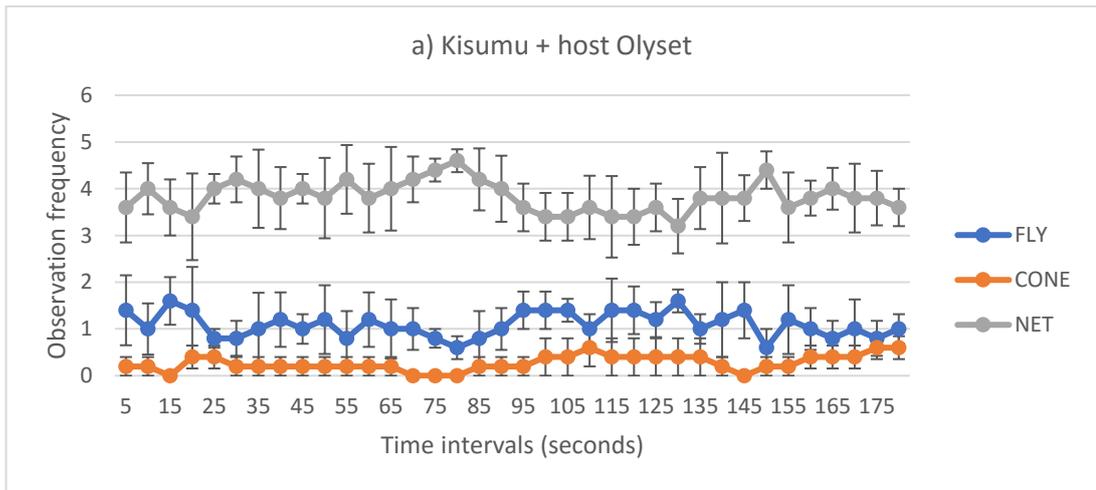


Figure 7-7 (a–d) Line graphs for untreated net (plus host) - showing frequency patterns of activity across the 3-min (180 second) assay period after exposure in each susceptible and resistant strains of *An. gambiae* using WHO standard cone bioassay method in direct contact plus host experiments. At each interval mosquitoes are counted either positioned on the net (grey line), positioned on the cone (orange line) and active in flight (blue line). Standard error bars show variation around the mean.

7.3.7 Host – Olyset



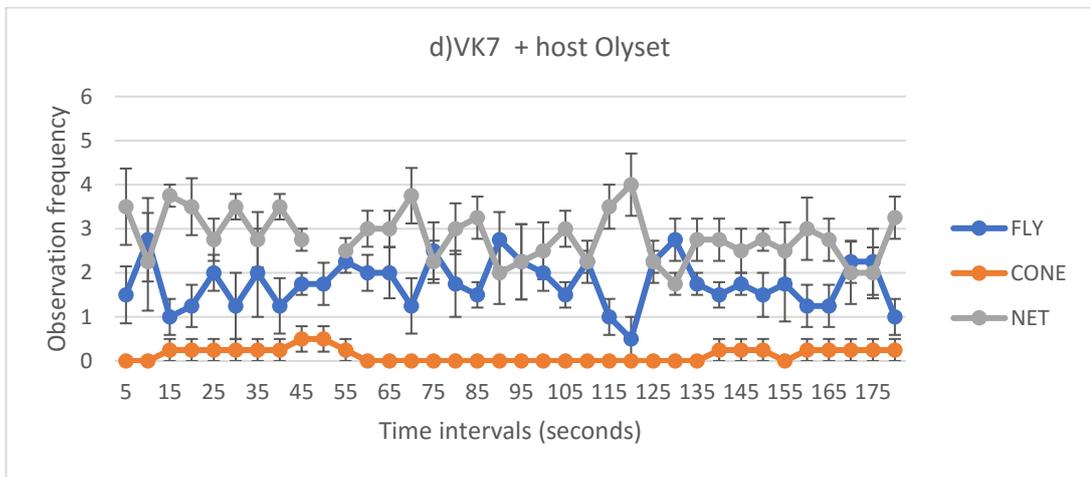
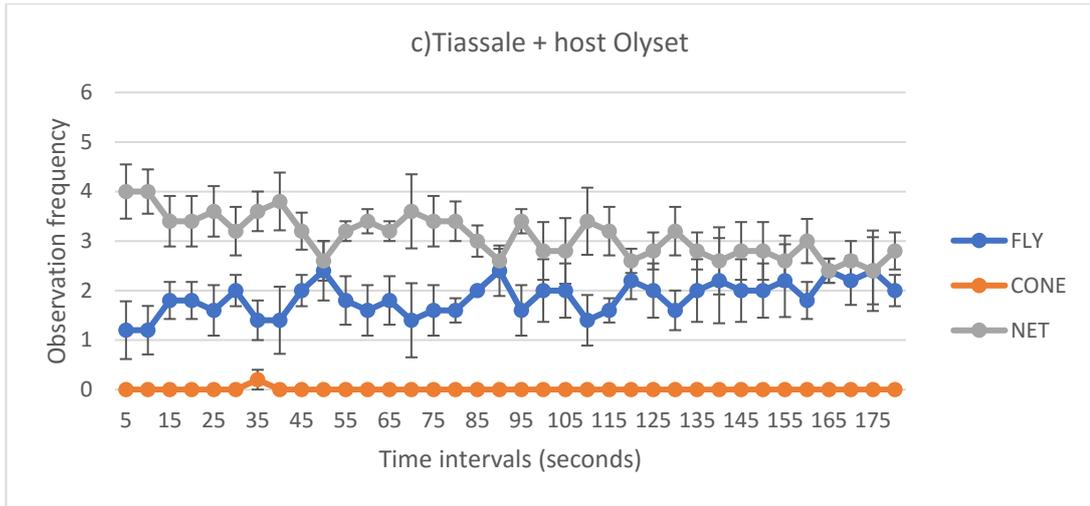
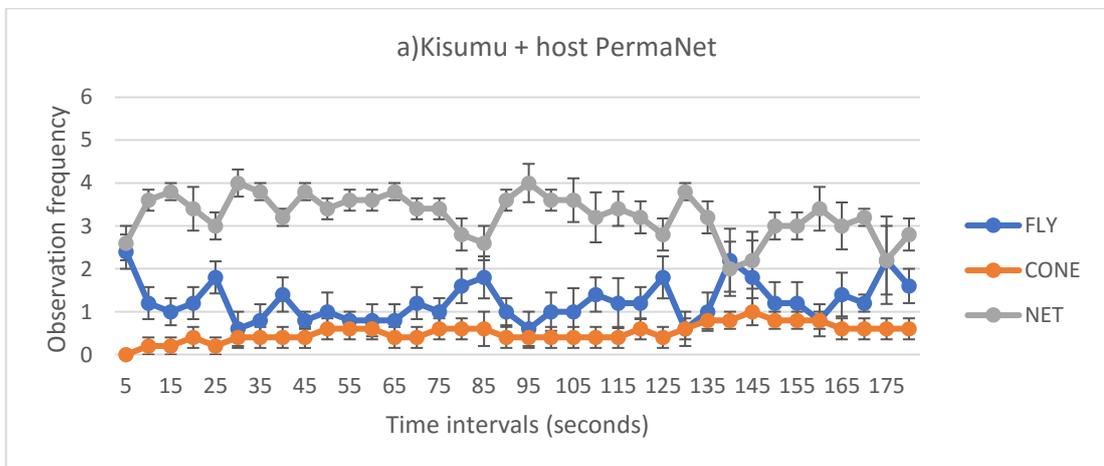


Figure 7-8 (a–d) Line graphs for Olyset (plus host) - showing frequency patterns of activity across the 3-min (180 second) assay period after exposure in each susceptible and resistant strain of *An. gambiae* using the WHO standard cone bioassay method in direct contact plus host experiments. At each interval mosquitoes are counted either positioned on the net (grey line), positioned on the cone (orange line) and active in flight (blue line). Standard error bars show variation around the mean.

7.3.8 Host – PermaNet



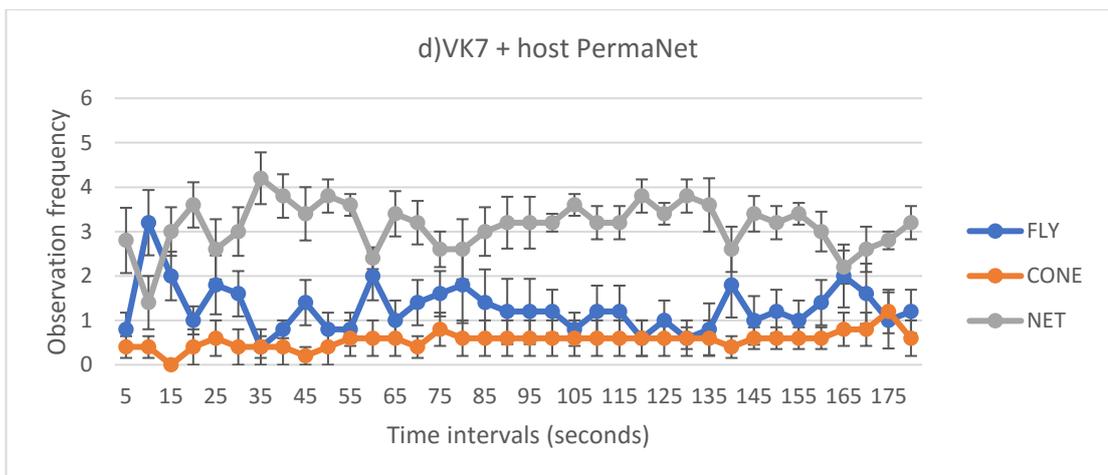
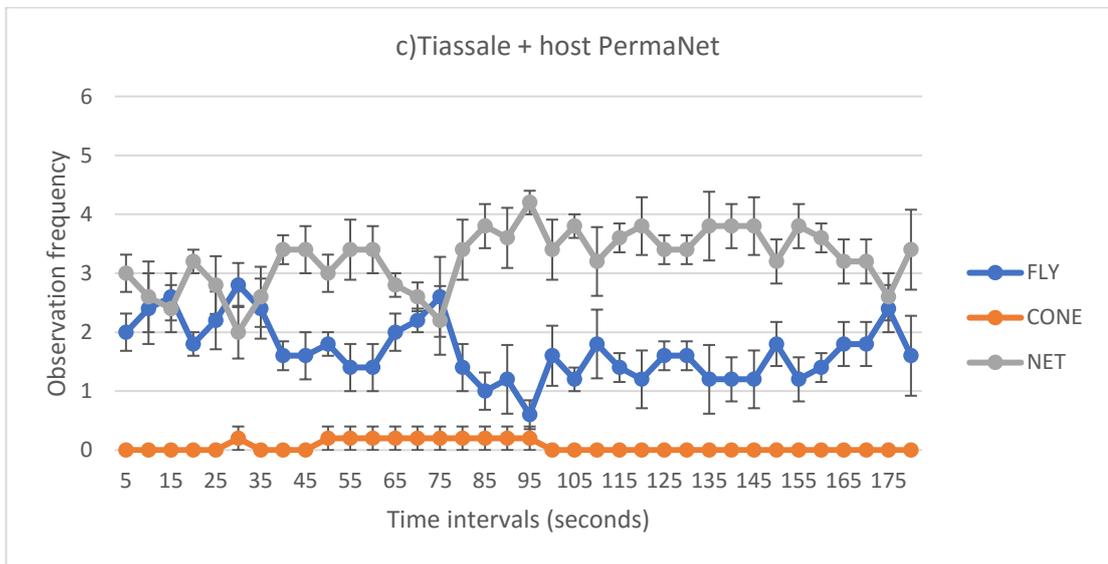
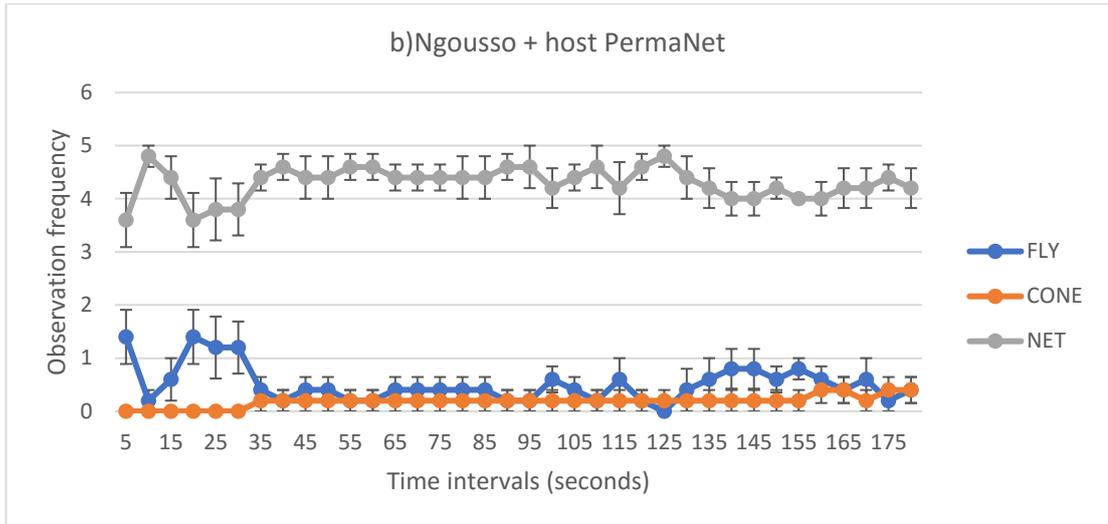
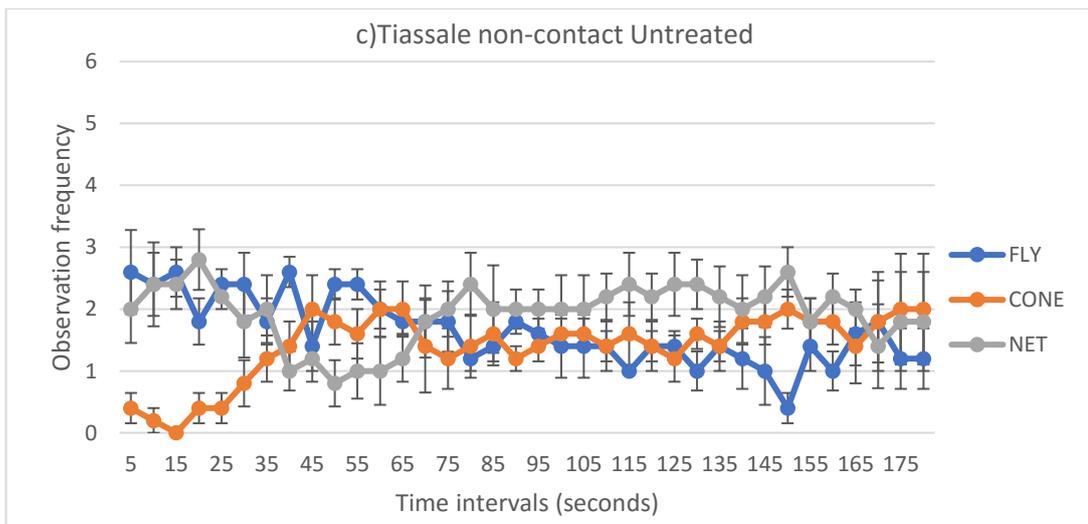
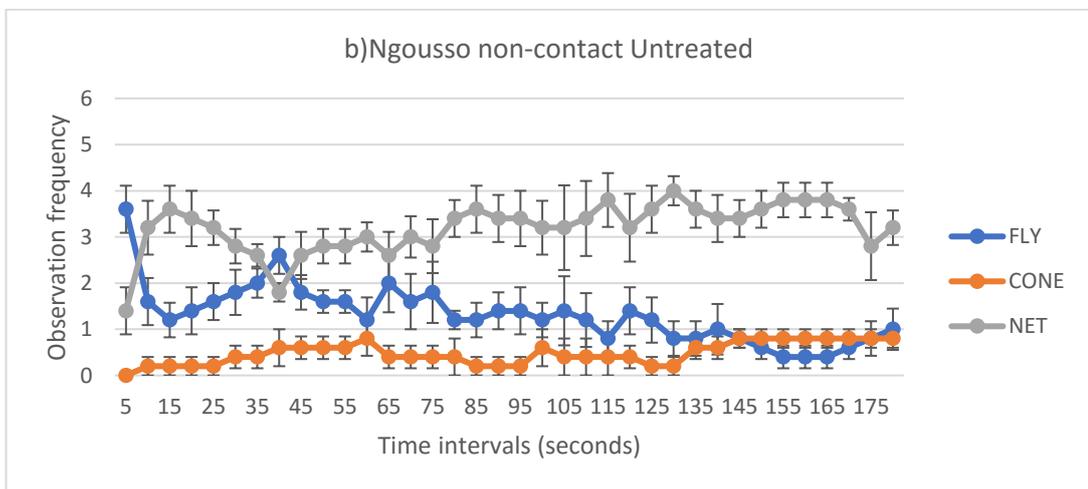
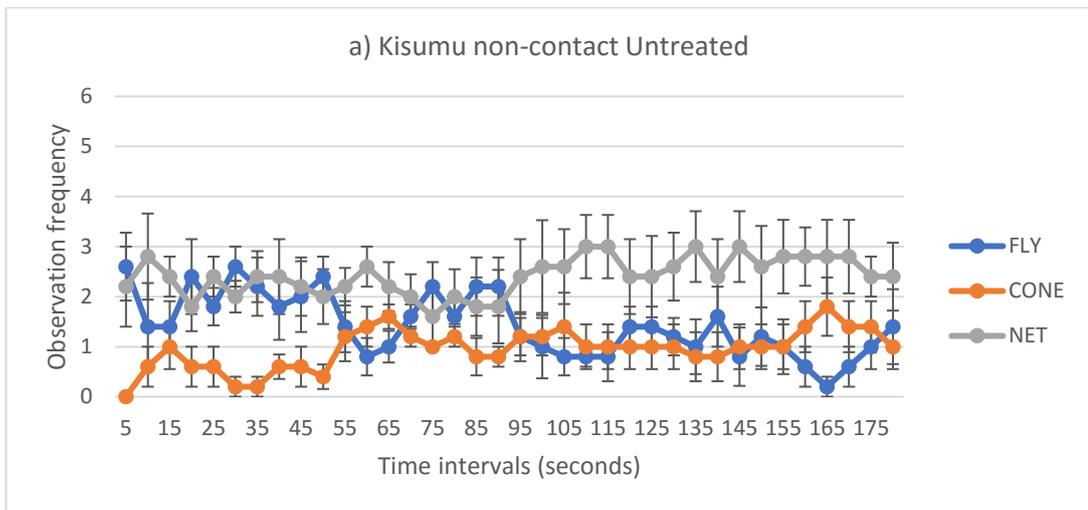


Figure 7-9 (a–d) Line graphs for PermaNet (plus host) - showing frequency patterns of activity across the 3-min (180 second) assay period after exposure in each susceptible and resistant strain of *An. gambiae* using the WHO standard cone bioassay method in direct contact plus host experiments. At each interval mosquitoes are counted either positioned on the net (grey line), positioned on the cone (orange line) and active in flight (blue line). Standard error bars show variation around the mean.

7.3.9 Non-contact Untreated



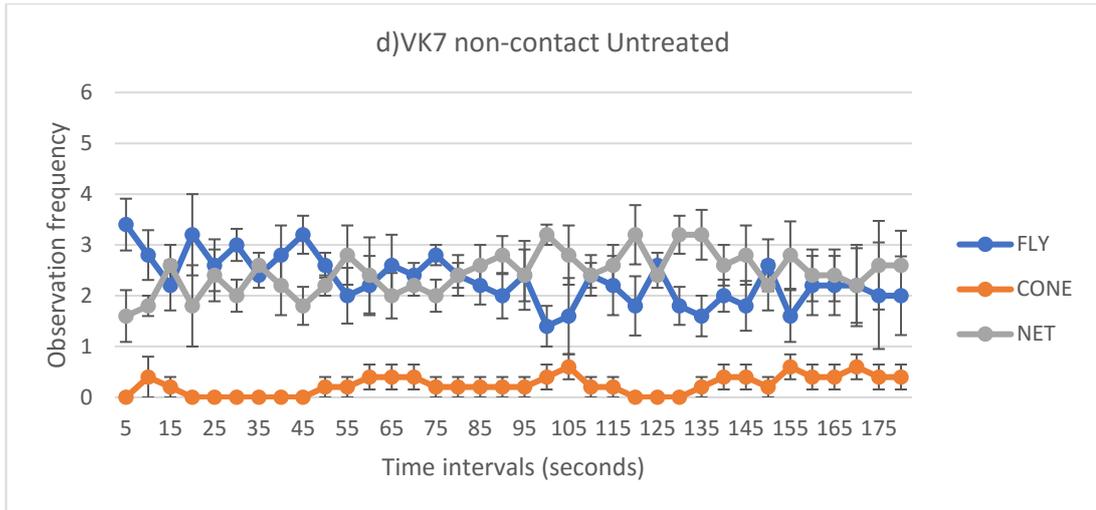
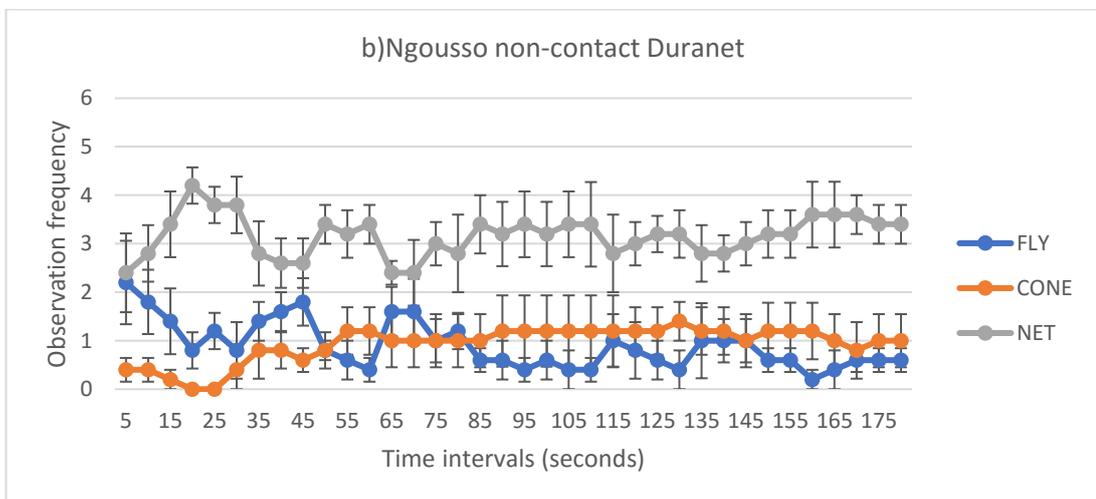
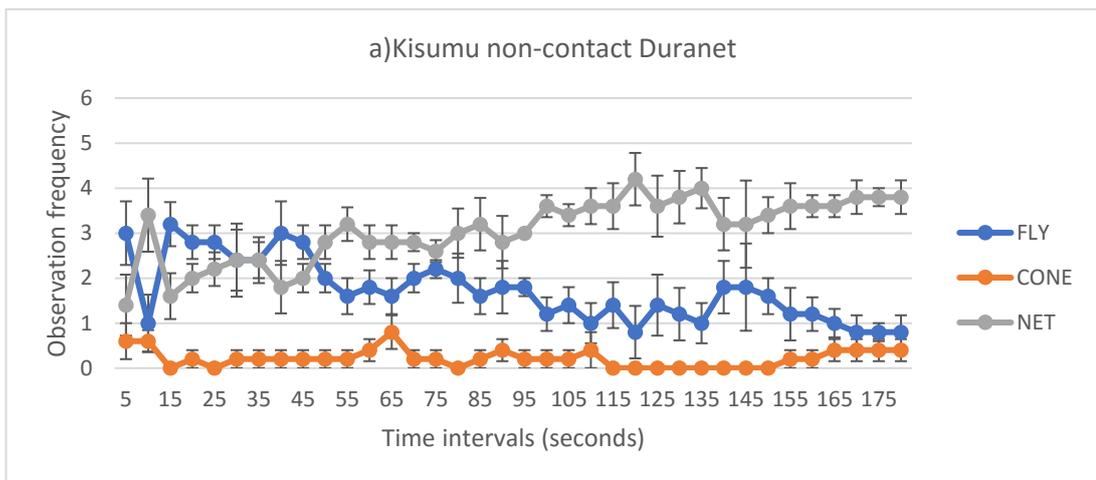


Figure 7-10 (a–d) Line graphs for untreated (non-contact) - showing frequency patterns of activity across the 3-min (180 second) assay period after exposure in each susceptible and resistant strain of *An. gambiae* using the WHO standard cone bioassay method in non-contact experiments. At each interval mosquitoes are counted either positioned on the net (grey line), positioned on the cone (orange line) and active in flight (blue line). Standard error bars show variation around the mean.

7.3.10 Non-contact Duranet



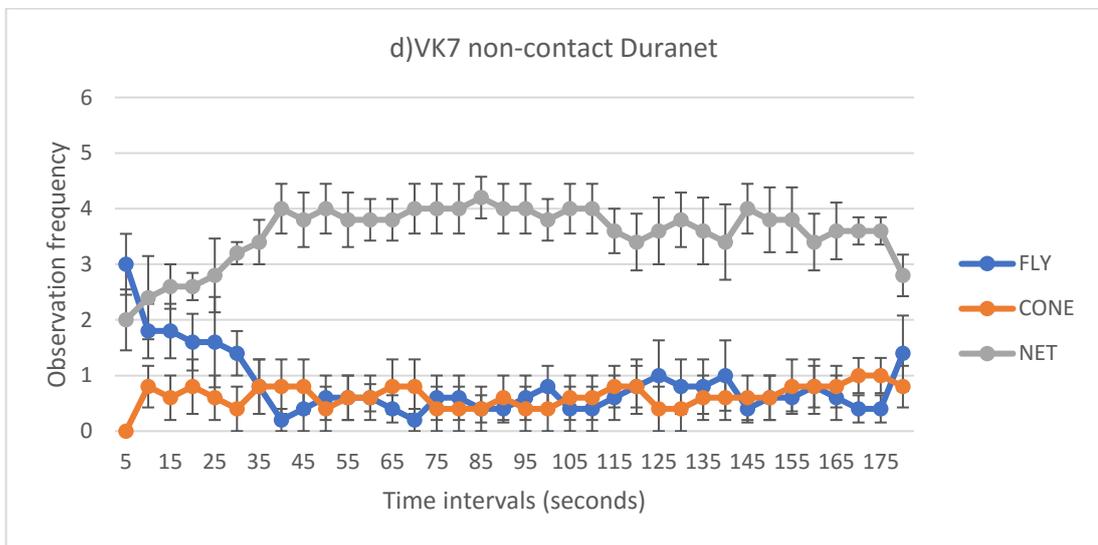
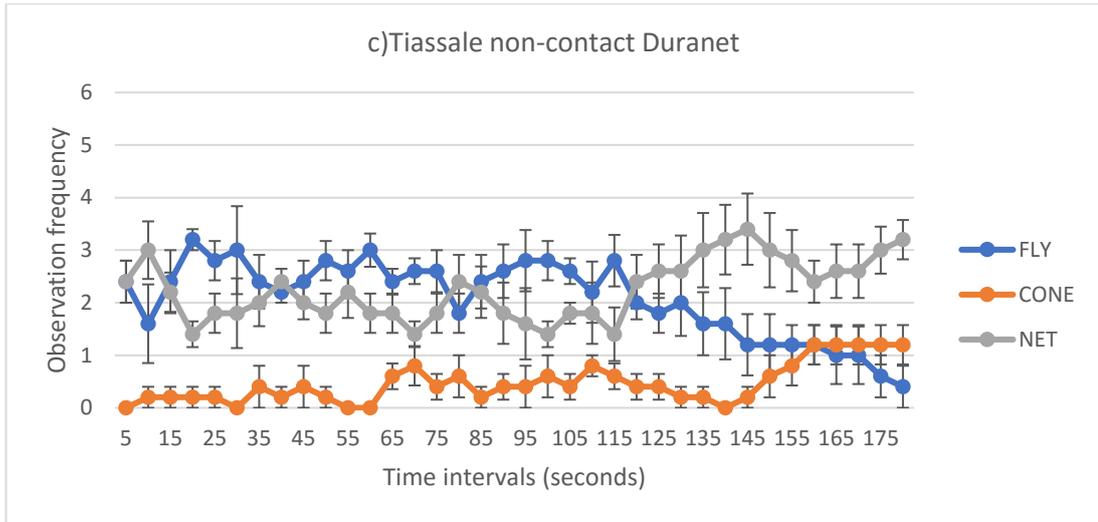
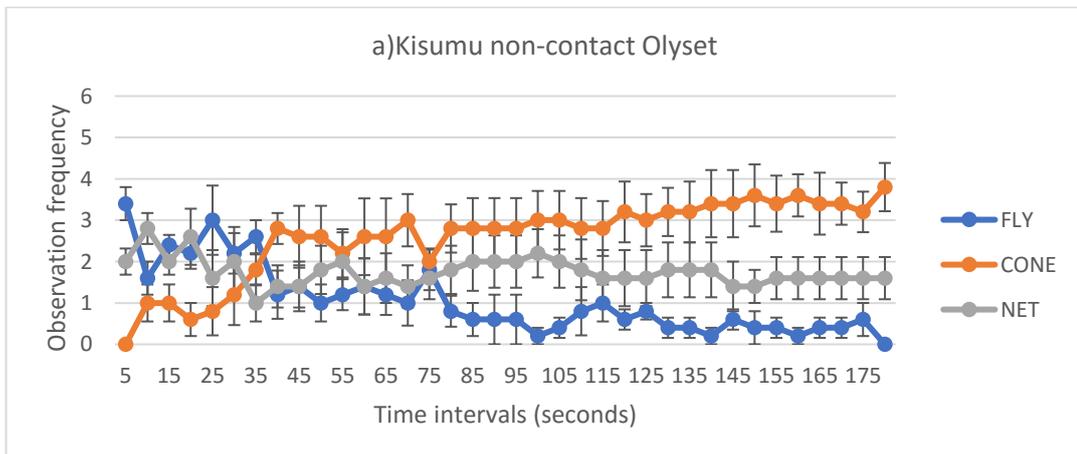


Figure 7-11 (a– d) Line graphs for Duranet (non-contact) - showing frequency patterns of activity across the 3-min (180 second) assay period after exposure in each susceptible and resistant strain of *An. gambiae* using the WHO standard cone bioassay method in non-contact experiments. At each interval mosquitoes are counted either positioned on the net (grey line), positioned on the cone (orange line) and active in flight (blue line). Standard error bars show variation around the mean.

7.3.11 Non-contact Olyset



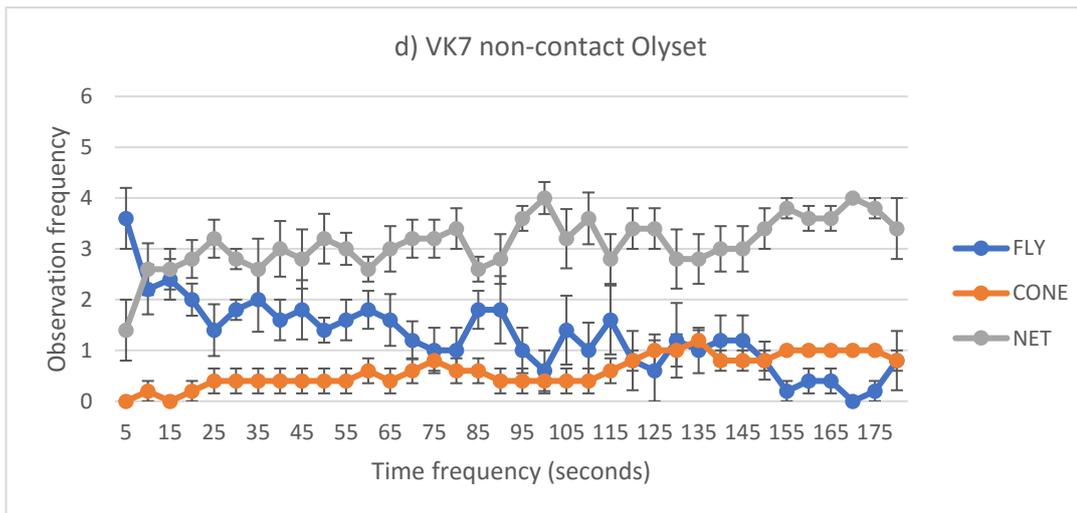
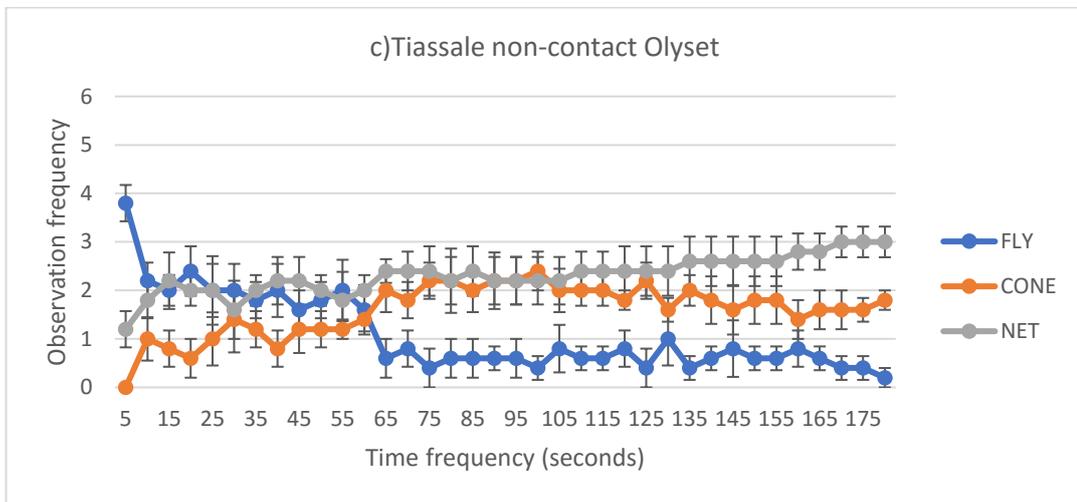
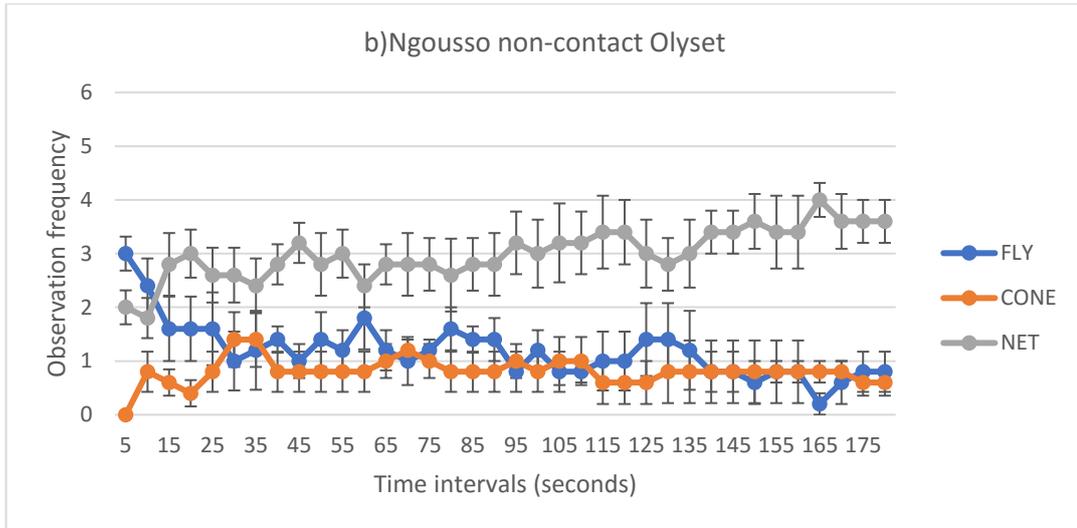
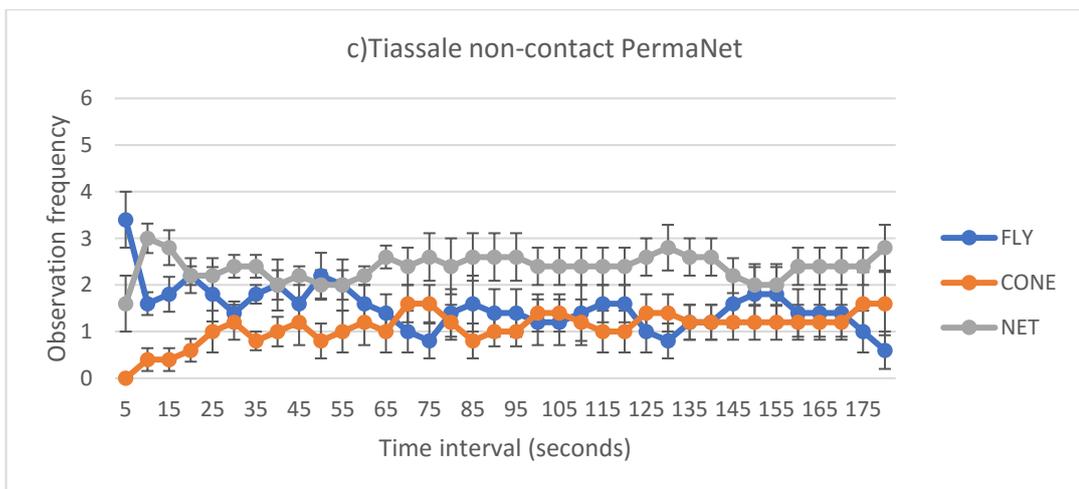
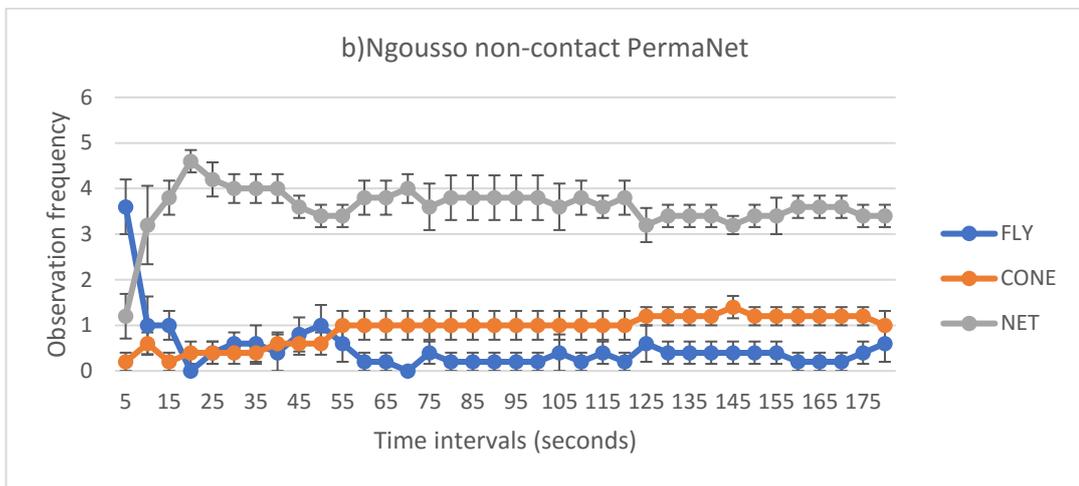
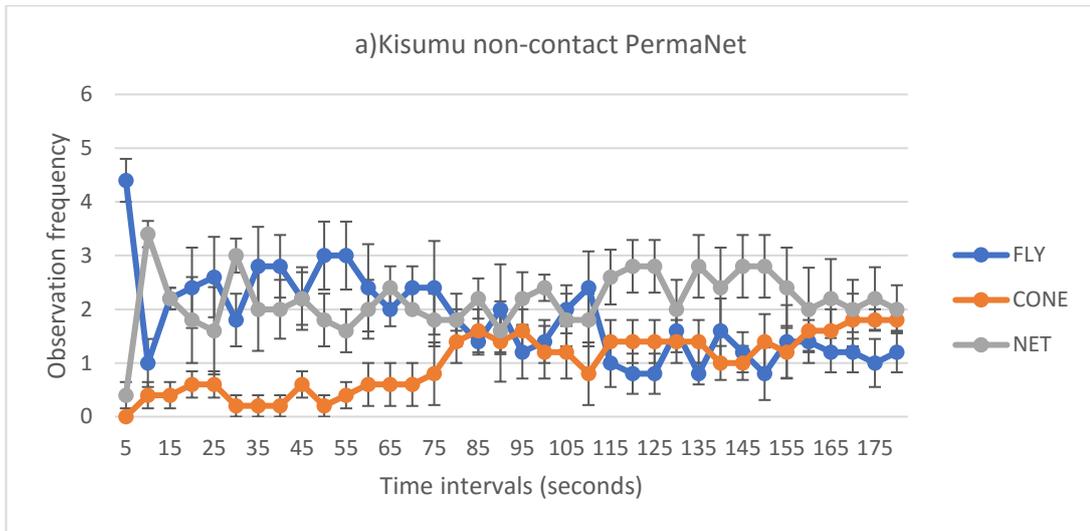


Figure 7-12 (a–d) Line graphs of Olyset (non-contact) - showing frequency patterns of activity across the 3-min (180 second) assay period after exposure to Olyset netting in each susceptible and resistant strain of *An. gambiae* using the WHO standard cone bioassay method in non-contact experiments. At each interval mosquitoes are counted either positioned on the net (grey line), positioned on the cone (orange line) and active in flight (blue line). Standard error bars show variation around the mean.

7.3.12 Non-contact PermaNet



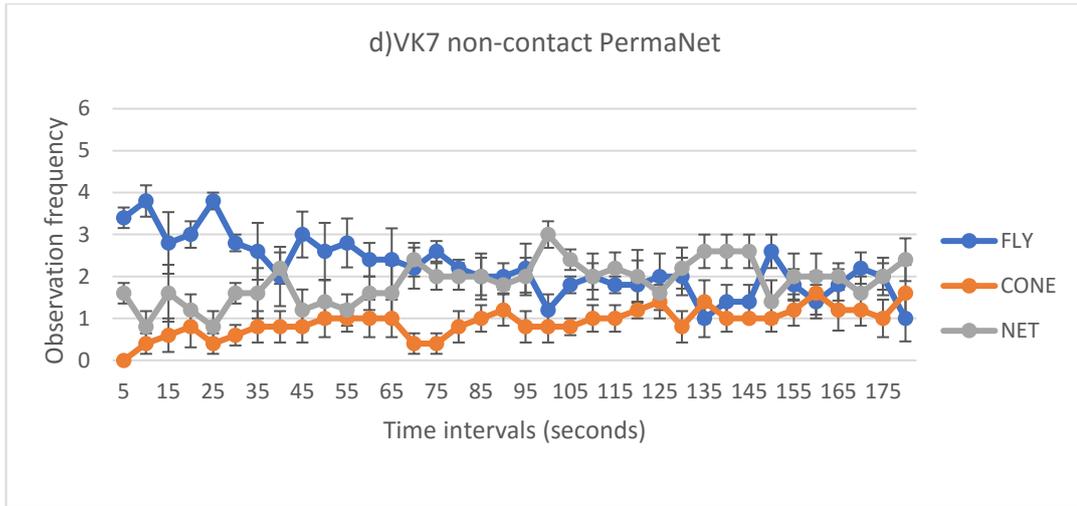


Figure 7-13 (a–d) Line graphs for PermaNet (non-contact) - showing frequency patterns of activity across the 3-min (180 second) assay period after exposure to PermaNet netting in each susceptible and resistant strain of *An. gambiae* using the WHO standard cone bioassay method in non-contact experiments. At each interval mosquitoes are counted either positioned on the net (grey line), positioned on the cone (orange line) and active in flight (blue line). Standard error bars show variation around the mean.

7.4 Appendix 4 – WHO tube bioassay (chapter 4)

Table 7-1 Table showing knockdown (KD) and mortality data of younger (3-5-day old post eclosion) versus older (17-19 days post eclosion) Tiassalé mosquitoes to Deltamethrin (0.05%) using the WHO tube bioassay.

Strain	Replicate	KD (60 mins)	Mortality (24 hours)	Total tested	% mortality each tube	% mortality overall
Tiassale	1	9	5	25	20	18
(Young)	2	9	3	25	12	
3-5 day	3	8	6	25	24	
	4	5	4	25	16	
	Control	0	0	25	0	
(Old)	1	10	14	25	56	55
17-19 day	2	12	13	25	52	
	3	12	17	25	68	
	4	7	11	25	44	
	Control	0	0	25	0	

7.5 Appendix 5 – Mean time for each behavioural event for all mosquito cohorts (chapter 4)

Table 7-2 showing mean time spent on each defined behavioural event after exposure of insecticide susceptible *An. gambiae* Kisumu strain individuals (n=20), young (3-5 days old) (n=20) and older (17-19 days old) (n=10) insecticide resistant *An. gambiae* Tiassalé strain individuals to untreated and PermaNet netting up to a maximum of 20 min (1200s) using the thumb box test system.

STRAIN/TREATMENT (AGE DAYS)	KISUMU/UNTREATED			TIASSALÉ/UNTREATED (3-5)			KISUMU/PERMANET			TIASSALÉ/PERMANET (3.5)			TIASSALÉ/UNTREATED (17-19)			TIASSALÉ/PERMANET (17-19)		
	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N
BEHAVIOURAL EVENT																		
Until appearance	71.9	70.1	20	73.7	78.6	20	94.2	84.8	20	50.5	64.2	20	97.1	101.1	10	53.3	39.0	10
Appearance to contact	10.5	19.3	20	21.7	35.4	20	19.9	38.2	20	21.6	52.0	20	16.7	30.3	10	42.9	93.5	10
Contact to landing	0.5	0.8	20	4.2	14.2	20	0.5	0.9	20	1.9	3.9	20	1.2	0.8	10	1.3	0.9	10
Landing to start point of probing	2.8	4.8	20	3.5	5.4	20	2.4	4.1	20	2.3	2.5	20	2.0	2.9	10	4.4	8.0	10
Probing to insert	34.1	36.9	20	49.6	63.5	20	35.8	39.7	20	40.1	41.6	20	73.0	75.2	10	53.8	39.7	10
Insert to start feeding visible	45.7	27.3	20	32.5	18.9	20	40.4	17.4	20	31.5	19.6	20	43.5	28.4	10	41.7	23.7	10
Feeding before defaecation	71.5	48.1	20	89.9	32.1	20	64.2	46.6	20	94.2	46.8	20	100.3	63.8	10	61.7	55.0	10
Feeding with defaecation until withdrawal	390.4	179.6	20	273.7	153.9	20	178.1	81.4	20	125.4	61.9	20	168.6	151.1	10	132.0	128.2	10
Resting on net before departure	420.0	298.0	20	505.2	301.4	20	108.6	209.0	20	115.8	190.5	20	357.1	285.6	10	152.5	169.4	10

7.6 Appendix 6 – GEE ANALYSIS results where contact on the net is compared between treatments/strain

Tables 7-3 Comparisons of net response of *Anopheles gambiae* insecticide susceptible strains A) Kisumu and B) Ngousso strain and insecticide resistant strains C) Tiassale and D) VK7 vs other strains and treatments. Green shading shows statistically significant difference between the two comparisons

STRAIN	LLIN		STRAIN	LLIN	NO HOST	HOST
A) KISUMU	OLYSET	VS	KISUMU	OLYSET	NA	<0.0001
				PERMANET	0.01	<0.0001
				UNTREATED	<0.0001	<0.0001
			NGUSSO	OLYSET	0.01	<0.0001
				PERMANET	<0.0001	<0.0001
				UNTREATED	<0.0001	<0.0001
			TIASSALE	OLYSET	<0.0001	<0.0001
				PERMANET	<0.0001	<0.0001
				UNTREATED	<0.0001	<0.0001
			VK7	OLYSET	<0.0001	<0.0001
				PERMANET	0.009	<0.0001
				UNTREATED	<0.0001	<0.0001
	PERMANET	VS	KISUMU	OLYSET	0.01	0.223
				PERMANET	NA	0.639
				UNTREATED	0.333	0.02
			NGUSSO	OLYSET	0.066	0.0319
				PERMANET	0.132	0.005
				UNTREATED	0.459	0.198
			TIASSALE	OLYSET	0.061	0.92
				PERMANET	0.01	0.649
				UNTREATED	0.002	0.399
			VK7	OLYSET	0.051	0.83
				PERMANET	0.524	0.666
				UNTREATED	0.845	0.846
	UNTREATED	VS	KISUMU	OLYSET	<0.0001	0.784
				PERMANET	0.333	0.352
				UNTREATED	NA	0.158
			NGUSSO	OLYSET	0.001	0.845
				PERMANET	0.541	0.042
				UNTREATED	0.652	0.901
			TIASSALE	OLYSET	0.352	0.207
				PERMANET	0.083	0.339
				UNTREATED	0.019	0.901
			VK7	OLYSET	0.364	0.069
				PERMANET	0.631	0.23
				UNTREATED	0.203	0.555

STRAIN	LLIN		STRAIN	LLIN	NO HOST	HOST
B) NGUSSO	OLYSET	VS	KISUMU	OLYSET	0.011	<0.0001
				PERMANET	0.166	<0.0001
				UNTREATED	0.001	<0.0001
			NGUSSO	OLYSET	NA	<0.0001
				PERMANET	<0.0001	<0.0001
				UNTREATED	<0.0001	<0.0001
			TIASSALE	OLYSET	<0.0001	0.002
				PERMANET	<0.0001	<0.0001
				UNTREATED	<0.0001	<0.0001
			VK7	OLYSET	<0.0001	<0.0001
				PERMANET	0.001	<0.0001
				UNTREATED	0.196	0.021
	PERMANET	VS	KISUMU	OLYSET	<0.0001	0.731
				PERMANET	0.32	0.074
				UNTREATED	0.541	0.555
			NGUSSO	OLYSET	<0.0001	0.326
				PERMANET	NA	0.196
				UNTREATED	0.231	0.489
			TIASSALE	OLYSET	0.828	0.041
				PERMANET	0.326	0.069
				UNTREATED	0.113	0.345
			VK7	OLYSET	0.958	0.007
				PERMANET	0.245	0.044
				UNTREATED	0.063	0.233
	UNTREATED	VS	KISUMU	OLYSET	<0.0001	0.427
				PERMANET	0.459	0.503
				UNTREATED	0.652	0.003
			NGUSSO	OLYSET	<0.0001	0.685
				PERMANET	0.231	<0.0001
				UNTREATED	NA	0.348
			TIASSALE	OLYSET	0.067	0.259
				PERMANET	0.002	0.476
				UNTREATED	<0.0001	0.83
			VK7	OLYSET	0.017	0.033
				PERMANET	0.931	0.29
				UNTREATED	0.276	0.785

STRAIN	LLIN		STRAIN	LLIN	NO HOST	HOST			
C) TIASSALE	OLYSET	VS	KISUMU	OLYSET	<0.0001	0.533			
				PERMANET	0.061	0.003			
				UNTREATED	0.352	0.631			
			NGUSSO	OLYSET	<0.0001	0.102			
				PERMANET	0.828	0.164			
				UNTREATED	0.067	0.193			
			TIASSALE	OLYSET	NA	0.003			
				PERMANET	0.322	0.003			
				UNTREATED	0.07	0.155			
			VK7	OLYSET	0.756	<0.0001			
				PERMANET	0.095	0.002			
				UNTREATED	0.019	0.111			
			PERMANET	VS	KISUMU	OLYSET	<0.0001	0.154	
							PERMANET	0.01	<0.0001
							UNTREATED	0.083	0.402
						NGUSSO	OLYSET	<0.0001	0.002
							PERMANET	0.326	0.624
							UNTREATED	0.002	0.004
						TIASSALE	OLYSET	0.322	<0.0001
							PERMANET	NA	<0.0001
							UNTREATED	0.352	0.013
						VK7	OLYSET	0.042	<0.0001
							PERMANET	0.006	<0.0001
							UNTREATED	0.002	0.016
						UNTREATED	VS	KISUMU	OLYSET
				PERMANET	0.002	<0.0001			
				UNTREATED	0.019	0.037			
			NGUSSO	OLYSET	<0.0001	<0.0001			
				PERMANET	0.113	0.696			
				UNTREATED	<0.0001	<0.0001			
			TIASSALE	OLYSET	0.07	<0.0001			
				PERMANET	0.352	<0.0001			
				UNTREATED	NA	0.001			
			VK7	OLYSET	<0.0001	<0.0001			
				PERMANET	<0.0001	<0.0001			
				UNTREATED	<0.0001	0.003			

STRAIN	LLIN		STRAIN	LLIN	NO HOST	HOST						
D) VK7	OLYSET	VS	KISUMU	OLYSET	<0.0001	0.588						
				PERMANET	0.051	<0.0001						
				UNTREATED	0.364	0.049						
			NGUSSO	OLYSET	<0.0001	0.018						
				PERMANET	0.958	0.01						
				UNTREATED	0.017	0.033						
			TIASSALE	OLYSET	0.756	<0.0001						
				PERMANET	0.042	<0.0001						
				UNTREATED	<0.0001	0.107						
			VK7	OLYSET	NA	<0.0001						
				PERMANET	0.055	<0.0001						
				UNTREATED	0.011	0.092						
			PERMANET	VS	KISUMU	OLYSET	<0.0001	0.428				
							PERMANET	0.534	0.676			
							UNTREATED	0.631	0.013			
						NGUSSO	OLYSET	0.001	0.688			
							PERMANET	0.245	0.002			
							UNTREATED	0.931	0.393			
						TIASSALE	OLYSET	0.095	0.392			
							PERMANET	0.006	0.656			
							UNTREATED	<0.0001	0.789			
						VK7	OLYSET	0.055	0.127			
							PERMANET	NA	0.441			
							UNTREATED	0.345	0.853			
						UNTREATED	VS	KISUMU	OLYSET	0.009	0.122	
										PERMANET	0.845	0.407
										UNTREATED	0.203	0.003
			NGUSSO	OLYSET	0.196				0.162			
				PERMANET	0.063				0.001			
UNTREATED	0.276	0.081										
TIASSALE	OLYSET	0.019	0.702									
	PERMANET	0.002	0.412									
	UNTREATED	<0.0001	0.239									
VK7	OLYSET	0.011	0.96									
	PERMANET	0.345	0.618									
	UNTREATED	NA	0.494									

