Studies on the impact of insecticide-treated nets on bloodfeeding and host seeking behaviour of pyrethroid resistant *Anopheles gambiae.s.l.*

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Abstract

Although the efficacy of insecticide-treated nets (ITNs) has been compromised by the emergence of resistance to pyrethroid insecticides in the primary African malaria vector species, epidemiological evidence indicates that pyrethroid ITNs still protect. This study investigated efficacy of standard ITNs, using methods that capture more precise information about mosquito behaviour at the net interface than standard WHO methods and measure a range of parameters including sublethal impacts on reproduction and longevity.

The study compared responses of four strains of *Anopheles gambiae.s.l.,* two insecticide susceptible (Kisumu and N'gousso) and two insecticide resistant (Banfora and VK7) to two pyrethroid nets, permethrin treated Olyset® Net and deltamethrin treated PermaNet 2.0. Bloodfeeding through the bednet was permitted or prevented and detailed behavioural events ranging from pre-contact repellency/contact-irritancy to duration of bloodfeeding were recorded for analysis. Video tracking experiments were conducted to characterise flight responses of mosquitoes to a human-occupied bednet and delayed or sublethal effects post-exposure were monitored.

In the baited box test, video recordings of mosquitoes feeding on a human host through an ITN, showed that for all ITNs, all mosquito strains landed and initiated bloodfeeding behaviour rapidly with no repellency evident. Bloodfeeding duration through the ITN was reduced in all strains compared to controls ($P<0.0001$). Post-feeding, all mosquitoes preferentially rested on untreated nets rather than Olyset or PermaNet 2.0 ($P<0.01$). When prevented from feeding through the ITN during exposure in the baited box assay, responses varied depending on ITN type, although generally mosquitoes spent less time in contact with treated nets. When tracking multiple free-flying *Anopheles gambiae* responding to a human-occupied bednet, activity predominantly occurred at the net roof regardless of treatment type. In the presence of an untreated net all strains displayed long-contact flight behaviours (bouncing). In comparison, flight behaviour around and Olyset net consisted of more short, infrequent contacts (visiting). These findings were true regardless of mosquito resistance profile, indicated contact irritancy to the Olyset net. Feeding rates after ITN exposure varied between the strains. Significantly less Banfora fed after exposure to Olyset in baited box assays compared to untreated controls. Significantly less VK7 also fed at 1-hour post-ITN exposure compared those exposed to untreated net in video tracking experiments. Although feeding duration was reduced through the ITN, when analysing the amount of blood ingested, results suggest that the resistant mosquito strains feed at a faster rate through both treated nets compared to untreated nets, with no significant difference in the bloodmeal volumes. No long-term detrimental effects on fertility and fecundity were observed post insecticide exposure for either resistant strain, with an observed increase in fertility after exposure to Olyset net in video tracking experiments. Finally, only those mosquitoes that did not take a blood meal had significantly shorter life spans when exposed to treated net compared to untreated. Results suggested that taking a blood meal enhanced survival chances post-ITN exposure, with no observed difference in the longevity of those mosquitoes that took a blood meal after exposure to either untreated or treated net.

Results from this study highlight the importance of assessing all impacts on behaviour and life history traits during and post ITN-exposure in order to gain a full understanding of net efficacy. Testing allowed correlation of the average amount of time spent in contact with a net and the delayed or sublethal effects post-exposure, allowing a more realistic picture of how ITNs perform in the field. Using more field relevant tests with a human host, comparison between responses of resistant and susceptible strains at different ITNs showed reduced contact and feeding duration in the presence of insecticide, something not detected by other standard tests. The detailed behavioural responses captured in these tests provide important insight into the entomological mode of action of each net type. Such tests are important for characterising the impacts of next-generation ITNs and overcoming insecticide resistance at the earliest opportunity.

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Chapter one: Introduction and Literature review

Malaria

Malaria elimination has been a goal set by the WHO since 2015, but despite progress, cases are now plateauing. Malaria is the most important vector transmitted disease, accounting for an estimated 229 million cases in 2019 (WHO, 2020) and approximately 409 000 deaths, the majority being children under the age of 15. The transmission is widespread with the most affected places being within the WHO African Region. The plateau in case numbers closely mirrors the rise of insecticide resistance in these regions (Churcher *et al.,* 2016), which has led to the WHO identifying insecticide resistance as the number one biological obstacle to malaria elimination (WHO, 2012). Resistance to insecticides can be both physiological and behavioural with most studies focusing on the former. In this thesis, the work is contributing to knowledge of behavioural resistance by developing and validating new assays to quantify the effects of this phenomenon in response to insecticide treated nets.

Figure 1.1 illustrates that it is mostly low-middle income countries affected by the disease. The disease is most prevalent in tropical and subtropical regions because of rainfall, consistent high temperatures and high humidity (Jamieson, 2006). Sub-Saharan Africa malaria mosquitoes with be the focus of this report as this is where the largest number of malaria cases and deaths occur.

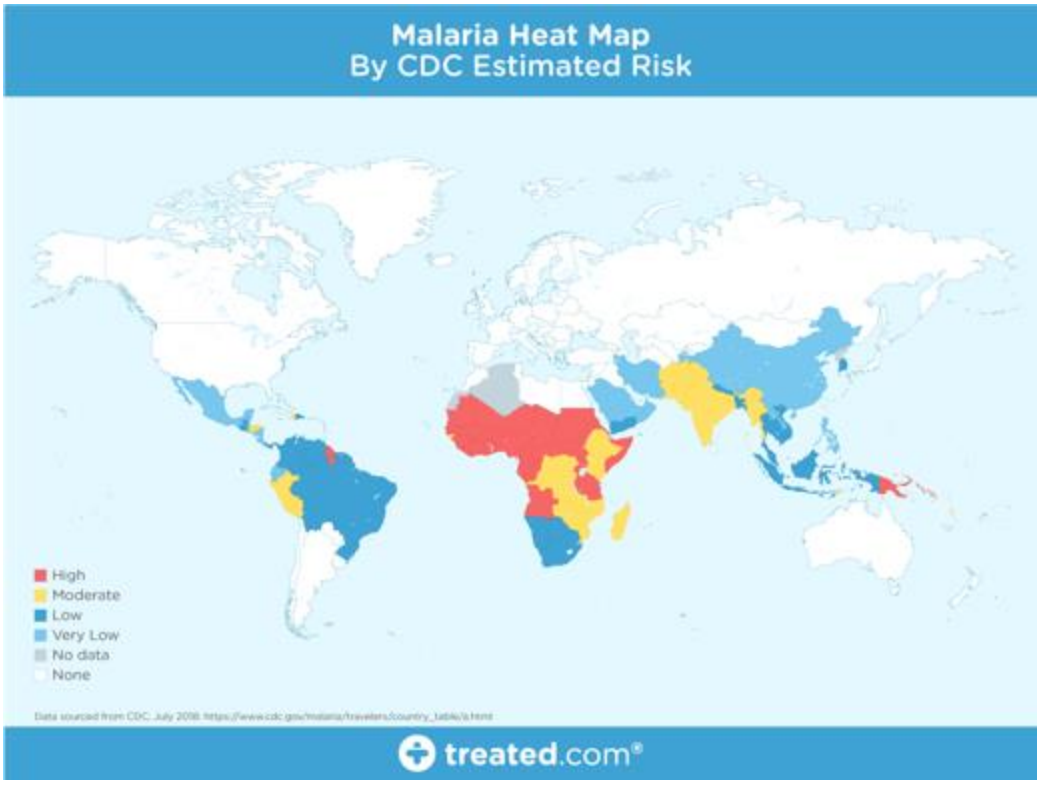


Figure 1.1: Malaria world map based on the estimated risk of malaria as defined by the US Centers for Disease Control and Prevention. CDC, 2018, where malaria occurs, viewed 20th April 2019, https://www.cdc.gov/malaria/malaria_worldwide/impact.html

Malaria is caused by the protozoan parasite *Plasmodium*. Symptoms can include severe anaemia, headaches and recurrent fever, with the worst infections progressing to cerebral malaria, which can be both disabling and life-threatening. Five species of *Plasmodium* are responsible for human disease: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi.* The malaria parasite is transmitted to the female mosquito as she takes up a bloodmeal from her host. Malarial gametocytes in the infected blood pass to the mosquito midgut where they develop into oocysts. Oocysts move through the midgut wall and once ruptured they release sporozoites which are then move to the salivary glands to be passed to a host during the next blood meal. This cycle takes around 9-14 days to complete (Ohm *et al.,* 2018; Venugopal *et al.,* 2020), depending on the species of parasite and the temperature (Figure 1.2).

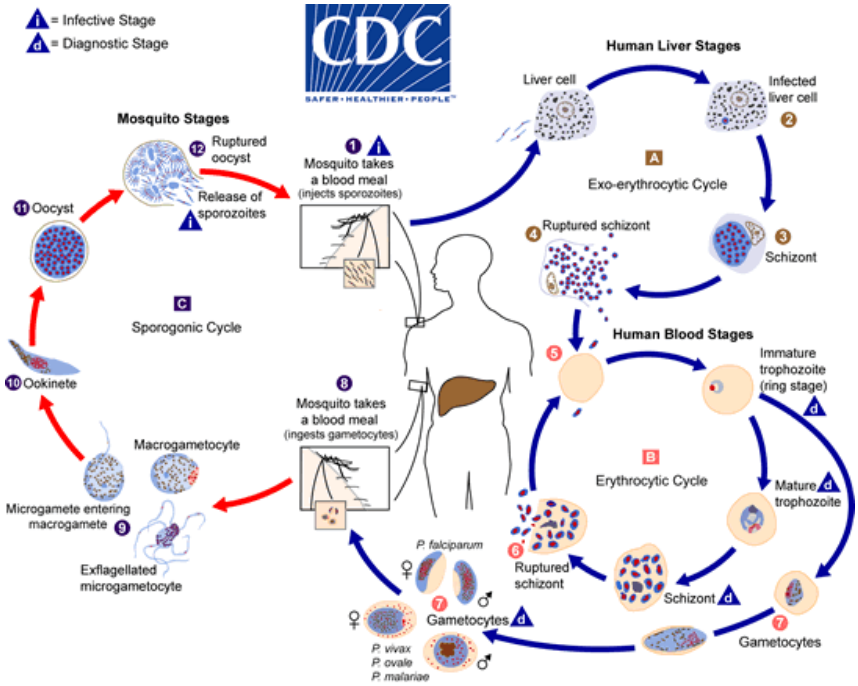


Figure 1.2: The malaria parasite lifecycle. CDC, 2018, biology, viewed on 20th April 2019 https://www.cdc.gov/malaria/about/biology/index.html

Plasmodium falciparum is the most prevalent and deadliest malaria parasite in sub-Saharan Africa, accounting for 99.7% of estimated malaria cases in 2018 (WHO, 2019). Outside of Africa, *P. vivax* is the predominant parasite, causing 75% of malaria cases in the WHO regions of America, and 53% in Southeast Asia (WHO, 2019).

Anopheles as vectors

Mosquitoes belong to the family Culicidae in the order Diptera or true flies (two-winged). There are two major subfamilies: the Anophelinae with three genera; *Bironella, Chagasia, Anopheles* and *Culicinae* with many genera including *Aedes, Mansonia* and *Culex* (Harbach, 2007).

Mosquitoes are found everywhere except Antarctica, however different areas support specific anopheline species because of the diverse environments and conditions across geographical regions. There are around 430 *Anopheles* species, with malaria being transmitted by approximately 40 different species (CDC 2021, Sinka *et al*., 2010, Sinka *et al*., 2011; Sinka *et al*., 2012).

The *Anopheles gambiae* complex comprises of eight species and includes the important African malaria vectors *Anopheles gambiae sensu stricto (s.s.), Anopheles arabiensis* and *Anopheles coluzzii* (Coetzee *et al* 2000, Gillies and Coetzee, 1987, Sinka *et al*., 2010, Thawornwattana 2018). The other

primary African malaria vector is *Anopheles funestus* which is also a species group containing eleven species including *An. funestus s.s., An. rivulorum, An. leesoni, An. vaneedeni, An. parensis, An. confusus, An. aruni, An*. *fuscivenosus*, and *An. brucei* (Coetzee and Fontenille 2004).

Anopheles sp. are also vectors of lymphatic filariasis (LF). LF is mainly caused by the infection of *Wuchereria bancrofti* throughout Africa and India, and *Brugia malayi* in the South and Southeast Asia (Simonsen*,* 2009; Simonsen *et al*., 2013). The only arbovirus to be consistently transmitted by an anopheline is O'nyong'nyong virus, which is a relatively mild infection, common throughout east Africa (Corbet *et al*., 1969; Williams *et al.,* 1965). Although studies to date have not yet provided evidence that Anopheles can maintain transmission of other viruses, a systematic review did demonstrate the potential for this species to transmit a range of other viruses to vertebrate hosts (Minkeu *et al*., 2018).

Biology and behaviour of *Anopheles* sp. mosquitoes

Life cycle

The mosquito life cycle involves four morphological stages: egg, larvae, pupae and adult. The duration and conditions of each stage ranges between the species. Anopheline mosquitoes' eggs are singularly laid on the surface of freshwater pools. The larvae have four instars and are surface feeders, they can develop into pupae as soon as 2-3 days in the tropics and 9-12 days in cooler climates (Bayoh and Lindsay 2004).

The female mosquito typically mates soon after emergence. She may not need to mate again as she can store enough sperm to fertilise all eggs she might produce throughout her life. After a blood meal, the time taken to digest and produce eggs is dependent on the temperature. Quicker lifecycles occur at higher temperatures (Service, 2012). Once gravid, the female will search for a suitable oviposition site. *Anopheles* sp. mainly lay their eggs in temporary water pools such as puddles but some species have been recorded in more stable water bodies (Ndenga *et al.,* 2011; Asmare *et al.,* 2017). The female will lay between 30-300 eggs which hatch within 2-3 days (Clements, 1999).

Female *Anopheles* are anautogenous and for development of their eggs they require a blood meal (Clements, 1999). The female mosquito takes a bloodmeal from a host and finds a preferred location to rest for a few days and digest her meal. As she digests her blood meal, the eggs develop, and the mosquito becomes gravid. When ready, she leaves the resting location to oviposit at a preferred waterbody. Once she has laid those eggs, she immediately switches to host seeking behaviour and the cycle begins again. The duration of one gonotrophic cycle (from egg-laying (*gono*) to feeding (*trophic*)) is temperature dependent, but in sub-Saharan Africa, with a temperature range of 25-36^{o}C, the gonotrophic cycle for *An. gambiae s.l.* is typically between 2 to 3 days (Lehane 1991). Malaria vectors

therefore take a blood meal approximately every three days (depending on species and number of cycles), (Scott *et al*., 2012) if the mosquito picks up a malaria infection during this feed, they must then survive past 9-14 days to pass the infection on.

Mosquito species vary in their host preference, the time and place they feed and resting location. These behaviours can vary between populations of the same species, however, most anophelines are anthropophilic, nocturnal blood feeders and many rest indoors after the completion of their feed (endophilic) (Kabbale 2013; Sinka *et al*., 2010). Some malaria vectors, such as *An. arabiensis*, mainly rest outdoors (exophilic) (Tirados *et al*., 2006; Gillies & Coetzee, 1987) and exhibit behavioural plasticity in feeding preferences. *Anopheles arabiensis* are reported to be more zoophilic than *An. gambiae s.s.*, feeding indoor and out, with biting times varying from early evening to early morning (Tirados *et al*., 2006).

An. funestus are highly anthropophilic (Temu *et al.,* 1998). This species shares late night biting patterns with *An. gambiae s.s* and is also commonly found resting indoors (Githeko *et al.,* 1996). Some species, such as *Anopheles nili* feeds both indoors and outdoors (Carnevale *et al*., 1992). Other species, such as *Anopheles moucheti,* which are found mainly in forested areas are highly endophilic (Antonio-Nkondjio *et al.,* 2002).

When exiting a house *An. gambiae* s.l. prefer to leave via the eaves (Spitzen *et al*., 2016). *Anopheles s.l* generally prefer to lay their eggs in sunlit, shallow, temporary freshwater bodies. Oviposition preference in some species has shown to be based on the salinity content of the water body with *An. coluzzii* preferring less saline waterbodies (Nwaefuna *et al*., 2019). *Anopheles funestus* mainly prefer to lay in permanent freshwater bodies with lots of vegetation (Temu *et al*., 1998).

Malaria transmission and vectorial capacity

Members of the *An. gambiae s.l.* and *An. funestus* complex are considered the most important vectors of malaria (Sinka *et al*., 2010). As competent vectors, these species have high sporozoite rates of up to 10%, driving high transmission during the wet season. In some areas, however *An. arabiensis* can be the main drivers of transmission, if feeding all year round occurs. It is for these reasons, all Anopheles species mentioned above contribute to the malaria burden (Mzilahowa *et al*., 2012).

Vectorial capacity is defined as the ability of a mosquito to transmit an infectious pathogen as described in Equation 1:

$$C = \frac{ma^2p^n}{-\ln(p)} \quad (1)$$

The vectoral capacity (C) is calculated on the basis of; vector density (m), mosquito longevity (p), successful biting rate (a) the parasite's extrinsic incubation period (EIP, n days), (Dye, 1992; Garret-Jones & Shidrawi 1969).

The equation evaluates the overall potential number of infectious bites arising from one infected person per day. As discussed previously, the *Anopheles* mosquito is an important malaria vector as it takes a blood meal every 2 to 4 days and many of the *Anopheles* are anthropophilic. Development of the extrinsic stage of the *Plasmodium* parasite can take between 9 to 16 days (Beier, 1998; Paaijmans *et al.,* 2010; Vaughan, 2007) meaning only the older mosquitoes can transmit the disease. With vectorial capacity relying heavily on mosquito survival, control tools targeting the adult mosquito are highly effective (Brady 2016, Smith 2012).

Treated nets for example directly reduce the longevity of the mosquito due to insecticide induced toxicity (p). They are likely to reduce both the overall vector population density (m) and successful biting rates (a) with the later function of nets leading to a wider community effect, whereby non-net users are protected from a lower number of infectious bites (Okumu *et al*., 2013).

Malaria prevention and control in Africa

Although a range of methods are available and recommended variously in different contexts (WHO. 2019), current malaria prevention and control in Africa comprises of three core interventions, insecticide residual spraying (IRS), long lasting insecticide-treated nets (LLINs) and artemisinin-based combination therapy (ACT). These are considered to have made a major contribution to the reduction in malaria burden since 2000 (WHO, 2017). All three interventions were estimated to have prevented 663 million cases of clinical malaria between 2000 and 2015 (Figure 3; Bhatt *et al*., 2015)

ACT Is the administration of fast acting artemisinin-based compounds combined with another drug class such as lumefantrine, mefloquine, pyrimethamine or piperaquine. The use of ACTs has made important contributions to reducing prevalence and incidence of malaria cases. During 2010–2017, 1.45 billion ACT treatment courses were delivered in malaria endemic regions (WHO, 2018). However, although important to controlling the disease, the primary role of ACTs is in prevention of severe disease and death from uncomplicated infections, rather than reducing transmission.

IRS is a control intervention that involves spraying the inside walls of dwellings with insecticides. This control strategy targets the adult mosquito stages. The insecticide on the walls kills the mosquitoes

that contact the treated surface when resting indoors. IRS was estimated to provide protection for approximately 93 million people at risk of malaria infection in 2018, providing a global protection of around 2% (WHO, 2019). Unlike bednets, IRS does not directly prevent the mosquitoes from biting people, instead, this control tool targets recently fed mosquitoes resting inside the home. Therefore, to be an effective control measure, IRS programmes require high coverage (usually 80%) and retreatment is required once or twice a year (CDC 2019, WHO, 2019).

Bednets act as a physical barrier, preventing access of the mosquitoes and thus providing personal protection against malaria to the individual under the net. Treated nets can provide an extra chemical barrier, killing the mosquitoes that contact the net and enhancing the effectiveness of bednets, reducing further human-vector contacts by the induced mortality. Figure 1.3 represents the different methods both IRS and insecticide treated bed nets target the mosquitoes entering a human baited hut. Insecticide treated nets may also enhance protection by causing sublethal-lethal impacts that effect the mosquitoes vectoral capacity. These effects will be discussed in more detail below.

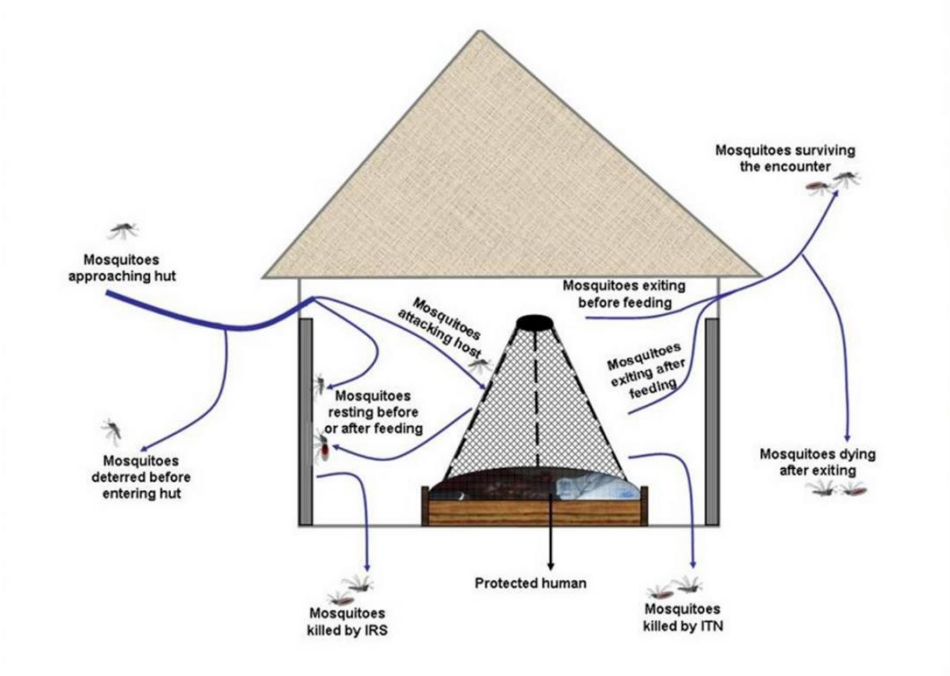


Figure 1.3: A diagrammatic representation of various effects of insecticide-treated nets (ITNs) and indoor residual spraying (IRS) on mosquitoes that enter or attempt to enter houses. Image taken from Okumu et al., 2020.

Bednets have been proven to be by far the most important control intervention across Africa (Figure 4; Bhatt *et al*., 2015). Between 2000 and 2015, 2 billion insecticide-treated mosquito nets (ITNs) were distributed globally. Of this, the majority were delivered in sub-Saharan Africa (WHO, 2017) where

malaria case incidence decreased by 50%. Bhatt *et al.* (2015) found that two thirds of this reduction were mainly attributed to treated net use, with nets contributing to 63% of averted cases (Bhatt *et al.,* 2015). Since then, in 2018, 197 million nets were distributed by manufacturers, with more than 87% of nets being delivered in sub-Saharan Africa countries (WHO 2019). The WHO estimated that half the at-risk population in sub-Saharan Africa slept underneath a bednet in 2018 and households owning at least one bednet increased in the past year to 72% (WHO 2019). Coverage of nets is increasing, but at a slower rate over recent years and the WHO states that they are far from reaching the universal coverage target of 80% (WHO 2019). Insecticide treated nets will be discussed in more detail below.

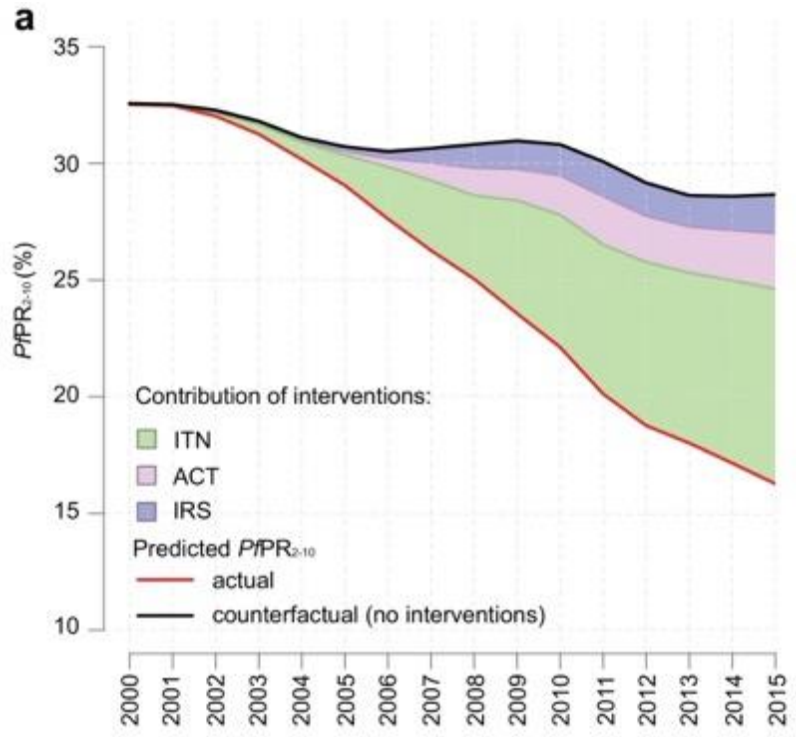


Figure 1.4: the predicted cumulative number of clinical cases averted by each intervention at the end of each year (Bhatt *et al*., 2015).

Insecticide residual spraying

As mentioned above indoor residual spraying involves the application of residual insecticide to the walls and other surfaces of a house and is used to target endophagic and endophilic adult mosquitoes. In the past year, global protection from IRS reduced to just 2%. (WHO 2019). Despite this, the core interventions recommended by WHO for malaria control continues to be either sleeping under a bednet or indoor residual spraying (IRS) (WHO 2019).

The launch of the Global Malaria Eradication Campaign recommended the use of indoor residual spraying (IRS), primarily with DDT together in 1955 (WHO 2008). this contributed to large efforts in the elimination and reduction of malaria (Casida & Quistad, 1998; Lengeler, 2003; Mabaso *et al*., 2004; Roberts *et al*., 2010). Despite this, IRS has declined over time, due to lack of government funding and rising concerns regarding reports on the harmful effects of DDT use on the environment (WHO 2000, Gunter, 1998). Although DDT was one the longest lasting IRS products in the past, remaining effective for over 6 months after application, the Stockholm Convention on Persistent Organic Pollutants (2010) encouraged a switch from the use of DDT to other available insecticides. By 2014, 80% of IRS campaigns used pyrethroids (Hemingway, 2014), although in current years the use of non-pyrethroid IRS has become more common due to the emergence of insecticide resistance (Haji, 2015; Oxborough, 2016).

Applying IRS to the walls, reduces the amount of human-insecticide contact compared to other control tools such as ITNs. IRS consequently has the advantage of being able to use a wider range of chemistries such as carbamates and organophosphates, to target pyrethroid-resistant mosquitoes than other control methods. A range of chemicals now have approval for use in IRS including pyrethroids, carbamates, organophosphates, organochlorines and neonicotinoids (WHO, 2016), although resistance to the majority of these chemistries does also exist (Ranson, 2016).

For IRS to be effective, high household coverage is required and with the residual bio-efficacy of 6-9 months for most of the commercially available products, retreatment is required multiple times a year (Sadasivaiah *et al*., 2007). Spraying IRS is expensive, logistically complicated and relies on collaboration of specially trained teams alongside community acceptance. As a result of all these factors, in order to maintain the effectiveness of an IRS program the timing of spray campaigns is extremely important. Spraying should be completed before transmission peaks, with early interventions proving more effective and cost efficient than the routine spraying which is performed every year (Worrall *et al*., 2007). With the correct coverage, IRS provides a high level of community protection, but unlike nets, it does not provide the personal protection of a barrier after the initial insecticidal effects are reduced (Protopopoff *et al*., 2018).

The development of new IRS products containing either repurposed active ingredients or a mixture of two compounds, aims to combat some of the challenges detailed above. Actellic® contains the organophosphate pirimiphos-methyl and with its launch in 2012, it became the first non-pyrethroid IRS promoted for tackling insecticide resistant mosquito populations. Sumishield® was the second to be released in 2017 (Sumitomo Chemical- containing clothianidin a neonicotinoid) and Fludora®

Fusion was the third in 2018 (Bayer - containing two active ingredients , clothianidin and deltamethrin).

IRS and ACT used directly with ITNs have shown to increase effectiveness of control (Bhatt *et al*., 2015; Fullman *et al*., 2013; Kleinschmidt, 2009). However, previous studies have shown that the addition of IRS in combination with ITNs has little to no effect on malaria incidence, parasite prevalence and anaemia incidence compared to ITN use alone and this is reflected in the WHO guidance (Choi *et al*., 2019; WHO 2019).

As mentioned previously, controlling the adult stage of the mosquito lifecycle is considered the most effective way of reducing malaria (Bhatt *et al*., 2015; Killeen *et al*., 2000, Koella, 1991; Giardina *et al*., 2014; Walker 2002). This technique is favoured over other techniques due to rapid success in reducing community-level transmission, the ease of implementation and cost effectiveness of these control techniques (Bhatt *et al*., 2015, Lengeler 2004, Hanson 2003, Goodman 1999, WHO 2015, WHO 2019). In a few specific settings and circumstances, ITNs, IRS and ACT use could also be supplemented by additional other vector control methods as described below.

Other vector control methods

Larval control

In some transmission settings control of the larval stage has been shown to be effective (Choi *et al*., 2019). Larval control of malaria vectors targets the oviposition sites of the mosquito. Larval control may be implemented through environmental modification, by draining or removing oviposition sites, through biological or microbial control, or using chemical applications to reduce the mosquito population density before they reach maturity.

Some studies have shown larval habitat spraying to be associated with lower malaria incidence (Choi *et al.,* 2019). This control is based on applying insecticides such as Temephos and Methoprene targeting the central nervous system or inhibiting growth of the immature mosquito stages. *Bacillus thuringiensis israelensis* (BTI) has also been used in the same way to reduce larval populations. BTI produces toxins which are effective in killing the immature stages of the mosquito (Lacey, 2007). BTI and temephos both need reapplication to remain effective (Shililu *et al*., 2003).

Draining of larval habitats has also been used in the attempt to reduce malaria transmission, however due to the large range of mosquito oviposition sites this method has proven unsuccessful over large regions. Ultimately this method of control is only capable of reducing larvae in localised areas, with

limited success when used alone over vast areas (Killeen *et al.,* 2002; Service, 2012; Shousha *et al*., 1948).

Larviciding is however recommended by the WHO as a supplementary control tool (WHO, 2013), but it is not widely adopted as a method due to the issues described above. The main advantage of this method is that it does target mosquitoes when biting outdoors, targeting residual malaria transmission (Choi *et al*., 2019; Killeen, 2014). In conclusion, it is not known if larviciding over large water bodies has any impact on malaria transmission (Choi *et al.,* 2019).

House screening

Anopheles gambiae s.l escape and enter through windows and openings in the home (Ogoma *et al.,* 2010). Evidence shows that they fly upwards when encountering the dwelling surface (Mahande *et al*., 2007; Snow *et al*., 1987) and odours rising from the hosts inside the home act as an attractant to mosquitoes entering between the eave gap between the wall and the roof (Lindsay & Snow 1988). Mosquito-proofing houses has been proposed as an effective way to reduce mosquito densities inside the home and ultimately reduce overall biting rates (Lindsay, 2003; Wanzirah *et al*., 2015). Specifically, house screening is thought to be effective against 'early exiting mosquitoes', those vectors entering houses but then rapidly exiting when encountering control tools such as IRS and ITNs, often not receiving a lethal dose of insecticide (Killeen, 2011). Studies have shown a positive correlation between city-wide window screening interventions and a reduction in malaria prevalence and vector biting densities in these areas (Chanda *et al.,* 2019)

Screening eaves has been proven an effective way to decrease malaria transmission indoors, with windows and door screens providing a reduction in mosquito densities inside the home (Lindsay *et al*., 2003; Njie *et al.,* 2014; Ogoma *et al*., 2010). The use of screening has also been investigated with pyrethroid (lambda-cypermethrin) and organophosphate (pirimiphos-methyl) treated netting. This was shown to be effective against *Anopheles funestus* mosquitoes resistant to pyrethroids, carbamates and organochlorines and to a greater extent, effective against pyrethroid-resistant, early exiting *An. arabiensis* mosquitoes (Killeen *et al*., 2017). Screens with pirimiphos-methyl killed greater proportions of both vectors than lambda-cyhalothrin alone or lambda-cyhalothrin plus pirimiphos-methyl (Killeen *et al*., 2017). This evidence has aided the development of control strategies to close eaves and the screening of doors and windows to reduce densities of indoor biting mosquitoes reducing human-mosquito contact (Mburu *et al.,* 2018; Ogoma *et al*., 2010; Wanzirah *et al*., 2015). More recently the development and evaluation of eave tubes has shown promising results in

suppressing mosquito populations (Barreaux *et al.,* 2019; Snetselaar *et al.,* 2017; Sternberg *et al.,* 2016; Sternberg *et al.,* 2018; Sternberg et al., 2021). Overall better housing can reduce risk of malaria (Lwetoijera *et al.,* 2013; Wanzirah *et al.,* 2015; Von Seidlein *et al.,* 2019) with studies in the past suggesting this control tool will work best in areas with low to moderate transmission and when used in combination with other malaria control strategies (Lindsay *et al*., 2003).

Endectocides

Endectocidal drugs are toxic to *Anopheles* mosquitoes when feeding on a host that has recently taken the drug (Chaccour *et al*., 2010, Foy *et al*., 2011). Endectocidal drugs such as ivermectin, diflubenzuron, eprinomectin and fipronil were effective against *Anopheles* in field assessments of treated cattle (Fritz et al., 2009; Poché *et al*., 2015). The drugs do not only cause mortality, but also affect fertility and fecundity of the adult mosquito (Pooda *et al*., 2015).

Domestic livestock treated with deltamethrin is also used as an effective control measure (Rowland *et al*., 2001). This control is thought to work best as part of an integrated disease management program and can also be used to target other neglected tropical diseases. A better understanding of the range of livestock treatments and the effects these have against disease vectors and how these then effect malaria ecology is needed (Foy *et al*., 2011).

Repellents

Insect repellents are defined as a substance with an active ingredient that deter the insect from approaching or settling on a host but does not necessarily kill them (WHO, 2017). Repellents are used for malaria control to reduce human-mosquito contact and reduce mosquito bites. There are three different types of repellent: topical, impregnated clothing and spatial repellents (Maia *et al.,* 2018).

Currently there is insufficient evidence that repellents have a large impact in preventing malaria transmission (Maia *et al*., 2018). It is hypothesized that since repellents do not always kill mosquitoes, there is increased risk to unprotected hosts (Moore *et al*., 2007). To understand the role and importance of repellents in controlling malaria infection there is a need for standardisation of the methodologies used when evaluating repellent products and a greater understanding required of the mode of action of these control tools (Maia *et al*., 2018; Ogoma *et al*., 2012).

Topical repellents

Topical repellents are applied directly to the skin and a wide range of formulations are available for mosquito control. Topical repellents commonly used include DEET, IR3535 and Icaridin (WHO, 2017). Approximately 200 million people worldwide use products containing DEET every year (Banard *et al.,* 2006; Klun *et al.,* 2000; WHOPES, 2000) but its mode of action remains poorly understood. Some studies indicate that DEET interferes with the mosquito's ability to locate lactic acid, a well-known host seeking cue (Dogan *et al.,* 1999), with other hypotheses suggesting that mosquitoes have olfactory receptors with the ability to detect DEET and ultimately avoid it (Syed & Leal, 2008).

Though topical repellents offer effective protection against outdoor biting mosquitoes during the day, protection is short-lived, lasting a maximum of 5-6 hours depending on formulation (Fradin, 2002; Lindsay *et al.* 1998). The efficacy of DEET varies depending on the age of the mosquito, with DEET inducing significantly higher blood-feeding inhibition in old females (Mulatier *et al.,* 2018).

Although topical repellents provide individual protection against mosquito bites, there is limited evidence to suggest they provide effective protection against malaria (Maia et al., 2018; Wilson *et al*., 2014).

Insecticide Impregnated clothing

Insecticide treated clothing (ITC) is most commonly used by military forces and used for outdoor recreational purposes (Croft *et al.,* 2001; Frances *et al.,* 2007; Kitchen *et al.,* 2009; Londono-Renteria *et al.,* 2015). Wearing permethrin-impregnated clothing reduces mosquito biting rates (Banks *et al*., 2014; Harbach *et al.,* 1990; Maia *et al.,* 2018; Orsborne *et al.,* 2016) and like topical repellents, ITC can be effective in providing personal protection. However, further investigations are required to accurately demonstrate the effect this control measure has on reducing malaria transmission (Banks *et al*., 2014; Maia *et al.,* 2018).

Spatial repellents

Spatial repellents provide a protective area, where the active ingredient is dispersed into the air. Spatial repellents, like topically applied repellents, are thought to interfere with the mosquito's ability to locate a host or reduce human-mosquito contact due to their excito-repellency effects (Maia *et al.,*

2018). Dispersal of spatial repellents is achieved via heat (coils and electric emanators) or evaporation (treated materials such as papers and gels).

Much like most control tools targeting the adult mosquito, the most commonly-used spatial repellents are pyrethroids. The unique benefit of spatial repellents is that not only can they be used to target outdoor and indoor biting mosquitoes, but they also create a protective area for all individuals occupying that space, creating greater protection against human-vector encounters (Achee *et al.,* 2012; Ogoma *et al.,* 2012). The volatile effects of spatial repellents are not only lethal to the mosquito but can affect their host seeking behaviours, ultimately reducing human-mosquito contacts. Metofluthrin has been shown to be effective in reducing landings and inducing mortality in outdoor biting mosquitoes (Charlwood *et al.,* 2016; Kawada *et al.,* 2004). Transfluthrin treated materials/coils are effective in delaying and ultimately reducing bloodfeeding activity (Andrés *et al.,* 2015; Ogoma *et al.,* 2014).

Even though spatial repellents offer a degree of personal protection to the individual, the overall protection this control tool provides against malaria infection is limited as these repellents can ultimately divert malaria vectors to those who are not protected by them (Maia *et al*., 2016). With most studies of special repellents missing epidemiological data post-treatment, there is the need for more trials integrating how these repellents work in the field and the effect they have on infection incidence (Achee *et al*., 2012; Maia *et al*., 2018).

Genetically-modified mosquitoes

The aim of genetically-modified (GM) mosquitoes as a malaria control intervention is to substitute the native or wild mosquito with mosquitoes unable to transmit the *Plasmodium* parasite or ultimately reduce the number of competent vectors (Scott *et al*., 2002). The success of this approach therefore relies on the competitiveness and survival of the GM mosquitoes, and ability to spread through the released environment (Boëte & Koella, 2002).

Anopheles mosquitoes have been engineered to express an antiparasitic gene in their midgut and salivary glands (Catteruccia *et al.,* 2003; Grossman *et al.,* 2001; Ito *et al*., 2002; Moreira *et al.,* 2002). This has been demonstrated to significantly reduce vector competence and make the mosquitoes inefficient disease vectors in the laboratory (Carballar-Lejarazú and James 2017; Catteruccia *et al*., 2000).

Other methods include the use of sterilised males to supress the mosquito population; releasing them into the wild reduces mating success of the wild type mosquitoes and therefore decreases the overall mosquito population. GM sterilised *Aedes aegypti* populations have been trailed in outdoor cages and open releases by Oxitec in Malaysia, Brazil and Cayman Islands between 2009- 2015 but as yet there have been no field releases of GM *Anopheles*.

Both techniques described above rely heavily on public acceptance in order to be used widely as a control tool. Some criticism has recently arisen based on the detection of transgenic genome within the natural population in Brazil (Evans *et al.,* 2019). To gain confidence and full public acceptance, further studies are needed to provide proof of concepts and accurately predict the effects of transgenic mosquitoes in the natural environments and the affect they can have on disease transmission (Evans *et al.,* 2019; Facchinelli *et al.,* 2019).

Wolbachia

Wolbachia is a symbiotic bacterium found in many diverse insects that can be used in vector control (Tantowijoyo *et al.,* 2020). The presence of *Wolbachia* has been shown to lower the transmission potential of many arboviruses (Moreira *et al.,* 2009; Walker *et al.,* 2011). *Aedes* mosquitoes infected with *Wolbachia* spp. displayed resistance to the dengue virus (Hoffmann *et al*., 2011, Xi *et al*., 2005) and *Wolbachia* wAlbB has also been shown to confer elevated resistance to *Plasmodium* infection in *An. stephensi* adults (Bian *et al*., 2013).

This control intervention has been tested in large field trials in a number of countries by the World Mosquito Program (WMP) but like GM, it relies on many factors such as the survival of the released mosquitoes and their ability to spread among the wild population (Hoffaman *et al.,* 2015). Public acceptance of this control tool is also an important factor (Hoffman *et al.,* 2015; Tantowijoyo *et al*., 2020).

After initial studies in northern Australia (Hoffmann *et al.,* 2014), in Yogyakarta, Indonesia, Tantowijoyo *et al.,* (2020) demonstrated the successful deployment of *Wolbachia* wMel strain for control of dengue with ample community support (Tantowijoyo *et al.,* 2020).

Insecticidal bednets

A successful bednet intervention targets the mosquito during host seeking, providing a physical barrier that reduces the sleeper's risk of being bitten. The host beneath the net also attracts the

mosquito to the insecticide treated net creating an effective human-baited lethal mosquito trap (Killeen *et al*., 2006; Snow, 1970).

Historically bednets were used as protection against mosquitoes during war periods to protect the soldiers against vector-borne diseases (Curtis *et al*., 1991 and Lindsay *et al*., 1988). In the late 1970s, entomologists discovered the use of synthetic pyrethroids which were found to be low in toxicity to mammals and high in insecticidal activity. The idea of impregnating bednets with pyrethroid insecticides was developed in the 1980s. Insecticide treated nets were proven to be safe for regular human contact and domestic handling, they were rapidly acting and successful in reducing mosquito feeding through the netting (Curtis *et al*., 1992; Curtis *et al*., 1996; Lines *et al*., 1996 and Rozendaal *et al*., 1989).

A Cochrane review in 2004 showed treated nets provide 17% protective efficacy compared to no net and 23% compared to an untreated net when protecting children under 5. The review concluded that for every 1000 children protected by a net, an average of 5.5 lives can be saved in areas where there is a stable malaria infection rate (Lengeler *et al*., 2004), showing the massive impact ITNs can have in reducing malaria mortality especially for the vulnerable population (Lengeler *et al*., 2004).

Insecticide treated bednets, although effective, lose their activity after washing and general use (Gimnig, 2003; Vantandoost *et al*., 2009) leading to the need for retreatment. This subsequently resulted in the development of Long-Lasting Insecticide treated nets (LLINs). These nets contain insecticide incorporated within the fibres or soaked onto the surfaces of the fibres using resins. The WHO guidelines stipulate that an LLIN must sustain effective biological activity, by killing mosquitoes after use for 3 years and obviating the need for re-treatment for at least 20 washes (WHO, 2007). In practice, LLINs have been reported to last between 1-5 years, with an average survival of approximately two years (Ahogni *et al*., 2020; Gnanguenon *et al*., 2014; Hakizimana *et al*., 2014; Kilian *et al*., 2021; Solomon *et al*., 2018;). This lifespan of a net varied based on factors such as number of washes, maintenance and use of the nets (Gnanguenon *et al.,* 2014).

A full list of nets prequalified by WHO and their active ingredient is included in Table 1.1 and below in Table 1.2. These can be divided into standard pyrethroid-only nets (Table 1.1) and 'next generation' or 'dual active ingredient' nets (Table 2.1) that contain pyrethroids plus a second synergist or insecticide (see next generation nets section).

Table 1.1: A full list of pyrethroid only nets, their manufacturer and the active ingredient

Product name	Date of WHO PQ	Manufacturer	AI
Olyset Net	07/12/2017	Sumitomo Chemical Co., Ltd	Permethrin
Interceptor	08/12/2017	BASF SE	Alpha-cypermethrin
Royal Sentry	07/12/2017	Disease Control Technology, LLC	Alpha-cypermethrin
Royal Sentry 2.0	06/02/2019	Disease Control Technology, LLC	Alpha-cypermethrin
PermaNet 2.0	08/12/2017	Vestergaard S.A.	Deltamethrin
Duranet LLIN	07/12/2017	Shobikaa Impex Private Limited	Alpha-cypermethrin
MiraNet	21/02/2018	A to Z Textile Mills Ltd	Alpha-cypermethrin
MAGNet	19/02/2018	V.K.A. Polymers Pvt Ltd	Alpha-cypermethrin
Yahe LN	19/02/2018	Fujian Yamei Industry & Trade Co Ltd	Deltamethrin
SafeNet	19/02/2018	Mainpol GmbH	Alpha-cypermethrin
Yorkool LN	19/02/2018	Tianjin Yorkool International Trading Co., Ltd	Deltamethrin
Panda Net 2.0 LLIN	03/05/2018	LIFE IDEAS Biological Technology Co., Ltd.	Deltamethrin
Tsara	14/08/2020	NRS Moon netting FZE	Deltamethrin
Tsara Soft	09/10/2020	NRS Moon netting FZE	Deltamethrin

This thesis focused on two pyrethroid only nets (Olyset Net and PermaNet 2.0), to gain a baseline understanding of the standard bednets used in the field. PermaNet 2.0 (Vestergaard, Lausanne), a deltamethrin-treated polyester net, is one of the most widely used bednets worldwide. Olyset Net is also widely used in the field but is different to the PermaNet 2.0 as it is a polyethylene net coated with permethrin. Furthermore, permethrin and deltamethrin were chosen for comparison as previous studies have shown both pyrethroids to act differently against anopheline mosquitoes (Hodjati *et al*., 2003; Siegert *et al*., 2009). Regardless of the differences in materials, both nets have been very effective against pyrethroid-susceptible vectors (Haji *et al*., 2020; Malima *et al.,* 2008; Sood *et al.,* 2014; Tamari *et al.,* 2020) but still, a greater understanding is needed on how insecticide resistance effects net efficacy. The phenotypic profile of all four strains used in this thesis is completed routinely in LSTM, providing supporting information on the resistance against these two bednet types (Williams *et al*., 2019).

Olyset

Olyset was the first commercial LLIN net to be approved by the WHO in 2001. Olyset net is a polyethylene net with permethrin incorporated (800 mg/m²) into its fibres. It Is known as a category 2 net which means the netting is made of polyethylene monofilament yarn, with permethrin contained throughout the material.

Under laboratory conditions Olyset net was proven to be effective against a variety of different mosquito species (Ansari *et al*., 2006; Jeyalaksmi *et al*., 2006; Rafinejad *et al*., 2008; Sharma *et al*.,

2006; Sood *et al*., 2011; Sreehari *et al*., 2009). Semi field and experimental hut trails proved Olyset to be effective against dengue and malaria vectors even after repeated washing (Ikeshoji & Bakotee 1996; Itoh et & Okuno 1996; Itoh *et al*., 2009; Lengeler 1998; Maxwell *et al*., 2006; N'Guessan *et al*., 2001; Njunwa *et al*., 1996; Vythilingam *et al*., 1996) and the nets have proven to remain effective after extensive use in the field and washes (Dev *et al*., 2010; Jeyalakshmi *et al*., 2006; Sharma *et al*., 2009; Tami *et al*., 2004). However not all studies have shown this long-lasting effect in the laboratory or in the field, with some suggesting Olyset nets have a long regeneration time and must be heated to restore efficacy after washing (Gimnig *et al*., 2005; Haji *et al*., 2020; Lindblade *et al*., 2005; Mejía *et al*., 2013). The initial release of Olyset nets in the field had a major impact on malaria vectors and disease burden, inducing vector mortality and reducing overall mosquito blood-feeding rates (Dev *et al*., 2010; Djènontin, 2015; Gouissi *et al.,* 2012; Itoh *et al*., 2009; Kobayashi *et al*., 2004; Soleimani-Ahmadi *et al*., 2012; Sharma *et al*., 2009).

Over the years, Olyset nets have shown to have deterrent effects against wild *Anopheles*, with observations reporting reductions in indoor resting and house entry rates (Gunasekaran *et al*., 2016; Gouissi *et al*., 2012; N'Guessan *et al*., 2008; Sharma *et al*., 2009; Siegert *et al*., 2009; Soleimani-Ahmadi *et al*., 2012). Olyset results in significantly lower density of mosquitoes inside the home (Dabiré *et al*., 2006; Sharma *et al*., 2006) and evidence suggests exophilic behaviour increased in the presence of Olyset nets (Koffi *et al*., 2015; N'Guessan *et al*., 2008).

Pyrethroid resistance potentially impacts the effectiveness of Olyset nets. (Djènontin *et al*., 2015; Gunasekaran *et al*., 2016; Koffi *et al*., 2015; N'guessan *et al*., 2008; Ngufor *et al*., 2016; Okia *et al*., 2013; Tiono *et al*., 2018). However, despite growing concerns of insecticide resistance, some trials have shown that Olyset nets are still effective at reducing malaria transmission and host/vector contact (Dabiré *et al*., 2006; Henry *et al*., 1999; Kleinschmidt *et al*., 2018; Tamari *et al*., 2020), but this varies depending on region (Janko *et al*., 2018; Levitz *et al*., 2018).

PermaNet 2.0

The second LLIN to receive a full WHO recommendation was the PermaNet 2.0, in 2008. PermaNet is a polyester net coated with deltamethrin containing 55 mg/m². This is known as a Category 1 net, the netting is made of polyester 75 or 100 denier yarn that has insecticide coated onto its surface (Service, 2011).

This bednet has been shown to be effective with long-lasting bio-efficacy (Anshebo *et al*., 2014; Beng, 2014; Fettene *et al*., 2009; Graham *et al*., 2005; Jaramillo, 2011; Kayedi *et al*., 2017; Kroeger *et al*., 2004; Sreehari *et al*., 2007). Not all studies have shown PermaNet to be as effective after washing

(Agossa *et al*., 2014). PermaNet 2.0 has however been shown to remain serviceable longer than Olyset net in some field trails (Haji *et al*., 2020; Jaramillo, 2011).

As with Olyset net, PermaNet has been shown to inhibit bloodfeeding behaviour (Beng, 2014; Menze *et al*., 2020; Prakash *et al*., 2009). Experimental hut trials have reported lower entry rates into huts containing PermaNet compared with untreated nets alongside an increased rate of exophily in response to this net type (Menze *et al*., 2020). However, other studies have concluded that there are no spatial repellent effects of this net type (Cooperband and Allan, 2009; Parker *et al*., 2015; Parker *et al*., 2017; Spitzen *et al*., 2014; Spitzen *et al*., 2017). More details on the behavioural effects of ITN exposure will be discussed below.

PermaNet is effective at inducing high levels of mortality against susceptible mosquitoes (Kweka *et al*., 2017; Tungu *et al*., 2010), however as with other pyrethroid only nets this effect is reduced against resistant mosquito populations (Anshebo *et al*., 2014; Asale *et al*., 2014; Awolola *et al*., 2014; Bamou *et al*., 2021 ; Glunt *et al*., 2015; Koudou *et al*., 2011; Mahama *et al*., 2007; N'guessan *et al*., 2010; Riveron *et al*., 2018; Tchakounte *et al*., 2019), depending on the population (Bortel *et al*., 2009; Omondi *et al*., 2017). Glunt *et al*. (2017) suggest that exposure to bednets still reduces subsequent host seeking success of resistant populations, therefore still providing protection against resistant populations. This alongside other sublethal effects such as reduced longevity after ITN exposure may explain how pyrethroid only nets may still be providing a level of protection against insecticide resistance mosquito populations (Hughes *et al*., 2020; Tchakounte *et al*., 2019). Further studies are needed to evaluate the impact of treated nets on malaria infection in the areas where mosquito populations are now resistant to insecticides (Kleinschmidt *et al*., 2018).

LLIN integrity

Current malaria control strategies rely on the long-lasting bio-efficiency, or durability, of bednets. According to the WHO, long-lasting insecticidal nets (LLINs) are expected to retain their biological activity for at least 3 years when used under field conditions (WHO, 2017).

There are two factors that can affect the longevity of treated nets, aging of nets which can reduce the amount of insecticidal effects as well as physical damage caused to the net over time such as holes and tears in the side. Although as discussed above the insecticidal activity of standard LLINs has been shown to last longer than 3 years, studies in several countries have shown that the physical integrity of nets does not exceed that of the insecticidal activity (Gimnig *et al*., 2005; Kilian *et al.,*

2015; Mansiangi *et al*., 2020; Massue *et al*., 2016; Mejía *et al*., 2013; Morgan *et al.,* 2015; Tami *et al*., 2004; Tan *et al*., 2016). Morgan *et al*. (2015) and others have concluded physical damage to nets was evident after just 1 year of use, however as with other studies, variation is observed between brand of net (Haji *et al*., 2020; Morgan *et al*., 2015; Wills *et al*., 2013).

Holes in old nets have been suggested to reduce the effectiveness of ITNs (Asidi *et al.,* 2012; Irish *et al.,* 2008; Mejía *et al.,* 2013; Sutcliffe and Colborn 2015). The personal protection by both ITNs and untreated nets has been shown to decrease exponentially with increasing holed surface area (Randriamaherijaona, 2015). And there is a trend for increase infection in infection rates and the deterioration of nets (Rehman *et al.,* 2011). However, the overall impact that holed nets have on malaria transmission is still inconclusive, with location of the hole thought to play an important role when assessing the effectiveness of damaged nets (Sutcliffe *et al.,* 2017).

Insecticide resistance

Future success of malaria control is threatened by insecticide resistance. Insecticide resistance is the ability of a mosquito to survive the exposure of a standardised dose of insecticide (WHO, 2016). With a limited number of chemistries available for malaria control and the pyrethroid class currently applied on all insecticide treated bednets (GPIRM, 2012; Zaim *et al*., 2000), resistance poses a major threat to malaria control.

Resistance to DDT was first observed in the 1950s (Lividas, 1953). Pyrethroid resistance in *Anopheles* was first discovered in Côte d'Ivoire in 1993 (Elissa *et al*., 1993). Resistance to four classes of the WHO approved insecticides (carbamates, organochlorines, organophosphates, pyrethroids) commonly used in public health is now widespread (WHO, 2019). Between 2018–2020, 73 of 81 malaria endemic countries reported resistance to at least one insecticide class used for malaria control and 26 countries reported resistance to all four (WHO, 2019).

Insecticide resistance is classified into four main characterised mechanisms: target site, metabolic, cuticular and behavioural resistance (Ranson, 2011). Sequestration has also recently been shown to be a key mechanism in resistance to pyrethroids, including SAP2 and the hexamerin and a-crystallin families (Ingham *et al*., 2018; Ingham *et al*., 2020).

Target site resistance

Target site resistance occurs when there is a point mutation at the target site of the insecticide, reducing their toxicity by reduced binding efficiency (Ranson *et al*., 2000*). Para,* a voltage-gated sodium channel is the binding site of both pyrethroids and DDT (Davies, 2007). Mutations within this region are therefore associated with reduced sensitivity to pyrethroids and DDT in resistant insect strains. The two main mutations identified in *An. gambiae* that confer target site resistance to pyrethroids and DDT are the leucine–phenylalanine substitution at position 995 (L995F) and leucine–serine substitution (L995S), known as *kdr* West and East respectively due to historic geographical distribution on the African continent (Martinez-Torres *et al*., 1998; Ranson 2000). A further mutation N1575Y has been strongly associated with insecticide insensitivity (Edi *et al*., 2017; Jones *et al*., 2012). More recently, several SNPs in the voltage gated sodium channel gene have been identified in west African mosquitoes, including I1527T, V402L and P1874S/L (Clarkson *et al.,* 2018). Several studies have begun to investigate the contribution these SNPs might confer to pyrethroid resistance (Clarkson *et al*., 2018; Collins *et al.,* 2020; Yan *et al*., 2020).

Target site mutations are also important for resistance to other insecticide classes including *Rdl*, a mutation in the GABA-receptor targeted by Dieldrin (Wondji *et al*., 2011), and *ace-1*, a mutation in the acetylcholine esterase targeted by both the carbamate and organophosphate classes (Alou *et al*., 2010; Edi *et al*., 2012; Weill *et al*., 2003).

Metabolic resistance

Metabolic resistance is caused by up-regulation of detoxification proteins that metabolise the insecticide before it reaches its target site, stopping insecticide reaching toxic levels *in vivo*. There are five major detoxification gene families that play a key role in insecticide resistance; carboxylesterases, ABC transporters, UDP-glycosyltransferases, glutathione S-transferases and cytochrome P450s (Antonio-Nkondjio *et al*., 2016; Dermauw *et al*., 2014; Epis *et al*., 2014; Hemingway *et al*., 2004; Zhou *et al*., 2019). It is well established that overexpression of cytochrome P450 genes enhances rates of insecticide detoxification in many insect species (Bergé, 1998; David *et al*., 2013). Overexpression of cytochrome P450 genes, such as the direct pyrethroid metabolisers, CYP6P3 and CYP6M2 are commonly found pyrethroid resistant *An. gambiae* populations (David *et al*., 2013) and overexpression of CYP6P9a, CYP6P9b and CYP6M7 are found in resistant *An. funestus* (Riveron *et al*., 2013; Wondji *et al*., 2009). More recently, other members of this enzyme family have been shown to metabolise a wide range of insecticides currently used in malaria control (Yunta *et al*., 2019, Vontas *et al*., 2018). Worryingly, the overexpression of these proteins causes cross resistance with a number of chemistries including carbamates, organophosphates and a sterilising compound, pyriproxyfen, which has

recently been licensed for use as a second chemistry on bednets alongside a pyrethroid (Edi *et al*., 2014; Yunta *et al.,* 2016, Yunta *et al*., 2019).

In addition to cytochrome p450s, increased expression levels of glutathione S-transferases activity have also been linked with resistance to the major classes of insecticides used in malaria control (Ranson & Hemingway 2005). GSTE2 has been shown to directly impact pyrethroid resistance in *An. funestus* and *An. gambiae* (Ortelli *et al.,* 2003; Tchouakui *et al*., 2018).

Although cytochrome p450s and GSTs are the best characterised of the detoxification families, UGTs, ABCs and COEs are also known to play roles in resistance in both *An. gambiae* and other insect species. Indeed, there are also some reports of enhanced carboxylesterases such as esterase activities in permethrin-resistant *An. gambiae* (Vulule *et al.,* 1999); ABC transporters are often found differentially expressed in resistant mosquitoes (Pignatelli *et al*., 2018) and UGT transporters also play a key role in pyrethroid response in the Asian malaria vector, *An. sinesis* (Zhou *et al*., 2019).

Cuticular resistance

The mosquito contacts the insecticide through tarsal contact when in contact with a bednet or IRS. Cuticular resistance results in reduced uptake of the insecticide by the mosquito due to a thicker cuticular barrier (Balabanidou *et al*., 2018; Balabanidou *et al*., 2019; Wood *et al.,* 2010). Genes associated with increased cuticular thickening are upregulated in some pyrethroid resistant populations of *An. gambiae*; these genes include CYP4G16 and CYP4G17 that act at the terminal point in the cuticular hydrocarbon synthesis pathway (Kefi *et al.,* 2019). Both pyrethroid-resistant *An. funestus* (Wood *et al*., 2010) and *An. arabiensis* (Balabanidou *et al*., 2018; Jones *et al*., 2013) have also been shown to have thicker cuticles than those less tolerant to insecticides. Similar phenotypes have been observed in other mosquito species, for example attenuation of expression of CPLCG5 in *Culex pipiens* has been shown to increase susceptibility to pyrethroids through its role in rigid matrix formation within the cuticle. More recently, resistant *An. gambiae* have been shown to have thicker leg cuticles, with the upregulation of cuticular hydrocarbons transcripts thought to be linked with increased production of chitin (a component of the inner layer of the leg cuticle) in the legs of resistant strains (Balabanidou *et al*., 2019).

Behavioural resistance

Increased studies into feeding behaviour have highlighted that insecticide treated net use may be associated with changes in mosquito behaviour and some previous work has suggested that

widespread and sustained bednet and IRS use can select for behavioural resistance (Killeen *et al.,* 2016). There have been several reports suggesting that control programs that rely on ITNs are resulting in shifts in vector behaviour and/or species composition such that vector populations are less likely to encounter the insecticide.

Behavioural resistance refers to the modification of vectors behaviour resulting in avoidance of insecticides (Gatton *et al.,* 2013). One concern is the development of an early, outdoor feeding in anopheline populations previously reported to bite and rest inside houses, predominately at night (Russell *et al.,* 2011). Increased exophilic behaviour has been reported in association with IRS and ITN use, in some instances, reducing the effectiveness of these controls (Mbogo *et al.,* 1996; Molineaux & Gramiccia, 1980; Tirados *et al*., 2006; Reddy *et al.,* 2011; Russell *et al.,* 2010; Russell *et al.,* 2011). Govella *et al.,* (2010) extended this knowledge further using mathematical models to estimate that in places such as Dar es Salaam, Tanzania, around 50% of malaria transmission occurs outdoors. He suggests that this was due to extended bednet use in this area (Govella *et al*., 2010).

Changes in peak biting time has also been associated with extensive insecticide use (Dukeen *et al*., 1986; Mbogo *et al.,* 1996; Russell *et al.,* 2011; Taylor 1975). Feeding in some cases increased in the early evening, before potential hosts are protected by a bednet (Mbogo *et al.,* 1996; Russell *et al.,* 2011; Taylor, 1975). However, this finding is not universal and, in most cases, biting still occurs throughout the night even in the presence of insecticides (Dukeen & Omer 1986; Mbogo *et al.,* 1996; Russell *et al.,* 2011).

Changes in host-preference (e.g. a switch to biting non-human animals) has also been hypothesised as a behavioural resistance mechanism, caused by the increased pressure of insecticidal use with the home (Tirados *et al.,* 2006). Studies have suggested that this shift in biting behaviour is due to behavioural plasticity of the vector population instead of genetically distinct populations (Prussing, 2018).

In parts of Africa, shifts in vector composition after large net interventions have been reported, primarily from *An. gambiae*, which are described to be generally endophagic, to *An. arabiensis*, which are mainly exophagic (Lindblade *et al.,* 2006; Mutuku *et al.,* 2011; Russell *et al.,* 2011). Although with this in mind, these behaviours described are an overgeneralization, and in the field, there are much more complex behavioural differences between the species and within different populations. One study in Kenya suggested a shift from a population of mainly *An. gambiae s.s* to more *An. merus*, with an observed decrease in the rates of engorged females found indoors in the presence of bednets with no observed effect on *Plasmodium falciparum* sporozoite rates (Mbogo *et al.,* 1996). However, this study, investigating the changes in species composition lacks in evidence such as preintervention data

(Mbogo *et al.,* 1996), standardisation of collection methods, collection area and time of year, which is known to differentially influence species abundance (Cano *et al*., 2004; Killeen *et al.,* 2007; Wanji *et al*., 2003).

Currently there is a lack of evidence to assess whether these behavioural resistance traits are genetic or adaptive responses (Gatton *et al.,* 2013). Without this understanding it is harder to monitor changes in these traits and implement surveillance programs. Ultimately this knowledge gap makes behavioural resistance very difficult to target with control tools.

The effect of resistance on bednets as a control is not fully understood. Even with the emergence of insecticide resistant to pyrethroid treated bednets still remain an effective control too (Bradley *et al*., 2017; Kleinschmidt *et al*., 2018; Lindblade *et al*., 2015; Ochomo *et al*., 2017). A WHO-coordinated cohort study observed the effects of insecticide resistance at 279 locations in five countries, revealing ITN users had lower infection prevalence and disease incidence. The study concluded that irrespective of insecticide resistance, populations in malaria endemic areas reduced their risk of infection by using long-lasting insecticide treated nets (Kleinschmidt *et al*., 2018). However, with this in mind, Churcher *et al* (2016) used computer modelling to help predict how insecticide resistance might affect rates of malaria infection, showing the more mosquitos surviving bednet exposure is increasing the likelihood of unprotected hosts being bitten (Churcher *et al* 2016). Other models have also shown that the presence of physiological resistance reduces the impact of control interventions (Briët *et al.,* 2012; Griffin *et al.,* 2010;) and that increased exophagy will decrease the effectiveness of insecticide treated nets and IRS as a control (Briët *et al.,* 2012; Griffin *et al.,* 2010). These models suggests that behavioural resistance could be as serious or even pose a greater threat to malaria control than the emergence of physiological resistance in a population.

However, Killeen *et al*., (2016) concluded that regardless of behavioural resistance, bednets remain an important control tool even when outdoor biting occurs. This study stated that regardless of where and when *An. arabiensis* ultimately feeds, most mosquitoes have previously been inside an occupied house. The study suggested that two thirds of mosquitoes that take a blood meal do so after failing to feed on a protected host. Consequently, improved control of both indoor and outdoor biting mosquitoes can be achieved by improving the control tools already in use and using the extended knowledge of mosquito host seeking traits to manipulate these behaviours.

One way of improving control tools is to develop novel bednets. Modelling has also been used to help determine when it makes sense to switch to the new generation of nets, designed to help combat

the problem of insecticide resistance (Churcher *et al.,* 2016). It has been shown that these nets such as PBO nets, can be substantially better at killing insecticide resistant mosquitoes (Gleave *et al*., 2018). But this may not be the case in all areas and further investigation is continuing into the durability of these nets (Corbel *et al*., 2010; Tungu *et al.,* 2010). As the next generation nets are more expensive to make than standard ITNs there must be a clear advantage to rolling these nets out.

Next generation nets

For the prevention of malaria, the World Health Organization (WHO) has provisionally recommended or made policy recommendations for the use of a new generation of insecticide-treated mosquito nets (Table 1.2). As discussed previously all nets released before 2017 contained only pyrethroids.

Next-generation nets are nets treated with two or more active ingredients (also known as Dual AI nets or nets treated with an insecticide and the addition of a synergist such as PBO). Most of the next generation nets are treated with a pyrethroid plus synergist (PBO) which enhances the uptake of the insecticide. One net (IG2) contains a pro-insecticide tackling the challenges of insecticide resistance by applying a repurposed chemistry with a different mode of action to pyrethroids. Pyriproxyfen is also used to interfere with the maturity of a mosquito, preventing the development to adulthood and reducing reproduction output. More details on each net type are discussed below.

Table 2.2: A full list of dual AI nets, their manufacturer and the active ingredient

Product name	Date	Manufacturer	AI
Interceptor G2	29/01/2018	BASF SE	Alpha-cypermethrin; Chlorfenapyr (Pro-insecticide)
Royal Guard	29/03/2019	Disease Control Technology, LLC	Alpha-cypermethrin; Pyriproxyfen (hormone analogue)
Olyset Plus	29/01/2018	Sumitomo Chemical Co., Ltd	Permethrin; Piperonyl Butoxide (Synergist)
PermaNet 3.0	29/01/2018	Vestergaard S.A.	Deltamethrin; Piperonyl Butoxide (Synergist)
Tsara Boost	29/01/2018	NRS Moon netting FZE	Deltamethrin, Piperonyl butoxide (Synergist)
Tsara Plus	29/01/2018	NRS Moon netting FZE	Deltamethrin; Pyperonyl butoxide (Synergist)
Duranet plus	13/08/2020	Shobikaa Impex Private Limited	Alpha-cypermethrin; Piperonyl Butoxide (Synergist)
VEERALIN LLIN	29/01/2018	V.K.A. Polymers Pvt Ltd	Alpha-cypermethrin; Piperonyl butoxide (Synergist)

Pyrethroid plus a second insecticide

BASF have recently developed the Interceptor G2 (IG2) it is a polyester net containing two active ingredients, alpha-cypermethrin and chlorfenapyr. To combat the threat of insecticide resistance, chlorfenapyr is a member of the chemical class pyrroles. Chlorfenapyr is a repurposed pesticide, originally used to control termites and pests (Dekeyser, 2005; Pimprale *et al.,* 1997; Romero *et al.,* 2010; Rust *et al.,* 2006; Wang *et al.,* 2019). It is a pro-insecticide, resulting in disruption of ATP production and loss of energy, this leads to cell dysfunction and subsequently causes the mosquito to die. This chemical has low toxicity to mammals and according to WHO criterion is classified as a slightly hazardous insecticide (Tomlin, 2000). Because of chlorfenapyr's novel mode of action there is no evidence of cross resistance to insecticide classes normally used for vector control (Oliver *et al*., 2010; Oxborough *et al.,* 2010; Raghavendra *et al*., 2011).

Chlorfenapyr was evaluated against a range of *Anopheles* species for its possible use in vector control (Raghavendra *et al*., 2011). Cone test revealed that chlorfenapyr does not exhibits irritability effects when on both pyrethroid susceptible and resistant mosquito strains (Verma *et al*., 2015). Tunnel test data revealed chlorfenapyr nets were effective in producing higher mortality in pyrethroid resistant strains than permethrin treated nets (N'Guessan *et al*., 2007). Experimental hut trials have shown high mortality effects of IG2 against pyrethroid-resistant *An. funestus* in Tanzania (Tungu *et al*., 2021) and *An. gambiae s.s.* in Côte d'Ivoire (Camara *et al*., 2018). Ngufor *et al* (2017) concluded that the use of chlorfenapyr and alpha-cypermethrin together as a mixture on nets provides improved control of pyrethroid-resistant malaria vectors.

One of the main issues faced with the implementation of chlorfenapyr treated nets is how to assess the overall efficacy of these nets. The standard WHO tests for net efficacy do not produce results in agreement with experimental hut trials (Oxborough *et al*., 2015). Oxborough *et al* (2015) found exposure of mosquitoes to a chlorfenapyr treated surface in standard three minute bioassays resulted in considerably lower levels of mortality compared to standard tests performed on pyrethroid treatments. The impact of chlorfenapyr nets improved when tested at night, possibly the result of raised metabolism and flight activity in *Anopheles* at night (Oxborough *et al*., 2015). Further investigations are needed into the use of chlorfenapyr on the IG2 net in the field and importantly the most appropriate way to evaluate the effectiveness of this chemical when used in a control intervention.

Pyrethroid plus insect growth regulator

Pyriproxyfen (PPF) is a broad-spectrum insect growth regulator with insecticidal activity. It is a juvenile hormone equivalent that interferes with mosquito metamorphosis and prevents them from reaching maturity and reproducing (WHO, 1999). Nets coated with pyriproxifen (PPF) alone or in combination with permethrin have a significant impact on mosquito fertility (Grisales *et al*., 2021).

Olyset Duo and Royal Guard both contain PPF plus a pyrethroid. Studies have shown that Olyset Duo net is more effective against pyrethroid resistant populations mosquitoes, causing higher personal protection and mortality effects than the standard Olyset Net containing only permethrin (Koffi *et al*., 2015; Ngufor *et al*., 2014;). Royal Guard tested in hut trials however produced similar levels of mortality and bloodfeeding inhibition than a standard net, the PPF component of the net induced an 83% reduction in oviposition and 95% reduction in offspring (Ngufor *et al*., 2020).

Olyset Duo has been shown to strongly inhibit bloodfeeding rates of susceptible *Anopheles* strains, however this effect is reduced in highly resistant strains (Djènontin *et al*., 2015). It was shown that exophilic behaviour was higher in the presence of Olyset and Olyset Duo and was highest with the latter. (Koffi *et al*., 2015). Overall, Olyset Nets have shown to be effective in reducing bloodfeeding behaviour of malarial mosquitoes, the addition of PPF with pyrethroids can further reduce mosquito lifespan with significant reductions in reproductive output detectable for at least a year under operational settings (Grisales *et al*., 2021). Further research into the mode of action of PPF and impacts of PPF-treated nets on mosquito populations continues to examine the potential contribution of this new insecticide class for malaria control (Grisales *et al*., 2021).

Pyrethroid plus synergist

PBO targets specific metabolic enzymes (cytochrome P450s) within the mosquito, Alone PBO is not thought to cause mortality, however the synergist inhibits enzymes used to detoxify insecticides thus restoring the lethality of the pyrethroid. PBO works as an inhibitor of P450 enzymes which are implicated in pyrethroid resistance. It is also thought to increase the rate of the insecticide uptake through the mosquito cuticle. In areas where insecticide resistance is high, PBO nets increase mosquito mortality and reduce bloodfeeding rates compared to standard LLINS (Gleave *et al.,* 2018). In 2017, the WHO made a conditional recommendation that pyrethroid plus PBO nets should be considered for use in areas where pyrethroid resistance has been confirmed to be present in the main malaria vectors (WHO, 2017).

PermaNet 3.0 was the first insecticide-synergist combination bednet to be developed containing a mosaic of deltamethrin and PBO (Kweka *et al*., 2017; Tungu *et al.,* 2010). This was followed by other nets such as Olyset Plus, a polyethylene net incorporated with permethrin and the addition of PBO. Studies have compared the performance of these combination nets with standard pyrethroid only nets. Olyset Plus has been shown to perform better than the standard Olyset Net against a range of resistant populations of *Anopheles* in experimental hut trials, increasing mortality and bloodfeeding inhibition (Gunasekaran *et al.,* 2016; Pennetier *et al.,* 2013). As with Olyset Plus, PermaNet 3.0 has also been shown to be effective against pyrethroid resistant populations of *An. gambiae*, increasing mortality rates compared to standard ITNs (Gleave *et al.,* 2018). In areas with high levels of pyrethroid resistance, use of PermaNet 3.0 increased mosquito mortality by 81% compared to PermaNet 2.0 (Corbel *et al.,* 2010; Gleave *et al.,* 2018; Toé *et al.,* 2018).

PBO combination nets have also been shown to reduced parasite prevalence more effectively than standard pyrethroid ITNs in areas of high pyrethroid resistance (Gleave *et al*., 2018; Staedke *et al.,* 2020). When unwashed these combination nets remain effective in decreasing blood-feeding success, but the effectiveness of these nets once washed is still unknown (Gleave *et al.,* 2018).

Another important consideration for the next generation nets is how they should be deployed. Some laboratory studies have shown that PBO has an antagonistic effect on chlorfenapyr toxicity, due to the modes of action, PBO inhibits the metabolic enzymes needed to metabolize chlorfenapyr which causes the lethal effects (Raghavendra *et al*., 2011; Yuan *et al.,* 2015). With this in mind, the deployment of both chlorfenapyr and PBO incorporated controls could have adverse effects in the field. More information on this interaction is required, as well as a further understanding of how effective these new nets will be in the field.

Net design

The next generation of nets not only contain a mixture of insecticides and synergists, but they also vary in their design. Some of the new nets have higher doses of insecticide than the standard pyrethroid nets with the concentrations varying depending on location and denier of yarn filament.

Since previous studies have shown that the majority of mosquito activity is focused on the top of the net, with this being the first contact point for the majority of mosquitoes, (Lynd *et al*., 2013; Parker *et al*., 2015; Parker *et al*., 2017), some manufacturers have made the decision to include higher concentrations of insecticide/synergist only on the roof of the net, maximizing the safety of the net

and reducing costs. PermaNet 3.0 for example has varied concentration of deltamethrin depending on the location on the net; roof: 4.0 g/kg ± 25%, Sides: 2.8 g/kg - 2.1 g/kg ± 25% and the addition of PBO is on the roof panel only (Vestergaard 2015).

Building on this concept, additional ideas for the next-generation of nets include the development of barrier-bednets, a novel bednet with an additional panel across the roof of the net (Murray *et al.,* 2020). The addition of a barrier treated with an organophosphate was shown to be extremely effective at increasing mortality of pyrethroid resistant *Anopheles* in Burkina Faso. By only treating a smaller piece of net on the top of the bednet, this simple design would reduce costs, and the position of this barrier above the net may allow for the use of active ingredients not currently approved for the use on nets, increasing the potential range of chemistries for use against resistant mosquito populations (Murray *et al*., 2020). Other groups have also suggested the development of nets containing antimalarial compounds found to affect *Plasmodium* development (Paton *et al.,* 2019).

Both studies discussed above (Murray *et al.,* 2020 and Paton *et al*., 2019) demonstrated the potential for the use of new net designs in malaria control strategies to combat insecticide resistance. Paton *et al* (2019) modelling data demonstrated how the addition of antimalarial compounds could not only reduce malaria prevalence but extend the lifespan of the current insecticide-based control strategies. 3D models of mosquito flight behaviour around a human-baited net also aim to facilitate the development of these novel bednet designs, investigating the most cost-effective net designs and how these could most effectively be deployed in the field (Jones *et al.,* 2021; Murray *et al.,* 2020).

Mosquito host seeking behaviour

Understanding mosquito behaviour in response to insecticide treated nets is imperative when considering the development and evaluation of future control strategies.

Mosquitoes use a number of sensory cues to locate and select a host. Long-range host seeking is stimulated by visual and olfactory cues (Bowen 1991; Takken, 1991; Zwiebel & Takken 2004) with thermal cues being more important at short range (Cardé, 2015; Sutcliffe, 1994). It is known that carbon dioxide plays a role in short-range and long-range attraction of mosquitoes to a host (Dekker *et al.,* 2005; Gillies *et al.,* 1968; Majeed *et al.,* 2017; McMeniman *et al.,* 2014; Pombi *et al.,* 2014; Snow, 1970). Mosquitoes will fly upwind when olfactory neurons are stimulated by a source of carbon dioxide (Healy *et al.,* 1995). The presence of L- lactic acid, ammonia and carboxylic acids with carbon

dioxide also enhances a mosquito's preference to a particular host (Dekker *et al.,* 2002; Smallegange, 2005).

Body odour, such as volatiles from skin, sweat, and associated microbiota have also been shown to play an important role in host preference (Bernier *et al.,* 2000; Dormont *et al.,* 2013; Gallagher *et al.,* 2008; Takken & Knols, 1999). It is thought that odour cues play an important role in zoophilic and anthropophilic host preferences, with skin volatiles indicated to help guide mosquitoes with different host preferences. Variation in human attractiveness to mosquitoes is also affected by body odour cues (Dekker & Takken 1998; Lindsay *et al.,* 1993; Schreck *et al.,* 1990; Verhulst *et al.,* 2011; Zwiebel & Takken 2004).

Host-derived odour plumes help guide the mosquito toward the host. The mosquito navigates through the plume carried through air streams, making short turns, following the concentration of the plume as it increases as the mosquito becomes closer to the host. (Day 2005; Dekker *et al.,* 2011; Geier *et al.,* 1999; Van Breugel *et al.,* 2015).

Mosquitoes also rely on visual cues to help when following an odour plume (Kennedy, 1940). The mosquito eye is thought to be extremely sensitive, with day biting mosquitoes able to follow moving stripes (Kennedy, 1940) and the night biting *Anopheles* mosquitoes responding to stripes even at very low light intensities (Gibson, 1995). Diurnal species, such as *Ae. aegypti* have even been shown to respond to visual characteristics of hosts, such as colour brightness, patterns and shapes (Gibson & Torr 1999). However visual cues are not thought to be as important as odour signals. A study by Gillies *et al.* (1968) used calves as bait in hole below ground, which could not be seen by the released mosquitoes. This was done to show that it was olfactory cues that drew the mosquito to the host and not the visual cue of the cow (Gillies *et al.,* 1968).

Once the mosquito locates a blood meal, heat, moisture and carbon dioxide assist the orientation of the mosquito around the host. Convection currents arising from the warm-blooded host are important cues, assisting in this orientation (Dekker *et al.,* 1998; Khan *et al*., 1968;). *Anopheles* mosquitoes position themselves towards the base of a human host, using convection currents along the host following olfactory cues from the feet (De Jong *et al*., 1995; Dekker *et al.,* 1998). More recent research suggests a preference for feeding close to the ground in *Anopheles s.l* (Braack *et al.,* 2015).

Behavioural responses to insecticide treated nets

When a host is protected by a net, *Anopheles s.l* will preferentially contact the top of a bednet above the head and torso region of the net (Lynd & McCall, 2013). Lynd *et al*. (2013) used a sticky but non-

setting adhesive baited net to assess the first landing point of a mosquito, concluding that 74-87% of mosquitoes made first contact with the roof of the net above the head/torso region. Although this provides justification for the treatment of roof panels with higher insecticide and PBO, such as PermaNet 3.0, the study only recorded initial landing events and not the sequence of behaviours before or after, not allowing for investigation of host seeking and bloodfeeding behaviour.

More recently, video tracking experiments have provided much more information on how mosquitoes interact with the bednet interface. Sutcliffe *et al.* (2015) video recorded mosquito activity in small arena attached to a net surface, assessing the time spent in different activity modes and exit rates through a hole in the net. This study also identified higher levels of activity in regions above the head than on the side. By measuring the humidity in each arena on the net surface they demonstrated how the higher amount of activity above the head region is due to the rising heat and humidity plume from the volunteer beneath the net.

Due to the nocturnal feeding activity of *Anopheles*, studying this behaviour in a natural setting has been problematic. Recently, advanced technology has enabled the development of filming methods to observe undisturbed mosquito behaviour in a nocturnal setting (Angarita-Jaimes *et al*., 2016; Parker *et al*., 2015). Gibson *et al.,* (1995) discovered that *Anopheles gambiae* cannot see red light in a study using filming equipment and light cues. This information can then be used to set up filming in the dark to study feeding behaviour. A 3D filming system, with an infra-red lighting system was used to analyse *Anopheles gambiae s.s* host seeking behaviour. The study aimed to assess flight response to attractants mentioned above such as heat and human odour. It found that these factors are both important in mosquito host seeking. This new technology may therefore help in improving efficacy of traps used for the control of malaria mosquitoes (Spitzen *et al.,* 2013).

Parker *et al.* (2015) used the room scale video tracking system to explore how *Anopheles gambiae s.s.* behaves during approach, landing and feeding on a human host that is protected by an insecticide-treated net within a whole room, revealing four distinct behaviours: swooping, visiting, bouncing and resting. The majority of mosquito activity was observed on the roof of the net where mosquitoes made persistent attempts to reach the host beneath the net (Parker *et al*., 2015). The study method is providing new insight into the minutiae of behaviour at the bednet interface, with much-needed evidence regarding the repellent effects of insecticide treated nets, how effective they are in preventing bloodfeeding and effects they have on survival of vectors post-contact with the net surface. The study confirmed the majority of mosquito contact activity occurs on the roof of the net and this has led to the development of a novel bed-net design, the barrier bednet (Murray *et al*., 2020).

Treated nets are essentially baited traps, with the host luring the mosquito to make contact with the insecticide coating (Curtis *et al.,* 2006). Contact required to receive a lethal dose is very short for pyrethroid susceptible mosquitoes (Hauser *et al*., 2019; Hughes *et al.,* 2020; Parker *et al.,* 2015). The character of events for the pyrethroid susceptible Kisumu strain around a baited PermaNet 2.0 resembled that of an untreated net during initial exposure, with mosquitoes making reduced contact with the ITN (less than 1 minute) and contact reducing dramatically after 10-minutes of contact with the treated net (Parker *et al*., 2015). However, this is result is based on a pyrethroid susceptible mosquito strains and more work is required to characterise the behaviour of resistant strains at the net surface.

Repellency to insecticide treated nets has been described previously in the literature, from reduced landings on the nets (Siegert *et al*., 2009) to reports of less mosquitoes entering huts when insecticide nets are present (N'Guessan *et al.,* 2001; Spitzen, 2017). Repellent properties of treated nets may increase personal protection for the user but could reduce killing and increase diversion of mosquito to an unprotected host (Killeen & Chitnis, 2014). Other studies have shown no repellency to a baited treated net. Parker *et al*., (2015) showed that the lag time to contact and velocity measurements of mosquito flight before approaching a baited net, were identical prior to both ITN and untreated net contact, suggesting no repellency effect of the insecticide treated PermaNet 2.0 (Parker *et al*., 2015). However, repellent effects of ITNs have been shown to vary depending on the insecticide, with reports of permethrin treated nets being more repellent than deltamethrin and how many times the nets had been washed (Asidi *et al.,* 2004; Siegert *et al*., 2009).

Grieco *et al*., (2007) defined both irritancy and repellency; "a contact irritant stimulates directed movement away from the chemical source after the mosquito makes physical contact. A repellent stimulates directed movement away from the chemical source without the mosquito making physical contact with the treated surface".

A further consideration to the success of bednets is the existence of 'contact-irritancy', where a brief exposure to an insecticide results in mosquitoes exhibiting avoidance behaviour, before being exposed to a lethal dose. Contact irritancy or 'excito-repellency' can also result in increased house exiting and the diversion of a mosquito to an unprotected host. (Grieco *et al.,* 2000; Lindsay *et al.,* 1991; Maia *et al*., 2013; Maia *et al*., 2016; Roberts *et al.,* 1997; Spitzen *et al.,* 2017). The minimal contact that the mosquito has with insecticide treated nets and the ability to detect the net surface may allow entry to a house protected by an insecticidal net, with the ability to leave without fatal exposure to the insecticide (Killeen *et al.,* 2016; Parker *et al.,* 2015). Some studies have also reported shorter feeding durations through treated nets compared with untreated controls (Hauser *et al*., 2019, Hughes *et al*.,

2020). The relative importance of treated net repellency and contact irritancy may depend on the insecticide. Permethrin is thought to be slightly spatially repellent and a strong irritant to the mosquito (Dusfour *et al.,* 2009).

Although contact with nets is reported to reduce due to irritancy effects, some studies have reported that even with reduced exposure, this still results in delayed or sublethal effects that may still be reducing malaria transmission (Glunt *et al*, 2018; Liu *et al*., 1986; Takken *et al*., 2001; Viana *et al.,* 2016). Previously standard WHO testing of insecticide treated nets has always focused solely on 24-hour mortality, but it is key to look beyond this to understand the overall efficacy of a control tool. With the next generation of nets, some chemistries such as chlorfenapyr are slower acting insecticides and so the full mortality effects of these chemistries may not be apparent at 24-hours. Mosquito longevity should therefore be monitored as this could subsequently affect the ability of a mosquito to transmit malaria. Sublethal effects post insecticide exposure such as bloodfeeding inhibition or reduced host seeking may also affect malaria transmission and should therefore be evaluated when assessing the overall efficacy of insecticide treated nets. Post treated net exposure, Glunt *et al*., (2018) observed a reduction in "host-seeking" activity in resistant *Anopheles* strains and overall feeding rates were reduced by 60%. This study highlights the importance of assessing these factors when evaluating net efficacy to gain a greater understanding of the success of treated bednets as a control tool in the field.

Delayed and sublethal effects post ITN exposure will be discussed in more detail in Chapter 5.

Bioassays for measuring behavioural responses

The development of behavioural assays is key to enable more detailed observation of the behavioural interactions the mosquito makes when in contact with new insecticide treated nets. This research will be crucial to our understanding of insecticide treated nets as a vector control tool. To do this, we need the correct bioassays for evaluating how control tools affect mosquito behaviour, specifically capturing the impacts of newer compounds with novel modes of action.

Currently WHO standard methods to evaluate the effectiveness of insecticide treated netting are dependent on a few simple bioassays, which use exposure times that are not representative of the amount of time that a mosquito is in contact with a net on one-night times and report the data as percentage mortality and knockdown at 1hr to 24-hours post-test (WHO, 2016). The most common test used to monitor the effectiveness of insecticide treated nets is the WHO cone test. If a net fails to meet the criteria specified in the guidelines for WHO cone tests (≥ 80% mortality after 24 h or ≥ 95%

knock-down 60-minute after exposure before 20 washes) then tunnel tests are performed. The tunnel test is used to assess the efficacy of nets where properties of the insecticides, such as excito-repellency, may mean the effectiveness of the net are underestimated in a standard cone test. The test measures mortality effects and bloodfeeding success of host-seeking mosquitoes exposed to a baited (usually a guineapig or rabbit) ITN, in a 25 cm x 25 cm x 25cm glass chamber.

The standard cone test is a simple and quick bench top assay that exposes multiple mosquitoes to the formulated products (WHO, 2016). However, these assays provide limited information on frequency and duration of contact, delayed mortality, and sub-lethal fitness effects, which may reduce malaria transmission potential. Forced exposure in the cone test for a duration above that in the natural environment (Parker *et al.,* 2015; Parker *et al.,* 2017) may be producing enhanced mortality compared with those observed in the field. The lack of a human host in all standard WHO bioassays may also result in inadequate evaluation of the deterrent properties of a net during its intended use (Parker *et al*., 2015). Even when using a host for ITN testing, the WHO tunnel test mainly uses rodent and rabbit baits which are unrepresentative of the natural hosts of malaria vectors. Combining these basic assays with those designed to evaluate behavioural responses to ITNs would create a greater understanding of mosquito and net interactions, which is not only important for the evaluation of current control tools but can help in the novel designs for further control products.

Some small-scale assays have been developed to look at contact irritancy or 'excitorepellecy' effects of treated nets. 'Time to first take-off' was assessed using a modified cone test, this assay measured the time taken to take off after first landing on the treated net surface (Hougard *et al*., 2003). Grieco *et al*., (2005) also developed a small tube assay to assess special repellent or irritant effects of insecticides. Though, successfully used to distinguish the repellent and irritant impacts of a variety of chemistries, these assays were considerably small scale with a lack of any live or artificial bait (Grieco *et al*., 2007). While it has been found that the field trials compared well to lab findings, supporting the reliability of this testing method (Grieco *et al*., 2007), other studies have shown that a mosquito's response to insecticide treated material is affected by the presence or absence of a bait (Kongmee *et al.,* 2012; Siegert *et al.,* 2009).

A behavioural assay designed to demonstrate the efficacy of insecticides used in bednet treatment must include a bait to simulate host seeking. Many tests use artificial or inadequate attractants to those they would encounter in the field, such as human breath or limited body parts, simple odour blends or animal hosts, rather than an entire human (Dekker *et al.,* 2011; Hughes *et al.,* 2020; Spitzen *et al.,* 2013). Characterisation of mosquito behaviour at the bednet interface also requires

observations under conditions that are more representative of the natural environment and not restrained to small test arenas.

The behavioural research group at LSTM has developed a number of benchtop assays to assess the behaviour of mosquitoes at the net interface. All are designed to quantify exposure more accurately than previous tests did.

The Video Cone Test (Emery *et al*., 2019; Foster *et al*., unpublished) is a simple upgrade of the standard WHO cone test that introduces a host and a camera for recording the mosquito activity during exposure. Recordings are done using a standard iPhone so the test can be used in any laboratory environment.

The baited box is used to evaluate the behaviour of individual mosquitoes at a baited net interface (Hughes *et al.,* 2019) and complements the room-scale tracking system (Angarita-Jaimes *et al.,* 2016) by providing detailed information as the mosquito reaches the bednet. All mosquitoes tested in this way are then followed up through a sublethal effects pipeline (discussed in Chapter 5) to detect any delayed or sub-lethal of the treatment.

This study used the baited box (Chapter 2 and 3) and a room scale video tracking system (Chapter 4) with follow-up through the sublethal pipeline (Chapter 5) to study the behaviour of four strains of *An. gambiae s.l* at human baited bednets.

1.2 Aims and objectives.

The work presented in this thesis was undertaken as part of a larger project 'Accelerating time to market of new vector control tools by strengthening the phase 1 evaluation'. The main aim of the overall project was to develop new methods for evaluating the effectiveness and the modes of actions of novel insecticides.

At a time when resistance to pyrethroids occurs in *An. gambiae* throughout Africa, this project used the new Liverpool tests to study the interactions between pyrethroid susceptible and resistant *An. gambiae s.l* and human baited ITNs. The overarching aim was to develop a more comprehensive understanding of the behaviour of mosquitoes at standard pyrethroid nets, and of how insecticide resistance status could affect mosquito-net efficacy. The specific aims were:

- To describe the detailed behavioural responses of pyrethroid resistant and susceptible *An. gambiae* at the surface of pyrethroid-treated bednets, when bloodfeeding through the net is possible or prevented.

- To characterise the flight behaviour responses of *Anopheles gambiae s.l* seeking hosts protected by a permethrin treated net.

- To determine whether exposure to pyrethroid-treated bednets can result in sub-lethal impacts on resistant and susceptible mosquitoes.

Chapter 2: Quantifying mosquito bloodfeeding behaviour at the Human-ITN interface

2.1 Introduction:

Control of the malaria mosquito continues to be reliant on a limited number of insecticides. Evaluating the suitability of alternative insecticides for use on ITNs and understanding the impact of insecticide resistance on ITN performance requires robust bioassays that can determine the mosquito's response to ITN surfaces under natural conditions. Currently there are a limited number of standard tests routinely used to evaluate the performance of insecticide treated materials; mainly the WHO Cone and the Tunnel test (WHO, 2013 and WHO, 2019). These tests measure knockdown and mortality at zero to 24-hours post-test. The Cone test involves three minutes of near-forced contact with the net; it is simple and rapid but does not involve a host. The tunnel test runs overnight and requires the use of a tethered guinea pig on which mosquitoes feed, an aspect of the test that prevents its use in some countries due to the ethics of using animals as a bait. Moreover, they provide limited or no information on frequency and duration of exposure, providing no information on the behaviour at the net interface, which may reduce malaria transmission potential (Viana *et al.,* 2016). Using assays as rapid and as simple as the cone test alongside more complex tests that allow more natural exposure and have the ability to record behavioral responses to ITNs in some detail would be very valuable and improve our understanding of mosquito and net interactions. This is not only important for the evaluation of the impact of insecticide resistance on current control tools but can help in assessment of novel chemistries for use in future control products.

The tests above provide a good indication of whether a chemical is fast-acting, like pyrethroids, however due to complex modes of action, the insecticidal effects of the next generation of insecticides such as chlorfenapyr may be underestimated using these tests. Chlorfenapyr is a pro-insecticide, targeting the oxidative pathways in the mosquito mitochondria (Black *et al.,* 1984). Therefore, it is thought that the activity of the mosquito during or after exposure could affect the impact of this insecticide. Current work has shown differences in mortality effects of insecticides depending on the test used (Owusu *et al.,* 2015). Oxborough *et al* (2015) found that standard three-minute bioassays of chlorfenapyr produced extremely low levels of mortality, however overnight tunnel tests increased the mortality to up to 100% (Oxborough, 2015). Conflicting results between test methods demonstrates the need for tests that allow more natural exposure and the ability to assess whether these chemistries are affecting the behaviour of the mosquitoes. Further investigation is needed into

how these chemicals work and development of the most appropriate way to test these new chemicals in order to evaluate how they would best be deployed in the field.

Remarkably little is known about how mosquitoes interact with ITNs. Understanding the behavioural interactions mosquitoes have with insecticide treated materials is important for the success of current malaria control tools, and imperative in considering the much-needed development of future control strategies (Killeen *et al.,* 2011; Killeen *et al.,* 2014; Ogoma *et al.,* 2010). Anything that affects that interaction potentially enhances or compromises an ITN's performance.

Ideally, a behavioural assay designed to demonstrate the efficacy of an ITN should include a bait to stimulate host seeking. However many tests use artificial or alternative baits such as animal hosts rather than an entire human (Dekker *et al.,* 2011; Hol *et al.,* 2020; Spitzen *et al.,* 2013). For example, studies such as Dekker *et al* (2011) and Spitzen *et al* (2013) used wind tunnel experiments to assess the response of mosquitoes to odour plumes from a human arm collected in a tube, odour collected from a worn sock, or CO_2 pumped out at varying concentrations. Other studies do not use a human host at all and instead artificial baits such as commonly used in insectaries with a parafilm membrane and a heat source (Hol *et al.,* 2020)

Using a lethal capture method (a sticky net), Lynd *et al* (2011) observed that mosquitoes predominantly contact the top of a bednet above the head/torso of the host sleeping under the net, something not identified in standard test procedures. This information provides evidence for the use of the combination bednet PermaNet 3.0® (PN3), a net coated with a deltamethrin and a synergist plus insecticide present on the roof of the net. The data collected from Lynd *et al* (2011) and others showing high levels of contact on the roof, highlights how information from these behavioural studies can be useful when developing new net types, such as the PN3. The lethal nature of the capture technique meant the method only recorded initial landing events and not the sequence of behaviours before or after. More recently, advances in technology have allowed the development of recording methods to observe undisturbed mosquito behaviour in a nocturnal setting (Angarita-Jaimes *et al.,* 2016; Parker *et al.,* 2015). LSTM have developed a video tracking system to explore how mosquitoes behave during approach, landing and feeding on a human host when protected by an insecticide-treated net (Parker *et al*., 2015). The system has revealed more complex interactions with the bet net such as the irritancy and repellent effects of ITNs and how effective they are in preventing blood-feeding. These net tests can be used to characterise behaviour to a level not observed previously in other tests and aide in the development of more effective and novel bednet designs (Parker *et al.*, 2015). The results from this system are described further in Chapter 4.

While useful in the evaluation of insecticide treated nets, the video tracking system is labour intensive and expensive to set up. The data collected can define a large range of host-seeking behaviours around a baited net, however, information on how the mosquito interacts with the bednet interface when in close proximity with the net, at the last stages of host seeking, is required to gain knowledge on the overall effectiveness of each net type.

To investigate the short-range effects of insecticide treated nets on host seeking and bloodfeeding behaviour we developed a simple laboratory bench top assay 'the baited box' at LSTM (Hughes *et al.,* 2020). The test uses an infra-red camera to assess mosquito behaviour in response to a human host (thumb) at a net interface. The test is used to investigate the impact of current ITNs on *An. gambiae s.l.* behaviours, such as host seeking, bloodfeeding and any sublethal impacts post-exposure. Individual mosquitoes are used for each test, meaning the exact duration of ITN contact can be accounted for when assessing the delayed or sublethal effects of that exposure. During the test, bloodfeeding through the bednet is permitted or prevented, and detailed behavioural events ranging from pre-contact repellency/ contact-irritancy to duration of bloodfeeding are recorded for analysis. The results of the Bloodfeeding prevented analysis will be discussed in Chapter 3.

The work in this chapter builds on the previously published work, where the baited box was co-developed as part of my MSc project. All the new data generated below is not included in Hughes *et al* (2020), although supports the previous findings. The baited box was used to assess overall impact of exposure to standard 'first generation' pyrethroid ITNs on host seeking and bloodfeeding duration in four strains of *An. gambiae s.l*. (two strains resistant to pyrethroids and two susceptible). Longevity and fecundity impact post exposure to these ITNs was evaluated and reported in Chapter 5.

The specific aim of the experiment was to describe the detailed behavioural response of pyrethroid resistant and susceptible *An. gambiae s.l* when blood feeding through pyrethroid treated nets.

The hypothesis explored where:

1) There will be no significant difference in the response to a treated and untreated net for each strain.
2) There will be no significant difference in the duration of feeding through the three nets.
3) Post feeding there will be no significant difference in the amount of time spent resting on each net type.

2.2 Materials and Methods:

2.2.1 Mosquito colonies

For all experiments conducted, two insecticide susceptible and two insecticide resistant strains of *An. gambiae s.l.* were used (Table 2.1). The phenotypic susceptibility of each colony is checked annually using standard WHO insecticide susceptibility tube assays at LSTM. The Kisumu strain (*An. gambiae s.s)* was originally collected from Kenya in 1960, colonised at LSTM in 1975 and is susceptible to pyrethroids and all other classes of insecticides (DDT, deildrin, bendiocarb, proproxur and fenitrothion) tested by Williams *et al.,* 2019 (Williams *et al*., 2019, Weill *et al*., 2004). The N'gousso strain of *An. coluzzii*, originally colonised from Cameroon in 2006 (Harris *et al.,* 2010) is also susceptible to pyrethroids (1-hour WHO tube exposure 2019) and with some resistance to organochlorides, DDT (61% mortality; 1-hour exposure in WHO tube test, 2019) and dieldrin (39% mortality; 1-hour exposure in WHO tube test 2019). The resistant strains used were VK7 and Banfora, both of which are *An. coluzzii* populations from Burkina Faso, colonised in LSTM in 2014, and resistant to pyrethroids (Toe, 2014; Williams *et al*., 2019).

Table 2.1 Insecticide susceptibility status including resistant ratios (RR) based on topical application of permethrin calculated by Williams *et al*., 2019 and origin of the *Anopheles gambiae s.l.* colonies.

Name	Species	Phenotype	RR	Origin
Kisumu	*An. gambiae s.s*	Susceptible to pyrethroids	N/a	Kenya
N'gousso	*An. coluzzii*	Susceptible to pyrethroids	N/a	Cameroon
Vk7	*An. coluzzii*	Pyrethroid & DDT Resistant	145	Burkina Faso
Banfora	*An. coluzzii*	Pyrethroid & DDT Resistant	222	Burkina Faso

Non-blood fed female mosquitoes, aged 3-7 days post emergence, were used in all tests. All mosquito colonies were maintained at 27±2°C and 70±10% relative humidity under a 12hr light/12hr dark cycle. Adult mosquitoes were fed on 10% sugar solution and maintained on human blood. Larvae were fed on ground fish food (Premium Tropical Flake, Aquarama). All assays were performed between 0 and 7 hours after the start of the scotophase.

One day prior to testing, mosquitoes' access to sugar was removed and replaced with distilled water and three to five hours prior to testing, the water was removed from the mosquito cage. Female mosquitoes were removed from the stock cage one hour before the test and placed in holding cups in the experimental laboratory to acclimatise to the testing laboratory environment (27±2°C and 70±10% relative humidity under a 12hr light/12hr dark cycle).

2.2.2 Test materials

Mosquito behaviour was measured in response to PermaNet 2.0® subsequently referred to as PermaNet (55mg/m² deltamethrin; Vestergaard, Lausanne Switzerland), Olyset® Net (2% Permethrin; Sumitomo Chemical Co. Ltd, Tokyo, Japan) and an untreated polyester net (Bayer AG, Leverkusen, Germany). The nets were aired indoors at ambient temperatures for approximately 4 weeks prior to use in bioassays. The nets were not aired for longer than 4 weeks but the minimum airing time was not recorded. Following airing, the nets were cut and stored at 4°C. Pieces of test netting were acclimatised at 27±2°C and 70±10% humidity 1 hour prior to testing.

2.2.3 Experimental set up

Experiments were performed in an LSTM insecticide testing room. All experiments were recorded under infra-red light in complete darkness. The test chamber was assembled with an entrance port and test netting attached (Figure 2.1) as described previously (Hughes *et al.,* 2020; Parker 2015) but with modification. Firstly, the durability of the baited box was improved by using Perspex boxes measuring 10cm^3 (Retailacyrlics, Wales, UK) and polypropylene mesh from Watkins and Doncaster (Watkins and Doncaster, Herefordshire, UK). Design modification was carried out in house by the LSTM maintenance department. Secondly the ventilation was improved by introducing four 1.5cm diameter ventilation holes in the sides and one 7cm diameter hole in each of the top and bottom of the box. Polypropylene mesh covered each hole preventing mosquito egress whilst maintaining airflow through the apparatus. The entrance port was modified to include extra gating to allow ease of mosquito introduction.

The baited box was positioned on an adjustable stand, modified in house at LSTM with a 7cm diameter hole to ensure air flow to the box, in the centre of the test set up (Figure 2.1). Infrared LED lighting (M850L2: wavelength spectrum from 790-885nm, ThorLabs Ltd, Ely, UK) was attached to a bench top tripod approximately 30 cm from the thumb box with the 16cm x 16cm acrylic diffuser (COMAR optics, Linton, Cambridge, UK) 12cm in front of the LED. On the opposite side of the box, the camera (Ximea MQ013RG-E2 1.3 Megapixel near infrared enhanced CMOS Camera, Ximea, Munster, Germany) and 60 mm lens (F2.8 Nikon camera lenses used at F8, Amazon.co.uk) were attached to an adjustable tripod approximately 86cm from the thumb box (Figure 2.1). Video recordings were captured on a Windows laptop (Lenovo P50, Hong Kong, China) using StreamPix recording software (StreamPix V.7,

Norpix, Montreal, Canada) at 10 frames per second. All recordings were stored on Seagate 5TB (Seagate, Thailand) external hard drives.

Figure 2.1: Baited box set up. Left: testing chamber placed on an adjustable stand, all sides made with Perspex, with attachable entrance tube, gate and attachable tube for test netting. Middle: whole set up including LED and diffuser, test box and stand and camera lens placed on an adjustable tripod. Right: close up image of the testing chamber with an operator's thumb in position against the test netting.

2.2.4 Experimental procedure

To begin an assay, a single mosquito was transferred from the holding cup to the baited box entrance tube. After attaching the entrance tube to the testing chamber, the mosquito was given an acclimatisation period of 1-minute during which the operator's thumb was placed against the test netting. The same operator was used for all tests. After acclimatisation, the operator began the recording, and the mosquito entry gate was removed to allow the mosquito access to the main test chamber.

A mosquito was classed as a responder if it approached the thumb and was able to begin bloodfeeding. If a mosquito did not exit the entrance tube within 3-minutes (or did not commence bloodfeeding within 10-minutes) of the start of the test, the test was abandoned, and the mosquito discarded as a non-responder.

Bloodfeeding mosquitoes were left to feed to repletion and rest for up to three minutes after withdrawal of the proboscis from the thumb, at which point the recording was terminated and the experiment declared complete. If the mosquito departed the net during the 3-minute resting period, recording was terminated immediately.

Post-test, the mosquito was removed using a mouth aspirator and transferred to a 50 ml Falcon tube with an untreated netting lid and provided with 10% glucose solution.

2.2.5 Video analysis

The video recordings were analysed using BORIS, a free online software (Friard, O. and Gamba, 2016). The recordings were analysed manually in slow motion. The behavioural events were classified using the categories detailed in Table 2.2. All data was exported to Microsoft Excel 2010.

The behavioural events of the responder mosquitoes were compiled into a summary stacked bar chart using the coded behaviours as per Table 2.2 with each bar showing the mean duration for each event, stratified by strain and treatment.

Table 2.2 Behavioural event classification.

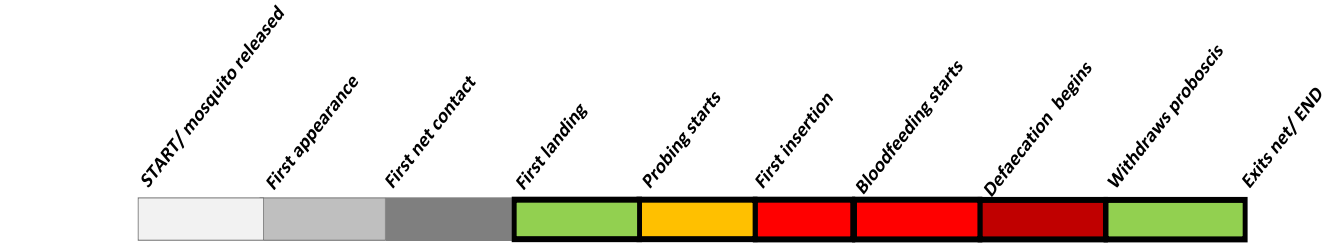
Step	Event	Definition
1	Appearance	The first point at which the mosquito is seen in the cameras field of view
2	Contact	The mosquito contacts the test netting, but wings don't stop
3	Landing	The mosquito lands on the net, the legs contact net; wings stop
4	Probing	The mosquito probes through the net, tilts forward; proboscis pushed through net
5	Inserts Proboscis	The mosquito begins feeding, inserts proboscis into the thumb and becomes still
6	Bloodfeeding before defaecation	Bloodfeeding is visible, the abdomen of the mosquito starts visibly expanding
7	Bloodfeeding with defaecation	Droplets appear at the tip of the mosquito abdomen
8	Withdraws proboscis	The tip of the proboscis is removed from the thumb and is visible above netting
9	Resting	The mosquito is standing still on the test net; not probing

2.2.6 Data analysis:

Response rates were analysed using a Chi squared test in SPSS (IBM SPSS statistics Version 25). Stacked bar charts of behavioural events were created in Microsoft Excel 2010. The nine behavioural events in Table 2.2 were combined for analyses into 7 biologically relevant 'Activities' as shown in Figure 2.2:

1. Prior to appearance – time from test start and mosquito's first entry into the field of view
2. Time to first net contact – time to approach net
3. Time to landing – willingness to land on the net
4. Landing to probing- time from landing to start of probing
5. Probing- Insert- time spent probing before bloodfeeding
6. Duration of bloodmeal – time from proboscis insertion into skin until withdrawal
7. Resting post feed – duration of net contact post bloodmeal

The duration of each behavioural event for every experiment was calculated. The time from 'open entry gate' until the mosquito appeared in the field of view was classified as prior to appearance. The duration of each event when exposed to two ITNs was analysed using Kruskal Wallis non-parametric test in SPSS (IBM SPSS statistics Version 25). This test was used due to the non-normal distribution of the data. Resting was separately as this behavioural event was confined to three minutes by ending the test after this time point. Resting was split into three categories, not resting (<0.1 minutes), Resting for less than 3-minutes (<3 minutes) and resting for three minutes/till the test was terminated (≥ 3 minutes). Resting behaviour was analysed using logistic regression in Statistical analysis software (SAS) (Version [9.4] of the SAS System for Unix 2002-2012).



EVENT	Time to Appearance	Appearance to 1st net contact	Contact to landing	Landing to probing	Probing to insertion	Insertion to visible feeding	Bloodfeeding pre-defaecation	Bloodfeeding with defaecation	Proboscis withdrawal and resting post feed
No.	1	2	3	4	5	6	7	8	9
Behaviour description	From release until first seen in field of view	Legs contact net but wings do not stop	Legs contact net and wings stop	Tilts forward, proboscis through netting	Probing ceases, proboscis remains inserted into thumb	Proboscis remains inserted, abdomen visibly expands	Continued feeding with no visible blood droplet	Visible droplet at abdomen posterior	Static or grooming on net until departure
Activity	< appearance								
	Time to first net contact								
	Time to landing								
				Landing- Probing					
					Probing - Insert				
						Bloodfeed duration			
									Rest post-feed

Figure 2.2: Ethogram of the nine behavioural events during bloodfeeding on human bait through insecticide-treated netting, showing the start and endpoint of each and how activities were grouped based on biologically relevant criteria. Grey sections with no border show activity prior to first net contact; coloured sections with black borders represent the behaviours involving contact with the test netting. Figure adapted from Hughes *et al*., 2020.

2.3 Results:

2.3.1 Response rates

A total of 1,032 mosquitoes were released individually into the baited box. The response rates varied depending on the strain and net type (Table 2.3), with Kisumu exposed to an untreated net being the most responsive (61%) and N'gousso exposed to the Olyset net the least responsive (16%). Response rates were similar when exposed to untreated and treated nets for both resistant strains with no significant difference in the response rates (Banfora and VK7). The susceptible strains, N'gousso and Kisumu, responded more frequently to the untreated net compared to an Olyset net ($P<0.0001$, $P= 0.01$), however there was no difference in the response when comparing untreated net and PermaNet.

Table 2.3: Mosquito response rates of *An. gambiae s.l* (resistant and susceptible) when exposed to three net types; Untreated, PermaNet and Olyset.

		Response rate (%) No. mosquitoes responding/ no. tested			
Mosquito strain		Kisumu	N'gousso	Banfora	VK7
Treatment	**Untreated**	**61%** (30/49)	**47%** (27/57)	**33%** (30/91)	**21%** (27/126)
	PermaNet	**58%** (21/36)	**31%** (22/70)	**20%** (24/119)	**32%** (23/73)
	Olyset	**35%** (22/63)	**16%** (26/164)	**22%** (22/99)	**26%** (22/85)
Total no. tested		148	291	309	284

All mosquitoes that responded went through a series of behaviours, from landing, probing, to bloodfeeding, with some immediately departing the net and other resting for up to three minutes before the test was terminated. Figure 2.3 shows an example of the different events observed.

The average amount of time (seconds) each strain spent in each behavioural event is recorded in Figure 2.4. Behaviours that take place prior to the first landing event are shaded in grey. Bloodfeeding duration is shown by red and brown shades and rest periods on the net in green.

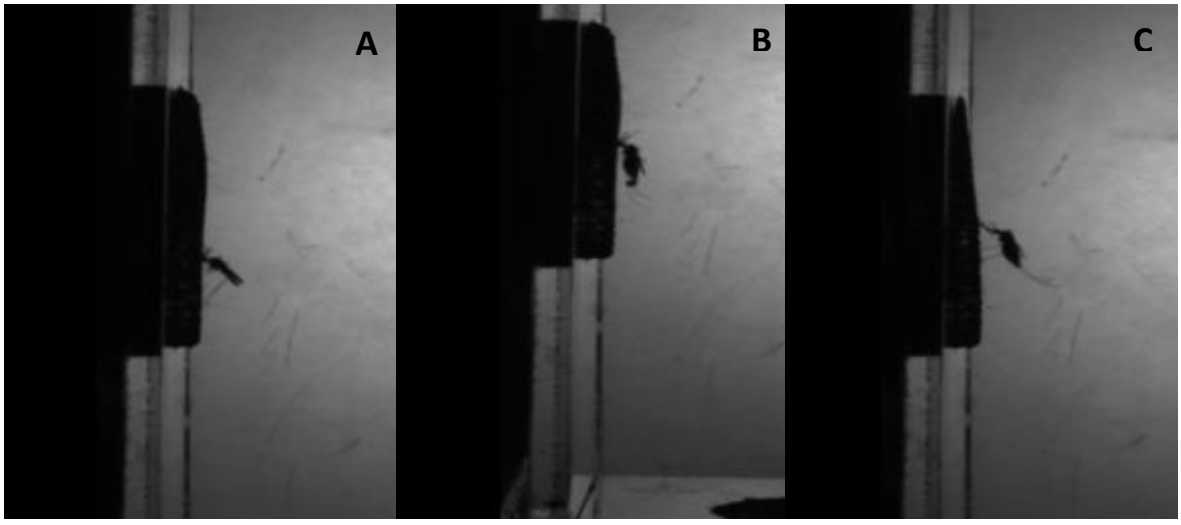


Figure 2.3: Behavioural events. A: Probing; The mosquito tilts forward; proboscis pushed through net. B: Bloodfeeding; The mosquito inserts proboscis into the thumb and becomes still. The abdomen of the mosquito starts visibly expanding and then droplets appear at the tip of the mosquito abdomen. C: Resting; The mosquito is standing still on the test net; not probing post feed.

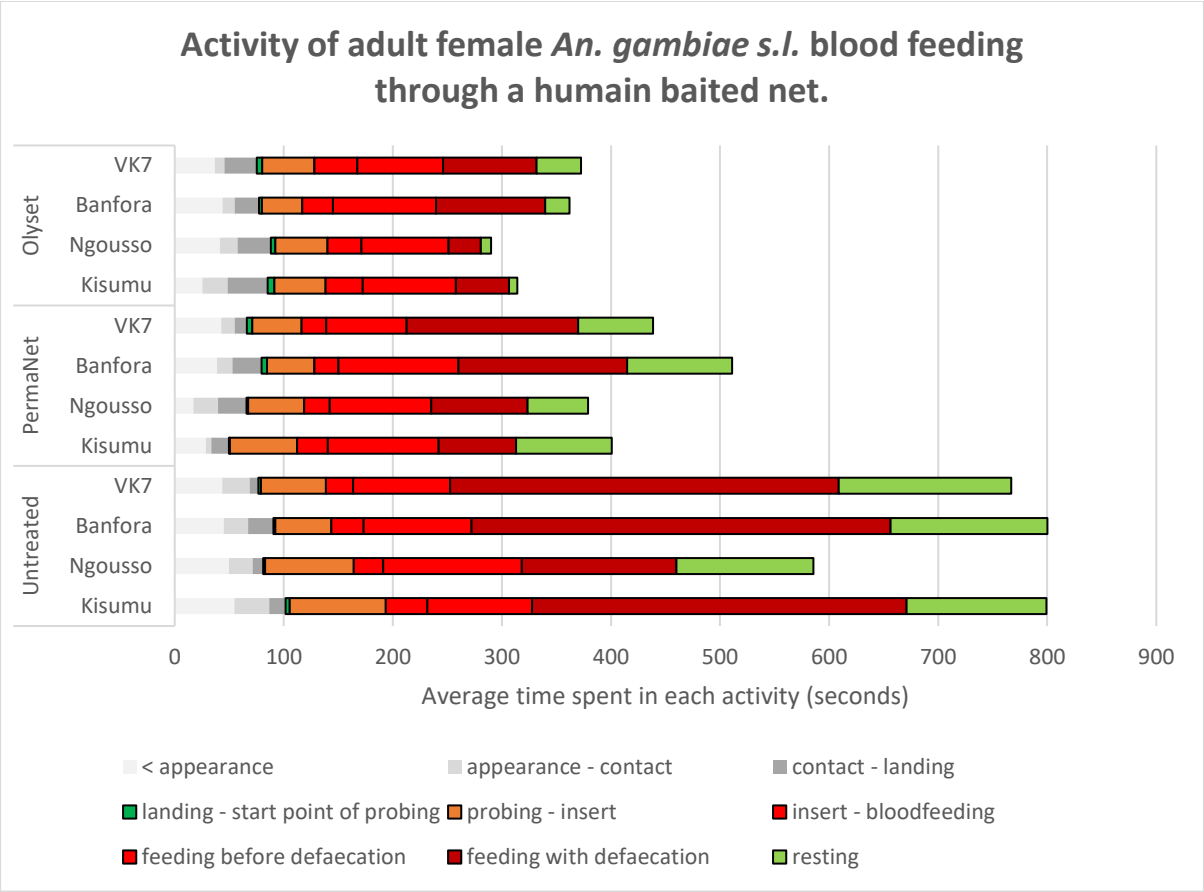


Figure 2.4: Behavioural events during feeding at human-baited net, treated and untreated for 4 strains of *An. gambiae S.l.* Mean time (seconds) spent in each behavioural event is plotted for each strain and net type. See Table 2.4 for total mosquito numbers for each net/strain combination.

2.3.2 Behaviour of pyrethroid susceptible *An. gambiae s.l* when bloodfeeding through an untreated net and two ITNs

Both susceptible strains showed a trend for higher response rates to untreated than treated nets (Table 2.3), however of those responders, there was no significant difference in the overall time it took both susceptible strains to enter the field of view when exposed to all treatments (Kisumu; P= 0.113 and N'gousso; P=0.172). At all net types, activity after initial time to respond, comprised of the same sequence of events, from initially contacting the net then landing on the net, to probing. A small subset of mosquitoes left the net after landing, before starting to probe, or during probing activity for 1-2 seconds, but this data is not included in the final dataset. After probing through the net, mosquitoes inserted the proboscis and began to bloodfeed before finally resting on, or departing from, the surface of the net, depending on the treatment.

For untreated nets, Kisumu and N'gousso spent an average of 101 and 81 seconds before initially landing on the net. At the untreated net surface, probing times were very similar for both strains (88 and 81 seconds; Table 2.4), but overall feeding time was much greater in Kisumu (478 seconds) than N'gousso (296 seconds). Kisumu also had a much wider range of overall bloodfeeding times through the untreated net, ranging from 191 up to 1935 seconds (Table 2.4), compared with N'gousso, ranging between 146 and 644 seconds.

Despite lower initial response rates to Olyset net, exposure to both ITNs had no effect on the time taken for the N'gousso strain to contact (P = 0.343) or land on the test netting compared to an untreated net (P=0.363). The Kisumu strain, however, took longer to contact (P=0.012) and land on untreated net than PermaNet (P= 0.021), but there was no difference in the amount of time observed for this strain to make initial contact with (P= 0.693) or land on the Olyset net (P= 1.000).

Once in contact with the nets, all mosquitoes spent little time on the net surface before beginning to probe, spending an average of 1 to 4 seconds on all net types (Table 2.4). No difference was observed in the amount of time spent probing through untreated net and PermaNet for either strain (Table 2.4; P= 0.507, P= 0.075). However, the amount of time spent probing before beginning to bloodfeed was significantly shorter for both susceptible strains when exposed to Olyset net compared to an untreated net (Table 2.4; Kisumu; P=0.013, N'gousso; P=0.033). Probing time for the Kisumu strain reduced from an average of 88 seconds when exposed to untreated net to 47 seconds when exposed to Olyset net. The N'gousso strain spent an average of 81 seconds probing through an untreated net but this also reduced to an average of 48 seconds when exposed to the Olyset net.

When feeding through treated net, bloodfeeding duration was significantly reduced for both susceptible strains compared to untreated net (Figure 2.3, Table 2.5; P<0.0001). For both strains feeding duration was shortest when exposed to an Olyset net, reducing to an average of 169 and 141 seconds for both Kisumu and N'gousso strains (compared to 478 and 296 seconds for untreated nets).

2.3.3 Behaviour of pyrethroid resistant *An. gambiae s.l* when bloodfeeding through untreated and treated netting

When exposed to all net types, the sequence of events was the same for both resistant strains as with the susceptible strains (Figure 2.4). All mosquitoes that responded bloodfed through both untreated and treated nets with no observed short range repellency effects for either ITN.

At an untreated net the time taken to contact the net ranged between and average of 67-72 seconds for both strains. Both resistant strains spent a similar amount of time feeding through the untreated net; on average the Banfora spent 513 seconds and VK7 spent 464 seconds. This is similar to the 478 seconds that the Kisumu spent on average feeding but longer than the N'gousso strain (296 seconds). Both resistant strains spent between 50-60 seconds on average probing through the untreated net before beginning to feed, slightly reduced compared to the susceptible strains (Table 2.4).

Similar to the susceptible strains there was no significant difference in the overall time it took both resistant strains to enter the field of view when exposed to treated compared to untreated net (Table 2.4; Banfora P = 0.595, VK7 P = 0.614). No repellency effects for either ITN were observed at this range, with exposure having no effect on the time taken for both resistant strains to contact (Table 2.4; Banfora P = 0.245, VK7 P = 0.184). and land (Table 2.4; Banfora P = 0.565, VK7 P = 0.405) on the test netting. As with the susceptible strains, both VK7 and Banfora spent little time on the net before beginning to probe. However, unlike the susceptible strains, there was no significant difference in the amount of time spent probing on an untreated or treated net for both resistant strains (Table 2.4; Banfora P = 0.069, VK7 P = 0.183).

Overall bloodfeeding duration was similar for both resistant strains when exposed to untreated netting ranging from 129 to 1140 seconds for the Banfora strain and between 198 to 932 seconds for the VK7 strain. When bloodfeeding through both treated nets, overall bloodfeeding duration was significantly reduced for both resistant strains compared to untreated net (Table 2.5; PN2 $P<0.001$, Olyset $P<0.0001$). As with the susceptible strains, feeding duration was shortest for each strain when exposed to an Olyset net (Table 2.4).

Table 2.4: Duration of events for insecticide susceptible and insecticide resistant *An. gambiae s.l* when exposed to PermaNet (P2) and Olyset net (Oly). The average amount of time spent in minutes for each behavioural event is shown with the standard deviations and range. Untreated net (UT) data is included for comparison purposes. Resting is not recorded in the table due to separate analysis.

EVENT / ACTIVITY		< Appearance	Time before first contact	Time before landing	Landing - Probing	Probing-insert	Overall Blood-feeding
STRAIN	**NET**	***mean duration*** *(Standard deviation), range*					
Kisumu	UT	**54** (66) 1.8-277.8	**87** (84) 4.2-315	**101.4** (84) 7.2- 319.2	**4.02** (6) 0-43.2	**88.2** (66) 4.2- 286.2	**477.6** (330) 190.8- 1935
	P2	**28.6** (36) 0-135	**33.5** (36) 6-139.8	**49.6** (54) 12-154.2	**1.17** (1.2) 0 -4.2	**61.6** (24) 30-139.2	**200.9** (60) 97.2-321
	OLY	**25.2** (48) 30-109.2	**48.7** (66) 7.2-121.2	**85.4** (72) 10.2-270	**5.9** (18) 0 – 84	**46.9** (48) 0-97.8	**168.2** (258) 73.8-391.2
N'gousso	UT	**49.6** (60) 1.2-183	**71.4** (78) 4.2-340.2	**81.2** (90) 7.2-348	**1.6** (2.4) 0 – 1.2	**81.3** (60) 28.8-295.2	**295.9** (120) 145.8-643.8
	P2	**17** (18) 3-66	**41.2** (42) 4.8-165	**65.8** (60) 10.2-238.2	**1.6** (1.8) 0 – 7.2	**51.3** (24) 18- 120	**204.7** (72) 99-342
	OLY	**41.6** (42) 1.2-166.8	**57** (48) 3-222	**88** (66) 9-247.2	**4.1** (12) 0 – 61.8	**48.1** (30) 4.8-106.8	**140.5** (72) 31.8-379.8
Banfora	UT	**45** (54) 1.2-199.2	**67.3** (60) 1.8-199.8	**91.3** (72) 1.8-262.2	**1.9** (2.4) 0 -13.8	**51.3** (30) 12-130.2	**512.8** (210) 129-1140
	P2	**38.7** (54) 1.8-169.8	**53** (66) 3-220.8	**79.6** (90) 6-265.8	**5** (12) 0 – 61.8	**43.5** (24) 12-100.8	**286.6** (114) 118.2-520.8
	OLY	**42.7** (42) 1.2-163.2	**55** (48) 6-181.8	**77.3** (60) 7.8-4.10	**2.6** (4.2) 0 – 18	**36.9** (30) 6-118.2	**222.8** (78) 108-430.2
VK7	UT	**45.3** (42) 3-142.2	**71.5** (60) 4.8-261	**79.7** (60) 16.2-265.8	**2.2** (24) 0 – 7.2	**62** (36) 7.2- 160.8	**464.4** (240) 196-931.8
	P2	**42.7** (48) 0-170.8	**55** (54) 4.2-201	**66.3** (60) 4.8-202.8	**4.74** (12) 0 – 60	**45.2** (24) 1.8-118.2	**253.6** (108) 121.8-535.8
	OLY	**36.8** (42) 12-133.8	**45.5** (48) 1.2-157.8	**35.3** (78) 7.2-304.8	**49** (12) 0 -31.2	**47.9** (42) 4.8-199.2	**203.6** (96) 52.8-414

Table 2.5: Comparison of duration of events for insecticide susceptible and insecticide resistant *An. gambiae s.l* when exposed to untreated net compared to PermaNet 2.0 and Olyset Net. The duration of each event was compared using a non-parametric test Kruskall Wallis in SPSS. * show when comparisons were statistically significant (P < 0.05). All significant values indicate the mosquito spent significantly less time when exposed to the treated net compared to an untreated net.

Group		Before appearance	Time Before First contact	Time Before landing	Landing - Probing	Probing - Insert	Overall Blood-feeding
Kisumu	Olyset	P=0.113	P= 0.693	P= 1.000	P = 0.227	P= 0.013*	P< 0.0001*
	PermaNet		P= 0.012*	P= 0.021*	P = 0.295	P= 0.507	P<0.0001*
N'gousso	Olyset	P=0.172	P= 0.343	P= 0.363	P = 0.168	P= 0.033*	P< 0.0001*
	PermaNet					P = 0.075	P= 0.032*
Banfora	Olyset	P= 0.595	P= 0.245	P= 0.565	P = 0.897	P= 0.069	P<0.0001*
	PermaNet						P= 0.001*
VK7	Olyset	P= 0.614	P= 0.184	P= 0.405	P = 0.593	P=0.183	P<0.0001*
	PermaNet						P= 0.001*

2.3.5 Resting behaviour of *An. gambiae s.l* post bloodfeeding through untreated and treated netting

There was a significant effect of treatment on resting. Post-feeding, all mosquito strains, regardless of insecticide-resistance status, were more likely to rest on untreated nets than on Olyset (P<0.0001) or PermaNet (P<0.0001). The odds for resting for 3 minutes versus immediately departing the net post-feeding are 95% and 77% lower on Olyset and PermaNet, respectively compared to untreated net. As shown in Figure 2.5, the highest proportion of mosquitoes that departed the net immediately after feeding occurred when all strains were exposed to Olyset net. Resting for 3 minutes occurred most frequently when mosquitoes were in contact with an untreated net.

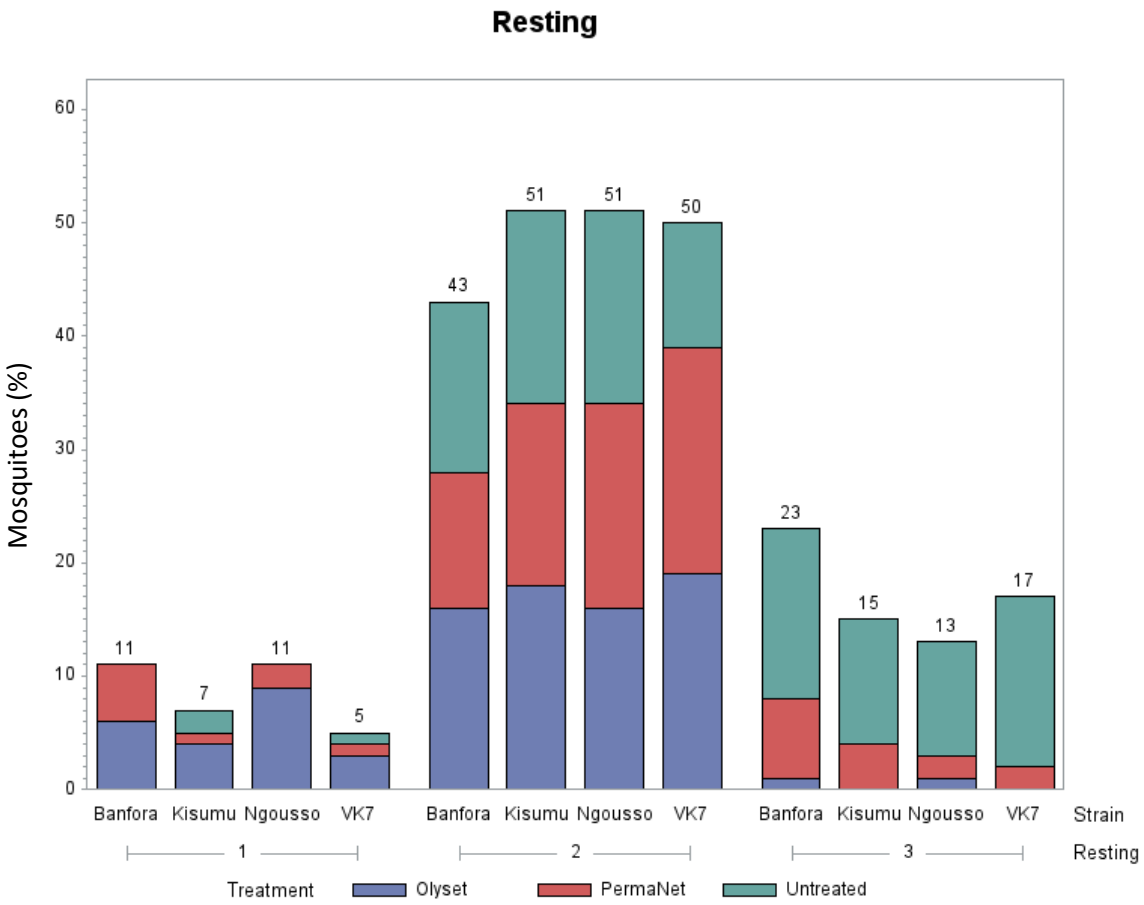


Figure 2.5: Time spent by *An. gambiae s.l.* resting on untreated and ITNs post feeding. The proportion of mosquitoes that spent time in each category with N shown at the top of each stacked bar: 1= departing net immediately (resting for 0 minutes), 2= resting for any time less than 3 minutes (0.10-2.59 minutes), 3 = resting for 3 minutes/the time the test was terminated, when exposed to each bednet type: Olyset = blue, PermaNet = red, untreated = green

2.4 Discussion:

The baited box test was developed to assess the behavior of *An. gambiae s.l.* at a treated net interface. The aim of the study was to describe the behavioral responses of resistant and susceptible *An. gambiae* when bloodfeeding through treated nets. This test provided key insights into the behavioural events and time frames involved in host seeking and bloodfeeding at a baited net interface, which current testing methods do not measure (Hughes *et al.,* 2020). The baited box gathers individualised data, allowing assessment of how reduced contact duration may impact subsequent insecticidal effects on behaviour and sublethal effects post exposure. Currently, WHO tunnel tests are used to assess the ability of a mosquito to reach a non-human host and feed after contact with a treated net, yet the key behavioural events are not measured in real time. The baited box was therefore used to gather overall understanding of the mosquito's interaction with the bednet interface. The sequence

of behavioural events from host seeking/bloodfeeding to resting post feed was similar in this study compared to previous tests (Hughes *et al.,* 2020).

In order to investigate the repellent effects of both ITNs, the first objective of the study was to assess whether there was any difference in the response rates of each strain to treated net compared to untreated net. Previous studies have shown response rates to differ depending on the operator of the baited box (Hughes *et al.,* 2020). Although this was beyond the scope of the current study, differences in host preference are an important consideration for the future application of this test method. However, the current study demonstrated, with a consistent host, some strain dependant differences in host seeking behaviour. With both Kisumu and N'gousso having the highest response rates to untreated net, these strain dependant responses could be explained by the fact that both are susceptible to insecticides therefore less likely to contact the treated net types. Another explanation for this result could be that this is the effect of long-term colonisation, as this has been shown to create physiological adaptations to an artificial laboratory environment over time (Benedict *et al*., 2009; Weill *et al.,* 2004).

Exposure to PermaNet may be slightly reduced the mosquito's response rate to a human host, with a reduction of between 3-16% in all strains compared to an untreated net. However, in the current study, with a small sample size, this reduction was not significant. Significantly lower response rates were observed in both susceptible strains when exposed to Olyset net compared to untreated net. There was no difference in the response rates of either resistant strain when exposed to untreated and treated netting.

Repellency to ITNs, as described by Dethier (1960), as a treatment which causes an insect to move away from its source, results in the mosquito making no contact with the net. In the past permethrin has been described repeatedly in scientific literature as having repellent properties (Brown *et al*., 1997; Cockcroft *et al.,* 1998; Sholdt, 1988) and a number of experimental hut trials have found permethrin treated nets to be repellent, with the use of Olyset nets increasing hut exiting and exophilic behaviour in *An. gambiae* s.l. species (Ansari *et al.,* 2006; Lindsay *et al*., 1991; Lines *et al.,* 1987). Results from the current study suggest Olyset nets have a repellent effect on the susceptible strains that is not present in resistant strains. Although a relationship between the kdr mutation and response rates to insecticides has been shown before, (Chandre *et al.,* 2000; Deletre *et al*., 2019; Porciani *et al.,* 2017), the complex mechanisms underlying the role of resistance on the responses to repellent insecticides are not yet understood.

Previously it has been shown that an innate drive to feed may override any repellency effects of an insecticide (Sungvornyothin *et al.,* 2001). In the current study, once host seeking was initiated, no

repellent effects were observed on the mosquito's willingness to contact and land on the net before beginning to probe. This was the case in all net types, across all four strains. Hughes *et al* (2020) also showed that at very close range, the mosquito's behaviour before net contact is indistinguishable to the behaviour observed at an untreated net interface.

Although permethrin has shown to have repellent properties, other pyrethroids, such as deltamethrin contained in PermaNet have shown low repellent effects (Mosha, 2008; Parker *et al*., 2015; Spitzen 2014). Findings from this study and previous video tracking experiments by Parker *et al* (2015) on PermaNet, found no repellent effects of this net type on host seeking mosquitoes although the mosquitoes spent less time in contact with the treated net. This test showed a difference in the behaviours prior and post contact with the net interface, which other studies have not described (Hauser *et al.,* 2019). The results from the baited box support the use of ITNs as a baited trap, showing no repellency when mosquitoes are actively host seeking at close range. However, the results do not show how effective a net is at deterring a mosquito at long range, with some experimental hut data showing deterrence effects of houses with ITNs (Koudou *et al.,* 2011; N'Guessan *et al.,* 2010).

Results from this study however to suggest Olyset nets may be increasing host diversion and increasing exophilic behaviours in *An. gambiae s.l* populations (Ansari *et al*., 2006; Lindsay *et al*., 1991). More data on longer range host seeking activity around a human baited Olyset net will be discussed in Chapter 4.

When in contact with the net, susceptible *An. gambiae* strains spent less time probing through an Olyset net compared with an untreated control. Previous research has shown reduced probing time through permethrin treated materials is the result of higher observed contact irritancy in susceptible mosquito strains compared to resistant strains (Cockcroft, 1998; Miller *et al.,* 2004; Hodjati, 1997; Sholdt, 1989). Contact irritancy effects of nets can result in the mosquito not receiving a lethal dose of insecticide, sometimes causing early disengagement with the net. The difference in probing times on an Olyset compared to an untreated net is only observed in the susceptible strains of *An. gambiae s.l* in both feeding permitted and feeding prevented baited box assays (Chapter 2 and 3), suggesting that susceptible strains of *An. gambiae s.l* spend less time probing in search of a blood meal due to the irritant effect of the insecticide.

Baited box results show that when a host is available all strains of *An. gambiae s.l* were willing to take a blood meal through the treated netting which is an extremely important finding when considering the efficacy of these net types.

Many hut trials have shown that ITN use reduces the number mosquitoes able to take a blood meal (Mathenge, 2001; Sood, 2011) as the person is protected by the net barrier. Other studies have also shown that exposure to insecticide treated nets may affect subsequent bloodfeeding attempts, ultimately reducing malaria transmission. Glunt *et al* (2018) reported a 60% reduction in successful feeding when *Anopheles spp* were offered a bloodmeal after ITN exposure.

The second objective of the study was to investigate whether there was a difference in feeding duration when mosquitoes fed through a treated ITN compared to an untreated net. The ability of insecticide susceptible mosquitoes to bite through treated nets has been shown previously (Hauser *et al.,* 2019; Hughes *et al.,* 2020). Not only has this been shown to reduce the personal protection provided by the ITN to the individual, but studies have also suggested that feeding directly through the net induces a protective effect on the mosquito's survival, decreasing the chances of insecticide induced mortality (Hauser *et al.,* 2019). This ability to bite and feed through bednets could seriously undermine the efficacy and success of ITNs and have detrimental effects on community protection provided by ITN interventions. These topics will be discussed in more detail in Chapter 5.

In the current study, although mosquitoes were able to bloodfeed through all nets, duration of the blood meal was significantly reduced in all strains when exposed to treated netting compared with untreated netting ($P<0001$). Hauser *et al* (2019) also reported that pyrethroid susceptible *An. gambiae* mosquitoes spent less time feeding through an Olyset Plus net than through untreated netting.

It has been suggested that differences in bloodfeeding behaviour between susceptible and resistant mosquito strains might occur due to differential expression of salivary proteins (Djegbe, 2011). However, the current study suggests all strains reduce the amount of time spent feeding when exposed to pyrethroids regardless of resistance status and could be therefore due to an adaptive response, or an irritant effect. It is likely that duration of feeding is reduced due to the irritation caused by the pyrethroid, suggesting that regardless of resistance status the pyrethroids still cause contact irritancy effects on the mosquito. Results suggest that resistant and susceptible mosquitoes can withstand the similar level of contact with an ITN when bloodfeeding, however in comparison to the susceptible strains, the resistant mosquitoes are more likely to survive this contact and ultimately reproduce and potentially pass on malaria infection. The exact mechanisms causing this reduction in feeding is unknown though and further research is required to investigate the effects of this behavioural change on malaria transmission. It is thought that lower inoculation rates are correlated a lower number of sporozoites in the salivary glands however it is unknown how the duration of blood feeding effects malaria infection (Rosenberg *et al.,* 1990).

Other studies with *Aedes aegypti* have shown that mosquitoes can increase or decrease feeding duration as required (Chadee *et al*., 2002; Gillett 1967). It was observed that wild populations of *Aedes aegypti* feed faster than the colonised colony (Gillett, 1967). These adaptations are thought to be a result of selection against slow feeders in the presence of predation. However other studies have shown bloodfeeding durations to be an adaptive response when the mosquito encounters physical interference during bloodfeeding (Chadee *et al.,* 2002). If the ability to adapt feeding behaviours is selected for, this may also enhance vector efficiency (Chadee *et al.,* 2002). Jin *et al* (2007) showed time spent feeding effects sporozoite delivery. Mosquitoes allowed to feed on a mouse ear for 3- and 15-minutes deposited means of 281 versus 452 sporozoites (Jin *et al.,* 2007). The study did not measure the volume of blood ingested and it is unknown whether volume of blood meal may also influence sporozoite delivery.

The third objective of the study was to investigate whether there was a significant difference in the amount of time spent each stain spent resting on each net type. Post feeding, mosquitoes preferentially rest on an untreated net than treated net. Previous cone tests performed at LSTM have shown that duration of resting on an Olyset net is significantly lower than untreated and PermaNet (Hughes, 2018). When contact with the net surface was prevented in the cone tests, and so exposure was restricted to any volatiles from the net, resting behaviour was re-established. Reduction of resting duration was therefore concluded to be due to contact irritancy of the insecticide treated net (Hughes, 2018). Levels of contact irritancy have been previously shown to vary with dose, mosquito species, and insecticide resistance status (Chandre *et al*., 2000; Hougard *et al*., 2003). In the current study the odds for resting for three minutes versus immediately departing the net post-feeding were 95% and 77% lower on Olyset and PermaNet, respectively versus untreated net. This reduction in resting duration was observed in all strains when exposed to treated net, regardless of their resistance status. With this in mind, contact irritancy of an ITN is an important consideration for bednet success as reduction in resting time contributes to an overall reduction in amount of contact the mosquito has with an ITN, ultimately reducing the overall chances of receiving a lethal dose of insecticide.

The baited box is not only a useful assay for assessing the standard pyrethroid ITNs, but the test also has the potential to contribute to the search for next-generation nets. Due to the complex modes of action and delayed mortality effects of the next generation of controls and bednets containing other insecticide classes, such as chlorfenapyr and clothianidin, current WHO methods inadequately predict the actual efficacy of these new chemistries when used in the field. The baited box gathers more detailed information, exposing mosquitoes in a more field relevant setting, in order enhance the understanding of the control measures performance in the field.

The sub-lethal effects after insecticide exposure was also measured with this method, something all current WHO standard test procedures omit. Sublethal impacts of ITN exposure is discussed in Chapter 5.

2.5 Conclusion:

The baited box was used to characterise the behaviour of individual free-flying mosquitoes attempting to bloodfeed through a human baited bednet, quantifying the behaviour in a field-relevant setup and providing a useful behavioural baseline for further studies. Results from this assay indicate that deltamethrin-treated ITNs are not repellent to host seeking mosquitoes at short range. Permethrin treated nets however displayed varied repellency/irritancy effects depending on the mosquito's resistance status. Exposure to both treated nets did reduce the overall amount of time spent bloodfeeding and resting on the net, with Olyset exposure causing the highest reductions. Determining how much contact a susceptible/resistant mosquito needs to induce mortality or cause delayed or sub-lethal effects will also be important when investigating the impact of non-pyrethroid nets. Results from this assay can be compared to large scale room tracking in Chapter 4, comparing behaviour of long- and short-range host seeking, gathering an overall insight into how susceptible and pyrethroid resistant *An. gambiae s.l.* interact with the bednet interface.

Chapter 3: Behaviour of *An. gambiae* at the human baited net interface when denied access to the host.

3.1 Introduction

To maximise performance and longevity, the next generation of ITNs must be founded on a comprehensive understanding of how bed nets perform, and how mosquitoes behave at the net interface. The standard WHO tests currently used for evaluating treated net performance may not be adequate in providing this information.

Previously, Hughes *et al*., (2020) used the baited box to create a better understanding of mosquito-ITN interactions and show how this new assay can be used to accelerate the development of more effective vector control tools. That was followed by work discussed in Chapter 2 where the test was used to characterise behaviour of resistant and susceptible strains of *Anopheles* as they bloodfed through standard pyrethroid nets. Results showed that both susceptible and resistant strains spent significantly less time in contact with treated nets compared with than an untreated control, also reported elsewhere (Hauser *et al*., 2019, Hughes *et al*., 2020, Parker *et al*., 2015). However, in reality, the majority of mosquitoes will neither feed through the net, nor enter the net to reach the host. It is important to understand the net's repellent or irritant properties, and when mosquitoes are denied access to the host, whether the mosquito is likely to contact the treated net for long enough to receive an effective or lethal dose, and to determine the minimum duration of ITN contact required to achieve that.

Large room-scale video tracking studies have been vital in exploring how mosquitoes interact with a human baited net. The first study by Parker *et al*. (2015) demonstrated how human-baited ITNs operate and how the mosquito makes brief but multiple contacts with the net, almost entirely on the net roof. This confirmed the results reported in an earlier study that first provided evidence for the importance of the net roof (Lynd & McCall, 2011). Subsequently Parker *et al*. (2015) used the video system to show that, during a test period of one-hour, individual *An. gambiae* (Kisumu susceptible strain) spent an average of less than 1-minute per mosquito in contact with a PermaNet. Repeating the study with a wild population of *An. arabiensis* in Tanzania found that the duration of contact any single mosquito had with the net was between 204–290 seconds on untreated nets and 46–82 seconds on PermaNet (Parker *et al*., 2017).

Observing mosquitoes at whole room scale cannot capture details of close-range behaviour during the final stages of host location as the mosquito encounters the bednet. This chapter reports on experiments to characterize the behaviour of mosquitoes prevented from feeding or touching the human host, by using a second untreated barrier blocking access to the test net and the host skin in the baited box assay (Figure 3.2). This was the only change from the experimental setup used in Chapter 2.

The specific aim of the experiment was to describe the short-range behavioural responses of pyrethroid resistant and susceptible An. gambiae when exposed to a baited pyrethroid treated net.

The hypothesis explored where:

1) There will be no significant difference in the response to a treated and untreated net for each strain.

2) There will be no significant difference in the duration of overall contact with the all three net types.

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3.2 Materials and Methods:

3.2.1 Mosquito colonies and test materials

In all experiments, two fully susceptible and two pyrethroid resistant strains of *An. gambiae s.l.* were exposed to untreated nets, PermaNet 2.0 or Olyset ITNs. All details were unchanged from those described in Chapter 2 (section 2.2).

3.2.2 Experimental set up and test procedure

The baited box apparatus and protocol were operated as described in Chapter 2, section 2.2.4 with the following described modifications. Unlike the bloodfeeding tests, where activity was almost entirely located at the test netting, preliminary tests showed that mosquito activity occurred throughout the box. Hence, to capture the entire testing arena (see Figure 3.1), a 50mm lens (Nikon f1.8 lens, aperture set at f2.8) was used instead of 60mm.

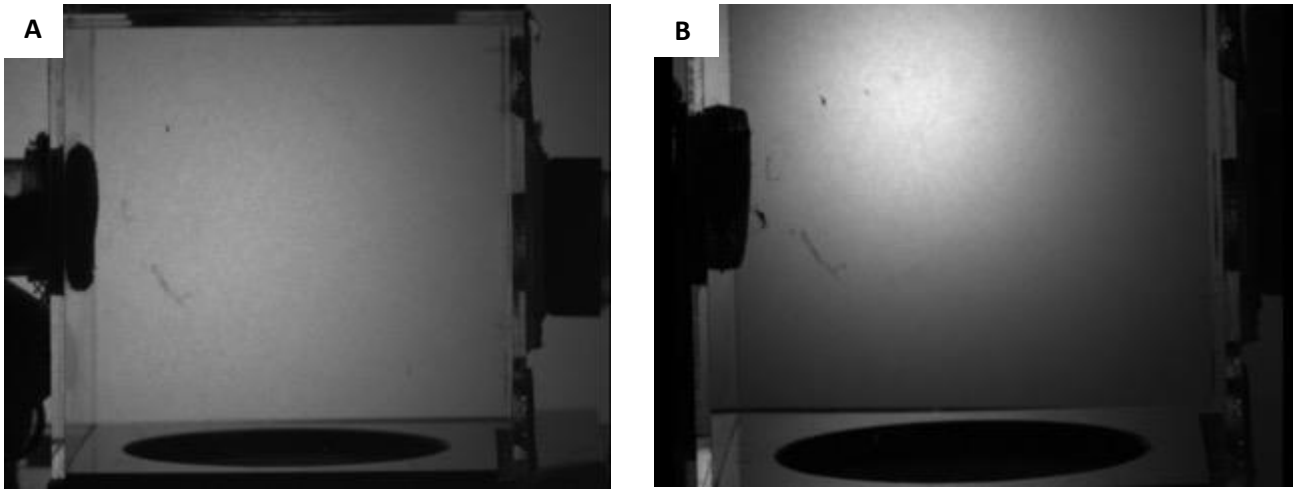


Figure 3.1: Example still image from test videos with the Baited box with different lenses A: The field of view with a 50mm lens, where the test net, entry port and walls of the test chamber are all visible B the narrower field with a 60 mm lens, allows more detail to be seen at the test netting but the walls are excluded.

Mosquitoes were prevented from reaching the host's skin through the test netting, by a polypropylene mesh barrier, placed 5mm between the test netting and thumb (Figure 3.2).

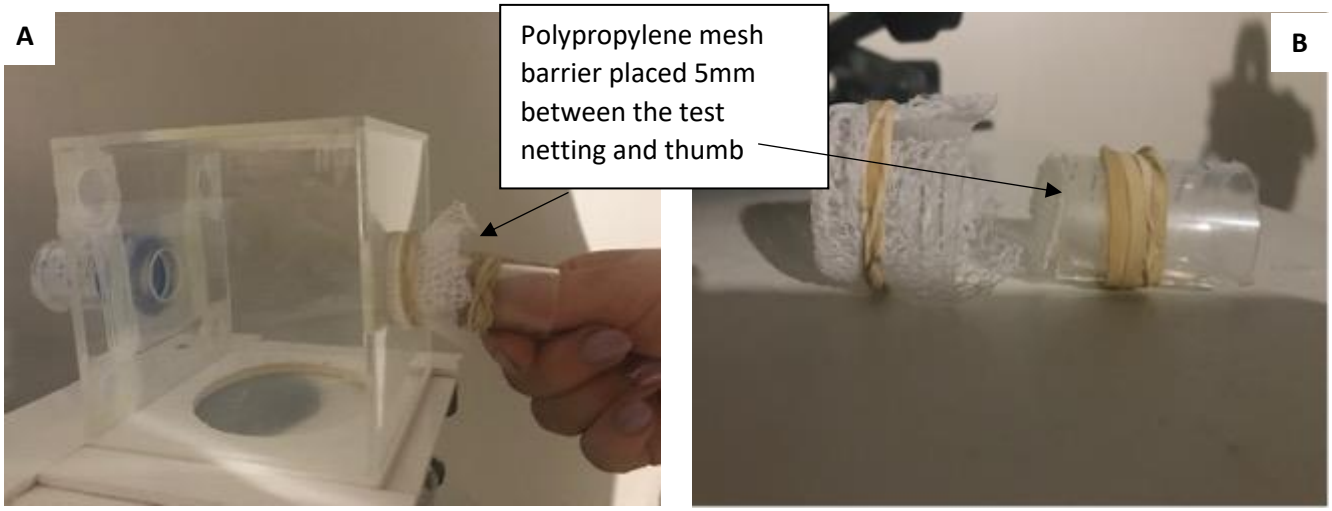


Figure 3.2: baited box for feeding prevented assay. A: Baited box apparatus with a polypropylene mesh barrier is placed 5mm between the test netting and thumb to prevent feeding through the net. B: close-up image of polypropylene barrier apparatus alongside test netting port

During tests, only mosquitoes that entered the arena and contacted the test netting within 10-minutes of the entry gate being opened were classed as responders and included in analyses. As bloodfeeding was not possible, the length of the experiment was standardised to 20-minutes.

3.2.3 Behaviour classification:

In order to explore the effects of ITN exposure on mosquito behaviour, mosquito behaviours that were

detectable and distinct from one another were identified. This was done by reviewing multiple assays with four mosquito strains and three net treatments. The duration of each behavioural event, as defined in Table 3.1, was calculated for every treatment and experiment. The time from 'open entry gate' (event 1) until the mosquito 'entered the field of view' (event 2) was classified as prior to appearance. Mosquitoes that re-entered the entry tube after appearance or remained still at the bottom of the box were lost form the field of view and recorded as 'Out of view'.

Table 3.1: Classification of events

Step	Event	Definition
1	Prior to appearance	The time before the first point at which the mosquito is seen in the cameras field of view
2	Visiting	The mosquito contacts the test netting, but wings don't stop
3	Flying	The mosquito is in flight
4	Contacting arena wall	Standing on any surface except the net
5	Probing	The mosquito probes through the net, tilts forward; proboscis pushed through net
6	Resting on net	Standing on net; not probing.
7	Out of view	Mosquito cannot be seen in field of view after first arrival

3.2.4 Video analysis:

All behaviour assays were recorded and stored on hard drives, and later analysed by reviewing the entire video in slow motion using BORIS (Friard, O. and Gamba, 2016). The behavioural events were classified in using the coding system set up in BORIS software and the categories detailed in section Table 3.1. All data were exported into Excel 2010.

3.2.5 Data analysis:

Originally 20 mosquitoes of each strain were exposed to each net type. Due to damage during storage for subsequent examination for sub-lethal analyses, additional replicates were completed, and

behaviour data was included in the analysis below. The sample sizes completed for each strain and treatment is detailed in Table 3.2.

Table 3.2: Sample Size for baited box assays per strain and net treatment. Total number of mosquitoes per strain and responding to Olyset, PermaNet and untreated control.

		Sample size			
		No. of responding mosquitoes			
Mosquito strain		Kisumu	N'gousso	Banfora	VK7
Treatment	**Untreated**	**25**	**24**	**29**	**22**
	PermaNet	**20**	**20**	**29**	**27**
	Olyset	**27**	**21**	**26**	**26**

Response rates were compared using a Chi squared test in SPSS (IBM SPSS statistics Version 25). Frequency plots were prepared with R (Team, 2017) using the package TraMineR (Gabadinho, *et al.*, 2011). As these data were not normally distributed, (Table 3.2; Figure 3.3) they were compared using the Kruskal Wallis non-parametric test in SPSS (IBM SPSS statistics Version 25).

Treated nets deliver insecticide by direct contact when the mosquito lands on the net, and the duration of contact is used as a proxy for insecticide exposure (and the dosage received) (Parker *et al*. 2015). For further analysis, behavioural events (Table 3.1) were grouped under contact events (probing, resting and visiting Table 3.1) or non-contact (all other events) and overall duration calculated. As these data were also not normally distributed, (Table 3.2; Figure 3.3) they were compared using the Kruskal Wallis non-parametric test in SPSS (IBM SPSS statistics Version 25).

To assess how interactions with each treatment changed over time, the duration of net contact was analysed for 5-minute time intervals using a GEE with Statistical analysis software (SAS), (Version 9.4 of the SAS System for Unix 2002-2012). PROC Genmod command was used to perform a GEE link function was specified with an exchangeable correlation structure and underlying binary distribution. The odds Ratio and 95% confidence intervals for response were computed from the final GEE model (Variable; contact vs no contact; Predictors; treatment, strain, and contact duration).

The correlation between longevity and contact duration was plotted in activities in GraphPad Prism

version 8.4.3 for Windows (GraphPad Prism version 8.4.3 2020).

	1	2	3	4	5	6	7
BEHAVIOUR EVENT	**START**	**Flying**	**Contacts arena wall**	**Visiting**	**Probing**	**Resting**	**Out of view**
	Time prior to appearance			**Contact with net**			
Behaviour description	Gate open (T_0) Time elapsed until mosquito enters the field of view	Mosquito in flight	Visiting and resting on any arena surface except the net	Touches the test net briefly, but wings don't stop	Tilts forward; proboscis pushed through net	Stands on net; no probing	Mosquito cannot be seen in field of view at any time after first appearance
Activity	Response lag time			Contact with insecticide			

Figure 3.3: **Ethogram of the behavioural events distinguishable in *An. gambiae s.l.* responding to a human host protected by an intact insecticide-treated netting**. The numbers are intended for ease of referral and do not suggest a fixed temporal sequence; events after #1 could occur in any order and at any frequency. The colour code corresponds with Figures 3.4 and 3.5: only events with solid black borders (events 4,5,6) represent behaviours involving net contact. All tests were stopped after 20-minutes. Modified from Hughes *et al* 2020.

3.3 Results

3.3.1 Response rates

Table 3.3: Response rates in the baited box assay of susceptible (Kisumu and N'gousso) and resistant (Banfora and VK7) strains of *An. gambiae* exposed to three net types; Untreated, PermaNet and Olyset net. The response rate for each strain exposed to treated net was compared to the untreated control. There were no significant differences between the response rate of each strain to the different treatments, with the exception of VK7 which was significantly higher when exposed to treated nets: the significant difference in response rates is labelled with different superscripts below (P<0.001).

		Response rate (%) No. mosquitoes responding/ no. tested			
Mosquito strain		*Kisumu*	*N'gousso*	*Banfora*	*VK7*
Treatment	**Untreated**	**56%** (25/45)	**47%** (24/51)	**23%** (29/127)	**13%** (22/174)[A]
	PermaNet 2.0	**45%** (20/44)	**49%** (20/41)	**29%** (29/101)	**46%** (27/59)[B]
	Olyset	**48%** (27/56)	**35%** (21/60)	**38%** (26/68)	**31%** (26/84)[B]
Total no. tested		145	152	296	317

A total of 910 mosquitoes were released and tested individually over a period of 36 months, of which 296 (33%) responded. The response rates ranged from 56% (Kisumu at untreated net) to 13% (VK7 at untreated net) (Table 3.3), varying with mosquito strain and net type. Within strains, there were no significant differences between response rates of Kisumu, N'gousso and Banfora at untreated *vs.* treated nets. However, significantly fewer VK7 responded to untreated nets than to Olyset (P<0.001) or PermaNet 2.0 (P<0.0001). The results of the analyses of mosquito behavioural responses as defined by the ethogram in Table 3.3, Table 3.4 Figures 3.3-3.5).

As in the bioassays where bloodfeeding was permitted, (Chapter 2) and as is clear from the data in Table 3.3, responses by individual mosquitoes, even within the same treatment or strain, were extremely variable. Moreover, in contrast to the conserved behavioural sequence observed when mosquitoes were able to bloodfeed, 6 out of the of the 7 activities (Table 3.1; time before appearance was only recorded once per test) occurred repeatedly throughout the assay, unless interrupted by the treated net. Despite the variability in the data, certain trends are apparent. For example, activity at the net surface (events 4, 5 and 6) constituted less than 5-minutes in all assays except the susceptible strains at untreated nets. Furthermore, the VK7 strain consistently shows a number of behaviour traits that differ markedly from the other strains, regardless of net treatment e.g. responses to the human host, as measured by probing and overall net contact time particularly probing at an untreated net, which VK7 spent much less time than all other strains.

Table 3.4: Duration of events for insecticide susceptible and insecticide resistant *An. gambiae s.l* when exposed to PermaNet and Olyset net. Untreated net data included for comparison purposes. The mean duration of time spent in each behavioural event (as classified in Table 3.1) with standard deviations are recorded. The range for all assays is included for each event.

EVENT / ACTIVITY		1-2	3	4	5	6	7	8
		Prior to appearance	**Visiting**	**Flying**	**Contacts arena wall**	**Probing**	**Resting on net**	**Out of View**
STRAIN	**NET**	***mean duration in seconds*** *(Standard deviation), range*						
Kisumu	UT	**35.0** (32.9) 2.8- 120.6	**16.8** (20.5) 1.9 -106	**300.6** (196.5) 49.8-767.9	**467.2** (329.7) 12.3- 991.3	**307.2** (217.8) 5.4- 817.8	**119.8** (206.6) 7 - 640.6	**53.9** (81.5) 0.8-
	P2	**33.7** (35.3) 3.3-102.3	**17.2** (13.8) 1- 49	**333.4** (187.5) 10.9- 798.2	**552.3** (234.4) 156.1-948.3	**106.7** (69.3) 18.1-	**63.5** (83) 1.2-284.4	**162.6** (231.5) 1.2- 866
	OLY	**52.8** (42.5) 2.8- 156.9	**11.3** (9.5) 0.6- 40.4	**301** (107.4) 50.1-538.9	**559.2** (164.2) 278.9-883.3	**83** (69.7) 1.9- 303.9	**47.6** (57.3) 1.9- 180.8	**183.8** (128) 1-405.1
N'gousso	UT	**55.5** (50.9) 7.6- 175.1	**17.9** (11) 5.2- 39.8	**324.2** (150.9) 55- 671	**406.6** (277.7) 9.4- 883	**341.5** (244) 53.4- 792.6	**18.1** 29.14 0.8- 61.6	**88.2** (188.4) 0.7-
	P2	**41.1** (37.3) 2.6- 148.1	**8.5** (6.2) 0.6- 18.6	**365.5** (231.1) 55.8-	**586.4** (250.1) 97- 942.3	**99.5** (93.6) 0.5- 274.1	**48** (129.5) 1.1- 393	**164.9** (219.5) 1.4-
	OLY	**36.1** (42.) 5.5- 170.7	**14.8** (16.8) 1.4- 65.4	**415.2** (210.3) 132.1-	**532.3** (200.1) 143.6- 852.7	**89.6** (64.6) 12.4-	**22.5** (0.8) 21.9-23	**115.6** (189.8) 1.3-
Banfora	UT	**39.1** (38.6) 1.6- 158.8	**19.1** (22.6) 0.3- 78.1	**264.3** (228.8) 3.3- 1085	**658.1** (348.6) 15.1- 1097.2	**138.2** (197.4) 0.4-	**121.2** (207.9) 4.3- 589.9	**97.4** (233) 1- 867.7
	P2	**32.6** (40.6) 2- 142.9	**8.8** (6.3) 0.8- 23.1	**224.6** (142.6) 0.2- 500.8	**741.9** (242.6) 171.7- 1120.2	**67.8** (91.2) 0.5- 460.2	**148.2** (302) 0.7- 1161.4	**126.3** (199.4) 0.5- 649
	OLY	**36.7** (41.9) 2- 175.6	**7.8** (9.1) 0.3-47	**253.4** (176.6) 32.9- 740.2	**804.9** (236.7) 276.2- 1142.5	**32.1** (35.1) 1.3- 139.1	**10.9** (10.5) 1-30	**111** (166) 0.6- 658.1
VK7	UT	**36.9** (36.1) 3- 139.5	**8.3** (9) 0.8- 43.7	**268.9** (272.6) 32.5- 967	**802.7** (309.7) 188.5- 1128.8	**36.9** (73.7) 0.8- 340.1	**84** (134.1) 4.1- 238.8	**93.6** (168.2) 4.8- 535.9

	P2	**24.1** (25.9) 0.2- 98.8	**6.9** (5.8) 0.9- 22.3	**423.4** (267.7) 77.9 -	**611.9** (270.9) 107.5- 1108.4	**40.5** (34.6) 0.5- 119	**64.4** (51.4) 15.7-120	**152.4** (224.6) 4-861.8
	OLY	**32.8** (32.6) 3.2- 142.7	**11.2** (8.4) 2.2- 36.4	**382.1** (184.9) 46.9- 779.2	**670.2** (221.5) 323.2- 1100.9	**55.7** (50.7) 1.9- 180.9	**33.7** (34.2) 6.4- 88.7	**60.2** (60.7) 0.9- 195.5

3.3.2 Behaviour of pyrethroid susceptible *An. gambiae s.l* at untreated nets.

Despite the high variability in individual mosquito responses (Table 3.3), both susceptible strains showed remarkably similar behaviour to untreated nets and in responses to treated nets. At untreated nets, a breakdown of activities during the 20-minute assay for Kisumu and N'gousso (Figure 3.4), comprised of, flight times of 301 and 324 seconds respectively, resting on the arena wall (467s and 407s), plus time to appearance (the initial lag time to respond) and periods when the mosquito was out of sight. At the net surface, probing times were also very similar at 307 seconds and 342 seconds, but resting time on the net was greater in Kisumu (120s) than N'gousso (18s).

In the Kisumu strain, the majority of resting occurred in the first 10-minutes. Flight levels were consistent throughout the 20-minute test. Changes in activity over time were also similar in both strains (Figure 3.5), with probing activity peaking at 2-3 minutes, and remaining high for the remainder of the 20-minutes. A slow decline in probing was observed towards the end. A corresponding increase in contact with the arena wall was observed for both strains when probing decreased.

3.3.3 Behaviour of pyrethroid susceptible *An. gambiae s.l* at pyrethroid-treated nets.

At the deltamethrin-treated PermaNet, a reduction in overall net contact, was immediately visible in both strains (Figure 3.5). For both susceptible strains' the duration of net contact (events 3, 6 and 7 combined) was significantly lower on treated than untreated nets, and on Olyset (Table 3.4: Kisumu; $P < 0.0001$ and N'gousso; $P < 0.0001$) more than PermaNet 2.0 (Table 3.4: Kisumu; $P = 0.035$ and N'gousso; $P < 0.0001$). Probing fell from 307 seconds and 341 seconds at untreated nets to 107 seconds and 100 seconds at PermaNet in Kisumu and N'gousso respectively. Probing duration was significantly lower at both treated nets (Table 3.4; $P < 0.0001$). Resting by Kisumu fell from 120 seconds at untreated net to 64 seconds at PermaNet with a corresponding increase in time out of sight (54s to 163 seconds) and resting on the net (467s to 552 seconds). Both increases were possibly due to insecticide intoxication or due to evading contact with the net. In N'gousso, the significant decrease in probing time also shifted activity to the arena wall (up from 407s to 586s) and out of sight (88s to 165s), but resting time increased from 18 seconds to 48 seconds on PermaNet. An explanation for this increase could be due paralysis from the toxicity of the net leaving the mosquito unable to escape the net surface. This is apparent in Figure 3.5.

Figure 3.5 shows how all behavioural events that occur post-release changed over the course of the 20-minute assay. Initial activity at treated nets is similar to that seen with untreated nets, but after 2-3 minutes, probing rates are lower and flight higher (especially in the N'gousso strain). After approximately 8-minutes, probing in N'gousso and resting in

Kisumu cease almost entirely and all activity at the net surface ceased by 15-minute. A gradual decrease is observed in flight activity from the 15-minute time interval, with a corresponding increase in time out of sight and time resting on the arena. The increase in time spent resting on the net by N'gousso on PermaNet occurred between 6 and 13 minutes and was most likely a result of pyrethroid intoxication on mosquitoes, halting the probing behaviour on the net (Figure 3.5).

With Olyset nets, total net contact was significantly lower than on untreated nets (p<0.0001) but not compared to contact with PermaNet, mainly from reduced probing duration (Table 3.4; from 307 seconds and 342 seconds at untreated nets to 83 seconds and 90 seconds at Olyset for Kisumu and N'gousso respectively; $P < 0.0001$). Probing never reached the levels of the untreated net and after 8-minutes had virtually ceased entirely in N'gousso with very low rates with Kisumu for the remainder (Figure 3.5).

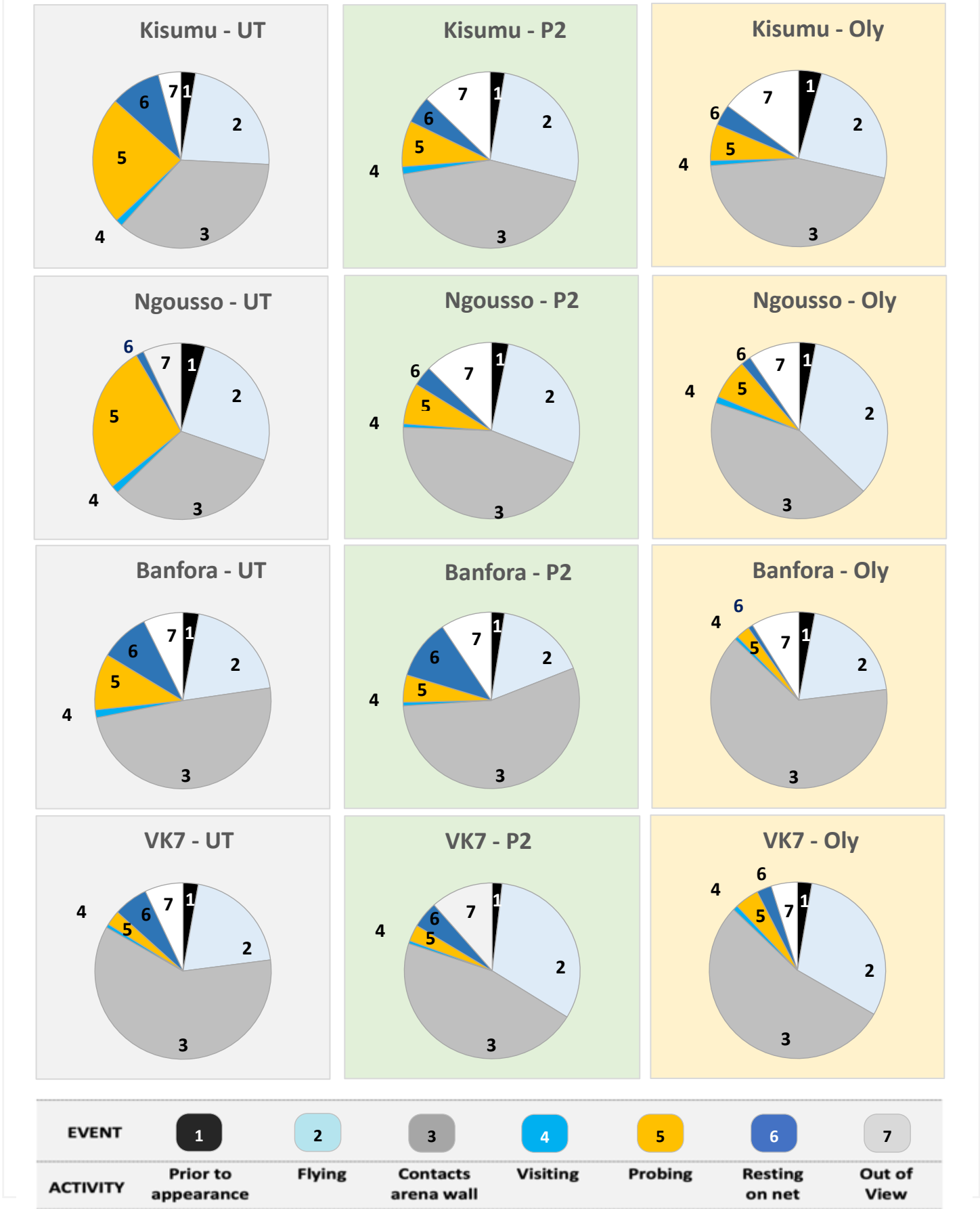


Figure 3.4: Summary pie charts of mean time proportions recorded for seven behavioural events observed in adult female *An. gambiae s.l.* denied access to the host by insecticide-treated netting in a human-baited box test. Each figure shows the mean proportion of time spent in each activity over the whole 20-minute assay.

3.3.4 Behaviour of pyrethroid resistant *An. gambiae s.l* at untreated nets

At untreated nets, the primary difference between the two insecticide resistant strains and the insecticide susceptible strains was the reduced duration in the resistant strains probing activity. Banfora and VK7 spent an average of 138 seconds and 37 seconds probing through untreated nets while, both Kisumu and N'gousso strains spent an average of 307 and 342 seconds, respectively. Banfora spent an average of 19 seconds visiting, and 121 seconds resting on the untreated net. VK7 spent slightly less time in both behaviours, spending an average of 8 seconds visiting and 84 seconds resting on the untreated net (Table 3.4).

For the Banfora strain, activity was consistent throughout the whole 20-minute assay, with resting and probing behaviours occurring throughout. Activity of the VK7 strains appears much lower than all other strains (Figure 3.5) with the majority of probing and resting time on the net occurring in the first 10-minutes. Flight levels were consistent throughout the 20-minute test for both strains when exposed to an untreated control net.

3.3.5 Behaviour of pyrethroid resistant *An. gambiae s.l* at pyrethroid-treated nets

The behaviour of pyrethroid resistant mosquitoes when exposed to treated and untreated nets differed between the two strains.

In the Banfora strain, contact with the untreated net and PermaNet remained constant throughout the 20-minute time- period. When exposed to Olyset net, similarly to both susceptible strains, Banfora's overall activity decreased compared to untreated and PermaNet, with most activity occurring in the first 8-minutes of the assay. Prior to appearance, the Banfora spent 32-39 seconds on average to leave the entry tube when exposed to all the three net types. At Olyset net, the amount of time Banfora spent in contact with the net significantly reduced (P= 0.002), probing significantly reduced from 138 seconds through and untreated to 32 seconds when exposed to Olyset net (P = 0.003), but there was no significant difference in the amount of time Banfora spent in overall contact with (P= 0.196), or probing through untreated and PermaNet (P= 0.194). Flight activity remained similar in Banfora when exposed to all three net types (P=0.935) spending an average of 225-264 seconds in flight throughout all assays.

Responses of the VK7 strain are the most aberrant of all tested. There was no difference in the amount of total contact (events 3, 6 and 7 combined) VK7 had with the untreated and treated net (P = 0.67). Broken down into different events, VK7 spent significantly more time in flight when exposed to PermaNet (Mean; 423-seconds) than untreated net (P= 0.039, Mean; 269 seconds), but there was no difference in the amount of flight activity when exposed to Olyset compared to untreated net (P= 0.075). The VK7 spent the least amount of time in the entry tube when exposed to PermaNet (Mean; 24 seconds) than untreated and Olyset net (Mean; 37 and 33 seconds; Table 3.4) and responded to this net more frequently than the other net types (Table 3.3). Overall, the VK7 strain exhibited very low levels of activity (Figure 3.5) with little probing behaviour through all three nets, with the most probing behaviour occurring through Olyset within the first 5-minutes (Figure 3.5).

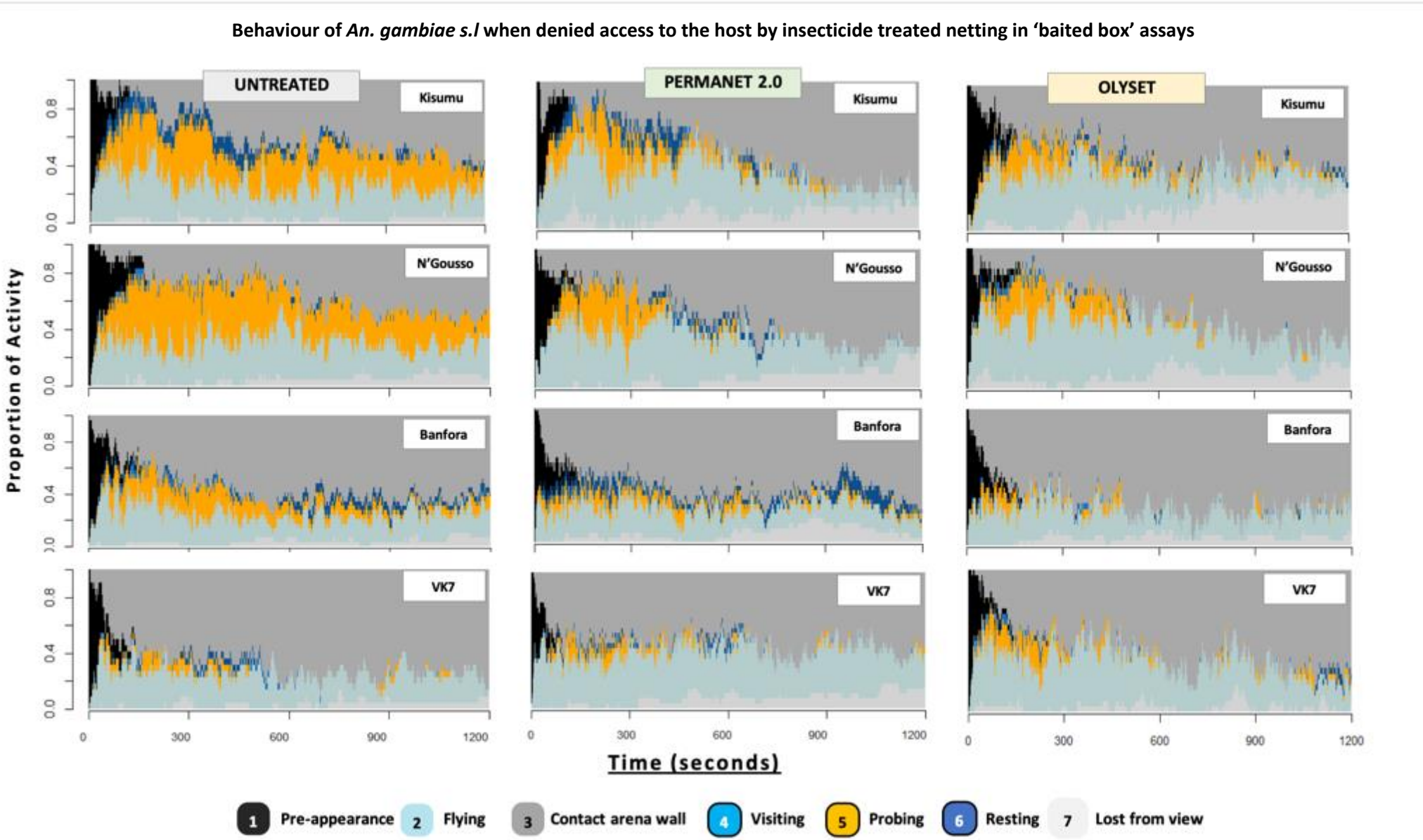


Figure 3.5: Temporal changes in the behaviour of adult female *An. gambiae s.l.* denied access to the host by insecticide-treated netting in a human-baited box test. Assays were performed for 20-minutes and the data (Table3.1) collected from the recorded video using BORIS (Friard & Gamba, 2016), Graphs created in R using the package TraMineR (Gabadinho, *et al*., 2011). Each figure shows the proportion of each activity observed every minute, over a 20-minute time-period.

3.3.6 Temporal changes in the behaviour of susceptible and resistant *An. gambiae s.l.*

To determine whether or not total contact with the net changed over time, total contact time was calculated per 5-minute intervals (<5, <10, <15, <20), and the probability of being in contact with the net vs. no net contact was determined for each net type.

0-5 minutes: The probability of being in contact with Olyset was significantly less in the first 5-minutes of the assay for all strains compared to not being in contact with the net ($P < 0.001$). Although there was less chance of being in contact with PermaNet for the first 5-minutes of the assay, this was only significant for the Kisumu and Banfora strain ($P = 0.043$, $P = 0.007$; Figure 3.6 and 3.8).

5- 10 minutes: Between 5 and 10-minutes of the assay, there was no significant difference in the amount of time spent in contact with treated and untreated net for both susceptible strains (Figure 3.6). For the resistant strains, contact with both treated nets at this time point in the assay differed depending on the strain. The chance of the Banfora strain being in contact with Olyset net was significantly less than untreated net at this time point ($P < 0.0001$), but there was no significant difference between PermaNet and untreated net contact ($P = 0.966$). Comparably the probability of VK7 being in contact with PermaNet was significantly less than untreated ($P = 0.008$) but there was no difference in the amount of time spent in contact with Olyset net compared to untreated net ($P = 0.900$) at this time point (Figure 3.6).

10- 15 minutes: Both susceptible strains spent less time in contact with Olyset and PermaNet than untreated net between 10 and 15-minutes of the assay ($P < 0.0001$) with the exception of N'gousso exposed to PermaNet ($P = 0.053$). Banfora spent significantly less time in contact with Olyset ($P < 0.001$) at this time point however there was no difference in the amount of contact this strain had with PermaNet ($P = 0.806$). Although not significant, VK7 was more likely to be in contact with both treated net than untreated net at this time-point in the assay.

15-20 minutes: Both susceptible strains were significantly less likely to be in contact with treated net compared to untreated net $P < 0.01$ (with the exception of Kisumu exposed to Olyset net $P = 0.393$) in the last 5-minutes of the assay. Banfora was less likely to be in contact with Olyset net at this time point than untreated ($P = 0.002$). There was no different in the amount of time spent on PermaNet compared with control netting within the last ten minutes for this strain. No significant difference was observed in the amount of time spent in contact with both treated nets compared with untreated for the VK7 strain at this time point.

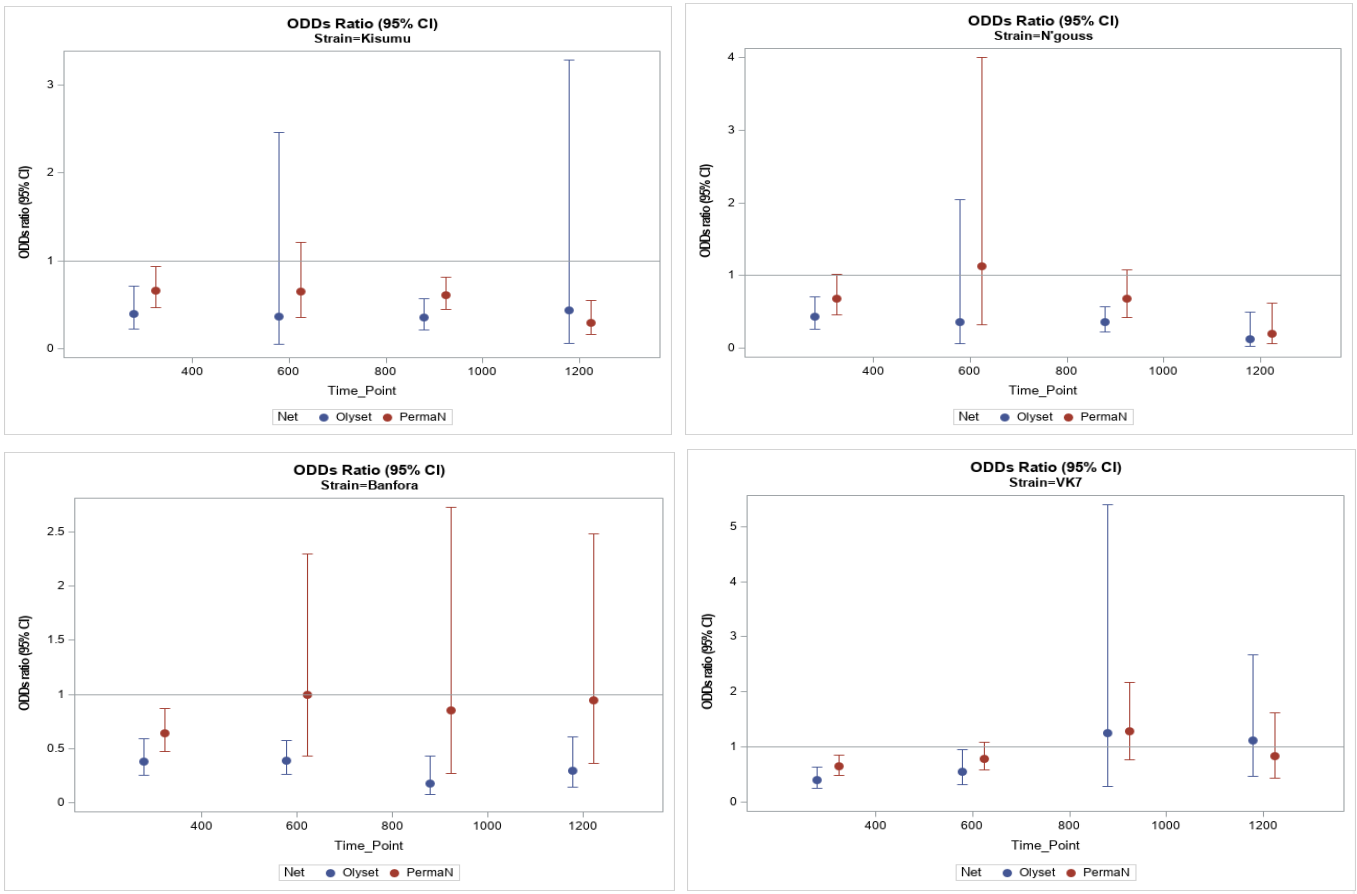


Figure 3.6: The probability of *An. gambiae s.l* female being in contact with an ITN compared with an untreated net. The probability of being in contact with Olyset (blue) or PermaNet 2.0 (red) compared to an untreated net (odds ratio) is shown for consecutive 5-min segments of the 20min assay for the pyrethroid susceptible Kisumu and N'gousso (top left and right) and resistant Banfora and VK7 strains (bottom row left and right, respectively).

The basic sequence of events for both susceptible strains was similar with an increase in contact in between 5-10 minutes, with significantly less contact with both nets for the remainder of the assay. For the Banfora there was no difference in contact duration when exposed to an untreated net compared to PermaNet after 5-minutes into the assay (Figure 3.4– 3.6; Table 3.4), however when exposed to Olyset net contact reduced over time, similarly to the susceptible strains. VK7 however, appears different to all other strains with a slight increase in contact over time at the ITN interfaces, decreasing slightly toward the end of the assay.

3.3.7 Impact of contact with ITN on mosquito survival

When exposed to treated net in the baited box assay immediate mortality of both susceptible strains was high (Chapter 5; Table 5.1: 64%-100%). Immediate mortality when exposed to treated net for both resistant strains was below 20% for both net types (Chapter 5 Table 5.1). There was no correlation between net contact and duration of survival subsequently (Appendix 2.3; Figure A2.1).

3.4 Discussion

The baited box assay was designed to study the behaviour of *An. gambiae* at a treated net interface. The overall aim of the study in detailed in this chapter was to assess the mosquito behaviour at a baited net interface without allowing access to the host. The assay provided key insights into the behavioural events and time frames involved in host-seeking and the persistence of activity at a baited net interface. Results identified a number of important behaviours at the net interface not possible to measure by other test methods.

All strains responded to the baited control net but at different rates. Both susceptible strains had higher response rates to the host than the resistant strains, as shown by Chapter 2 and others (Benedict *et al.,* 2009; Weill *et al.,* 2004).

The basic sequence of events was similar for both susceptible strains when exposed to an untreated net (Figure 3.4– 3.6; Table 3.4). At the net interface, pyrethroid susceptible mosquitoes probe persistently throughout the 20-minutes, reducing the amount of probing only in the latter minutes of the assay.

When exposed to the untreated control, both resistant strains displayed very different behaviour, with Banfora more similar to the susceptible strains than VK7. Both resistant strains showed a decrease in activity compared to susceptible strains on all nets. As discussed in the previous chapter, the differences observed could be accountable to physiological adaptations to the artificial laboratory environment over time (Benedict *et al.,* 2009; Weill *et al.,* 2004).

The first objective of the study was to measure the repellent effects of both treated nets by assessing the lag time from release of the mosquito to the time the mosquito began to host seek. For both susceptible strains responded to, and would land and probe through a baited ITN, and there was no significant difference in the response rates for Kisumu and N'gousso when exposed to a treated net compared to an untreated net.

When exposed to treated nets, there was no difference in the response rate for the Banfora strain. However, the VK7 strain responded more frequently to PermaNet and Olyset compared to untreated net ($P<0.0001$, $P<0.001$). It could be that the assay is 'noisy' and a perhaps the sample size could be larger to account for the variation in the data. However. previous research has reported that, mosquito's resistant to pyrethroids, with the Kdr mutation were more attracted to the host behind an Olyset net than an untreated net and showed no effect of net treatment as shown by susceptible strains (Porciani *et al.,* 2017). The strains used in the current study both have the kdr mutation, with the 1014F Kdr allele fixed in the VK7 population and the frequency of this allele around 60% in the Banfora population (Williams *et al.,* 2019). However, results from the current study do not suggest response rates are an effect of the presence of the kdr mutation, the VK7 responded at a significantly higher rate to PermaNet than any other net type, including Olyset nets.

The VK7 and Banfora although both phenotypically described as resistant, display a number of different behavioural traits. VK7 displayed very little contact with all net types, while Banfora appeared more similar to the susceptible strains, especially notable when exposed to the Olyset net. Further investigation could assess whether differences in the molecular biology of these two strains relate to these behavioural differences.

The resistance profile varies between the two strains used in this bioassay (Williams *et al.,* 2019). VK7's resistance to pyrethroids has remained stable in the population since 2014. In 2018 dose response curves demonstrated Banfora to be more resistant to pyrethroids than VK7 (Williams *et al*., 2019). Recently however, results from ongoing studies performed at LSTM have shown that the Banfora strain does not maintain stable resistance to pyrethroids in the laboratory environment (Ingham, unpublished). More details relating to this are discussed in Chapter 6.

The second objective of the study was to investigate the effects on overall contact duration when exposed to treated net compared to untreated net. Exposure to a treated net increased contact irritancy effects on both susceptible strains causing a decrease in all net contact activities over the twenty-minute time-period. Kisumu displayed more contact activities in the last 10-minutes of the assay than the N'gousso strain. At pyrethroid treated nets, 'normal' probing occurs for the initial 8-10 minutes after which it falls, eventually reaching only a third of the level seen at the untreated nets. Retaining a certain level of probing is important to ensure sufficient contact time is made to pick up a lethal dose. In both strains, with the reduced probing rates, there was a corresponding rise in 'out of view' or 'resting on the test area' activity.

Toxicity of the insecticide affected both susceptible strain's activity. Out of view, includes the area at the bottom of the thumb box and the entrance tube. Out of view increases over time for both treated

nets for both susceptible strains. This is likely due to the mosquitoes entering the entry tube to avoid the treated net or due to the toxicity of the net causing the mosquito to remain on the bottom of the box, if the latter mosquitoes in the wild these mosquitoes may be at risk of higher predation and mortality.

When exposed to a treated net, there was no significant difference in the way VK7 behaves when compared to exposure to an untreated net, with the exception of increased flight during PermaNet exposure. VK7 displayed little contact with all nets, perhaps demonstrating an innate avoidance developed to this control intervention. Banfora however showed no difference in behaviour when exposed to PermaNet compared with untreated net (see Figure 3.4 and 3.5) but, displayed remarkably similar behaviour when exposed to Olyset to that of the susceptible strains, with a significant decrease in the amount of time this strain spent probing through this net. This could be explained by the differences in the resistance Banfora has shown to permethrin compared to deltamethrin.

VK7 exhibited little contact with all three net types and displayed no difference in the level of contact over the 20-minute assay. VK7 displayed a slight increase in flight when exposed to PermaNet and responded more frequently to this net type. Overall, the resistant strains displayed lower levels of activity than the susceptible strains and less out of view behaviour was observed for both resistant strains when exposed to treated net compared to the susceptible strains, possibly due to less lethality effect of treated net on the resistant strains.

As displayed in the above frequency diagrams (Figure 3.5), activity at the net interface changed over time. A GEE model was used to assess the trend in contact duration over the twenty-minute assay for each strain (model output; Appendix 2.2; Table A2.2). Although not initially repelled by the net, all strains spent less time in contact with the treated nets then untreated net in the first 5-minutes (although not significantly less for VK7 and N'gousso exposed to PermaNet). Between 5 and 10 minutes of the assay both susceptible strains spent the same amount of time in contact with treated and untreated net. For the resistant strains, contact with both treated nets at this time point in the assay differed depending on the strain with Banfora having significantly less contact with Olyset and VK7 significantly less contact with PermaNet. As discussed previously, VK7 showed some slight irritancy to PermaNet and Banfora to Olyset net. Throughout the last 10-minutes of the assay Banfora spends less time in contact with Olyset net than untreated net, showing a preference to avoid this net type. VK7 however spends just as much time in contact with both treated nets than untreated net for the remainder of the assay. Both susceptible strains have less contact with treated compared with untreated net throughout the last 10-minutes of the assay, with the exception of Kisumu exposed to

Olyset. Activity decay after insecticide exposure has been shown when mosquitoes are susceptible to the insecticides (Parker *et al.,* 2015).

The current study not only identified differences between the different strains but highlights the importance of understanding the properties of each ITN. Not only are the nets treated with different insecticides, but they are also coated differently as well. The Olyset net is impregnated with permethrin and the PermaNet is coated with deltamethrin. This research, along with Chapter 2, has highlighted the increased irritancy effects of ITN exposure, with different levels observed by both net types. Decreased contact with PermaNet is observed however to a lesser extent than that of Olyset net, where contact reduces drastically for most strains (Chapter 2, Figure 3.5).

A number of experimental hut trials have found permethrin treated nets to be deterrent, with the use of Olyset nets increasing exophily by up to 19% in *An. gambiae s.l.* (Ansari *et al.,* 2006; Lindsay *et al.,* 1991; N'Guessan *et al.,* 2001). Tube tests have also shown repellency to Olyset nets to be equal to that of DEET in the presence of human breath (Giroux, 2006). In the current study, no significant difference was observed in the time taken to enter the field of view when exposed to treated compared to untreated net in the baited box assay indicating no short range repellency effect of these net types.

Sungvornyothin *et al*. (2001) found repellency to DDT was reduced when mosquitoes were unfed, showing blood fed and sugar fed mosquitoes displayed higher repellence to DDT (Sungvornyothin *et al.,* 2001). In the current study, repellency by the treated net could be overridden by the innate drive of a mosquito to take a blood meal on the available human host. Mosquitoes were unfed and starved of sugar for at least 4 hours before testing and so it is possible that any repellency effects of the ITNs may have been overridden by a drive to feed when exposed to a human bait. Future research may identify differences in the repellency effects of these nets when testing with previously blood fed and sugar fed mosquitoes.

In comparison to Olyset net, many studies have shown that deltamethrin treated nets have low deterrence (Mosha *et al.,* 2008; Parker *et al.,* 2015; Spitzen *et al.,* 2014), with some exceptions (Darriet *et al.,* 2000; Koudou *et al.,* 2011). Results from this study and Chapter 2 support the findings that deltamethrin treated PermaNet is not repellent to *An. gambiae s.l*. when host seeking, an important property when evaluating ITNs, as repellency to these nets would reduce the chance of mosquitoes receiving an effective dose of insecticide and could potentially divert them to unprotected hosts.

Given the all the above, little indication of detectable significant repellency effects of ITNs pre-contact (Table 3.3 and 3.4), with either net type or between the strains' responses. However less contact is

observed in the first 5-minutes of the assay, demonstrating perhaps reluctancy to remain in contact due to what has been described previously as contact irritancy effects of these net types (Miller *et al.,* 2004).

Before landing, mosquitoes made small frequent contacts with the net interface as described previously in Parker *et al*. *(*2015). The current study characterised this behaviour as visiting, any time the mosquito was contacting the net but wings still in motion. Parker *et al*. (2015), observed significantly more of this type of behaviour when a host was present behind the net than at an un-baited net and this behaviour reduced at an ITN compared to untreated net, however in the current study there was no difference in visiting behaviour at both untreated and treated net interfaces for all strains, with the exception of N'gousso exposed to PermaNet were the number of times spent visiting decreased compared to at an untreated net. Discrepancies between the tests may result from the difference in characterisation of this event.

Although not repelled by the treated net, the irritancy effects of both Olyset, and to a less extent PermaNet, are confirmed by the fact that the susceptible mosquitoes spent less time on the surface of the net compared to when exposed to untreated net. Of the time that was spent in contact with the net, the majority of activity time was spent probing, trying to increase chances of obtaining a bloodmeal.

Previous research has shown reduced probing time through permethrin treated clothing (Miller *et al.,* 2004; Sholdt, 1989; Cockcroft, 1998; Hodjati, 1997). The reduction in probing times on PermaNet (65%-71%) and Olyset net (73%-74%) observed in the current study, could have implications on disease transmission (Jin *et al*., 2007), however Li *et al* (1992), found that probing time had no effect on either the number of mosquitoes that subsequently delivered sporozoites during a blood meal or the mean number of sporozoites deposited (Li *at al.,* 1992).

In the current study, insecticide treated nets had no effect on the amount of probing activity observed for both resistant strains, with the exception of Banfora exposed to Olyset. Although not apparent from the mortality data collected in the current study, previous work performed in LSTM has shown that the Banfora's resistance to the diagnostic dose of permethrin varies when exposed in the WHO lab tube tests, despite remaining resistant to deltamethrin. For this reason, results for the Banfora strain must be evaluated with caution.

Flight activity remained the same when exposed to untreated and ITNs for most strains. Previous research has found that there was no significant difference in the speed or tortuosity of flight at baited ITNs and untreated nets (Parker *et al.,* 2017). VK7 however spent twice as long in flight when exposed to PermaNet compared to untreated net. VK7 spent more time in flight when exposed to treated net than Banfora. This is especially noticeable for PermaNet. Although there was no difference in probing, resting and visiting activity excess flight could be a response to contact irritancy of the net. Previous investigations of the escape responses of three strains of *An. albimanus* (Chareonviriyaphap *et al.,* 1997), found different levels of response in all three strains used, where deltamethrin and permethrin acted as contact irritants. Levels of contact irritancy have been previously shown to vary with dose, mosquito species, and insecticide resistance status (Chandre *et al.,* 2000; Hougard *et al.,* 2003).

As a result of reduced contact, baited box results also showed lower mortality than WHO cone tests (Emery, unpublished; Profiling completed yearly at LSTM; results available on the LITE website) and suggest avoidance behaviour may in fact be reducing the chance of mosquitoes receiving a lethal dose of insecticide. More detail on the sublethal/mortality effects of ITN exposure will be discussed in Chapter 5.

3.5 Conclusion

The results of this assay indicate that behavioural responses to baited ITNs are dependent on insecticide resistance status. All four strains of *An. gambiae* showed different levels of response, where deltamethrin and permethrin acted as contact irritants. High levels of contact irritancy were observed when the susceptible stains were exposed to both treated nets, dramatically reducing the amount of time spent in contact with these net types.

The behaviour of both resistant strains showed reduced contact to untreated nets compared to the susceptible strains. However, the behaviour of both strains varied when exposed to the treated nets, with Banfora displaying similar behaviour to the susceptible strains when exposed to Olyset and untreated net. VK7 was initially attracted to treated net yet spent little time on all net types with increased flight activity when exposed to PermaNet. On the other hand, although Banfora displayed little contact with the Olyset net, when exposed to the untreated net and PermaNet contact with these net types remained constant throughout the 20-minute time- period. Differences in results from different mosquito strains highlight the complex interactions mosquitoes have with the bednet surface and the importance of conducting tests in realistic behavioural bioassays using the insect strain or a range that an intervention is intended to be used against.

Chapter 4: Quantifying mosquito host seeking behaviour at Olyset, a permethrin treated net

4.1 Introduction

Until recently, characterisation of mosquito behavioural resistance to insecticides had been limited to behaviours considered likely to occur in response to widespread bednet use (Asidi *et al.,* 2005; Carrasco *et al.,* 2019; Chandre *et al.,* 2010; Gatton *et al*., 2013; Govella *et al.,* 2010; Killeen *et al.,* 2014; Killeen *et al.,* 2016; Lines *et al.,* 1987; Russell *et al*., 2011). Within the past 3 years, while completing the work for this thesis, evidence has suggested that large scale bed net use could potentially cause a shift in biting time and/or location of biting of pyrethroid resistant African vector populations (Githinji *et al.,* 2020; Kreppel *et al.,* 2020; Perugini *et al.,* 2020; Sanou, 2020), also raising the possibility that other less obvious changes may also be occurring unseen during host seeking inside a house (Machani *et al*., 2020).

An important advance in this knowledge came with the use of infrared video tracking by Parker and others (Parker *et al.,* 2015; Parker *et al*., 2017; Sutcliffe and Colborn 2015) used to explore the sequence of mid-range host seeking events around a whole human baited bednet.

Video tracking experiments have been used to characterise mosquito-ITN interactions and investigate the complex modes of action of ITNs and to aid in the development of the next generation of bednets. (Murray *et al.,* 2020; Parker *et al.,* 2015; Parker *et al.,* 2017). The research showed more complex interactions with the net than those observed in the other bench top assays, with more detail on the longer-range host seeking which had not been observed previously (Parker *et al.,* 2015). The study revealed four behavioural modes occur at the net interface, each involving different levels of net contact. The majority of net contact was observed at the roof of the bednet, involving brief multiple contacts focussed above the occupant's head/torso area. Exposure to an ITN reduced the overall flight and net contact times compared to a baited untreated net. PermaNet 2.0 did not initially repel the mosquitoes but impacted the susceptible strain rapidly, with less than one minute of contact with the ITN during the first ten minutes reducing all subsequent contact and flight activity (Parker *et al*., 2015)

Combined with the baited box study (Hughes *et al.,* 2020), those studies enabled the production of a detailed description of events immediately before, during and after a mosquito interacts with a PermaNet 2.0 bednet (Vestergaard, Lausanne), one of the most widely used bednets worldwide. The PermaNet 2.0 comprises deltamethrin-treated polyester. A second widely used bednet is the Olyset

bednet, (Sumitomo) a polyethylene net with permethrin. Regardless of the differences in materials, both nets have been very effective against pyrethroid susceptible vectors (Haji *et al.,* 2020; Malima *et al.,* 2008; Sood *et al.,* 2014; Tamari *et al*., 2020), but less is known of the impact pyrethroid resistance is having on these nets as a successful control tool. In this chapter, the behaviour of pyrethroid resistant *Anopheles gambiae s.l.* is investigated in response to a baited Olyset net.

Results from the baited box assays (Chapter 2 and 3, section 2.3) did not reveal any repellency effects of PermaNet and Olyset net with all mosquito strains responding to, landing, and probing through a baited ITN regardless of their resistance status. Both assays did show that exposure to the treated net increased contact irritancy effects, more notably when in contact with Olyset net (Chapter 2 and Chapter 3).

Previous to this work, the video tracking system has only been used to investigate the effects of PermaNet on susceptible *An. gambiae* and *An. arabiensis*. However, from the baited box assay it is apparent there are differences in the mosquito's repose to both PermaNet and Olyset net, with a larger reduction in contact observed when exposed to the latter. Other studies have reported that the two insecticides (Permethrin and Deltamethrin) act differently against Anopheles mosquitoes (Hodjati *et al.,* 2003; Siegert *et al.,* 2009). Previous work has found differences in the amount of time before first take-off when mosquitoes were exposed to a range of different pyrethroids (Hougard *et al.,* 2003). In agreement with baited box results, research has shown earlier disengagement when in contact with Permethrin compared to Deltamethrin (Hodjati *et al.,* 2003; Siegert *et al.,* 2009).

Although evidence suggests no spatial or close range repellency effect of deltamethrin impregnated nets on behaviour (Parker *et al*., 2015; Parker *et al*., 2017; Hughes *et al.,* 2020), some studies have suggested that pyrethroid coated ITNs repel mosquitoes prior to contact (Achee *et al.,* 2009; N'Guessan *et al.,* 2001; Soleimani-Ahmadi *et al*., 2012; Sharma *et al.,* 2009; Tungu *et al.,* 2010). Mosquitoes exhibiting avoidance behaviour, before being exposed to a lethal dose of insecticide is a major issue for the success of ITNs and understanding the magnitude of this is imperative for future control.

With this in mind, Siegert *et al* (2009) showed that when mosquitoes are confined to the standard cone test, lethality of both nets (PermaNet 2.0 and Olyset) appeared the same, but when mosquitoes were free-flying and a host hand was present exposure to PermaNet was more lethal than Olyset Net. The results displayed the importance of behavioural avoidance and on mortality effects (Siegert *et al.,* 2009). The results show that single reliance on one or two simple, small scale, bench top assays such as cone tests, could overemphasize the net's mortality effects on the mosquito and do not accurately

measure the subtle differences between the nets that will be important factors when assessing effectiveness in the field.

This chapter reports on the behaviour of insecticide susceptible and resistant *An. gambiae s.l* at a human baited Olyset net.

The main aim of the study was to characterise the flight response of *An. gambiae s.l* host seeking at a permethrin treated net.

The hypothesis tested where:

1) There will be no significant difference in the time taken to make first contact with the treated and untreated net for each strain
2) There will be no significant difference in the duration of overall contact
3) There will be no significant difference in the amount of time spent in each behavioural response

4.2 Materials and Methods:

4.2.1 Mosquito colonies

For all experiments conducted two insecticide susceptible (Kisumu and N'gousso) and two insecticide resistant strains (VK7 and Banfora) of *An. gambiae s.l.* were used as described in Chapter 2 (section 2.2.1). By 16:00 the day before testing, mosquitoes' access to sugar was removed and replaced with distilled water (20 hours before testing began). Water was removed from the mosquito cage three hours prior to testing. Mosquitoes were removed from the stock cage 1-hour before the test and 25 females were placed in a holding cup in the experimental laboratory to acclimatise to the test conditions.

4.2.2 Test materials

Behaviour of all four mosquito strains was studied in response to Olyset net (2% Permethrin; Sumitomo, Tokyo, Japan) and an untreated polyester control net (Bayer AG, Leverkusen, Germany). 3 Olyset nets were aired indoors for up to 4 weeks prior to testing. Following airing, the nets were adjusted in size. Adjustments were made to the bednet to ensure the net fitted to the frame to allow maximum visualisation of mosquito activity. The roof of the net was sewn to 90 cm x 180 cm, to fit tightly around the bed frame (refer to section 4.2.5) and excess netting from the side panels was

removed. Nets were stored at 4°C and acclimatised at 27±2°C and 70±10% humidity for at least 1-hour prior to testing.

4.2.3 Volunteers

All experiments required a human volunteer to act as a bait under the bednet. In total, 17 volunteers were used, based on their availability for testing, to complete the 42 tracking tests. Informed consent was obtained from all volunteers. The volunteers were asked to wear light clothing, to not wear perfume/cologne or any other strongly scented products on the day of the experiment, and not to bathe for at least 4-hours before the test. To prepare for the experiment they were asked to remove their shoes and socks before lying down, and during the experiment they were asked to lie as motionless as their comfort would permit. To control for any effect of body positioning, the orientation of the volunteer was alternated between each test, either with head or feet towards the mosquito release point.

4.2.4 Sample size

A total of 25 female mosquitoes were used per test. The video tracking system can track up to 100 mosquitoes per test and 25 individuals per test was previously found to generate an optimal number of flight tracks for analysis (Parker *et al*., 2015). A sample size for comparing net contact rates at different ITNs was calculated using previous data generated by this system, in the statistical program R (R Development Team, 2017), and using the phia (Rosario-Martinez, 2015) and pwr (Champely, 2017) packages. With a significance level of 0.05 and gives at least a power of 90% and using the default parameters, a minimum sample size was determined, inflating the sample size with 30% to adjust for any potential confounding factors. Standard deviation used was 562.14 (obtained from the previous study based on the ANOVA or t-test). Mean difference used was 4.54 times reduction in the PermaNet 2 group compared to the untreated and 5 times reduction in the Olyset group compared to untreated.

R Code used to calculate sample size:
power.anova.test (groups = length(groupmeans),between.var = var(groupmeans), within.var = power = .90)

A total of 18 replicates for three treatment groups, or 6 replicates per strain and treatment was the minimum requirement determined in order to compare net contact rates at different ITNs. This

sample size calculation was based on comparing contact duration with untreated, Olyset and PermaNet 2, but due to time constraints only the Untreated and Olyset data are shown in this thesis.

4.2.5 Experimental set up

Experiments were performed in the LSTM Accelerator building testing room (approx. 7 meters x 4.8 meters). All experiments were recorded under infra-red light at 27±2°C and 70% ±10% RH. The test nets were hung on a frame made of carbon rods, above the bed. The frame measured 45cm high at the front and 75cm at the rear. The roof of the net was tilted to allow observation of mosquito activity in this area. It is important to note that this set up is different than actual bed nets used in the field that are hung in all different positions, but the tilt was necessary for the methodology. Given that preliminary results showed a majority of contact on the roof, similar to other studies such as Lynd *et al.* 2011, the results do not suggest the slight tilt has an effect on mosquito behaviour. However, there is the potential that some behaviours would be slightly different when nets are hung differently in the field.

The frame holding the net was assembled with copper rods attached to the bedframe and a new frame was used for each net type (Figure 4.1).

Two fresnel lenses (1400 x 1050mm and 3 mm thick; NTKJ Co., Japan) were used to illuminate the large testing arena. A single Fresnel lens was used per camera (12 MPixel Ximea CB120RG-CM with 14 mm focal length lenses, positioned 100 cm apart on adjustable stands) (Voloshin *et al*., 2020), and was placed 121cm from the camera. The cameras recorded at 5 ms exposure time and -3.5 dB gain with lens aperture of F8.0.

To provide infrared light a custom ring light source was constructed by engineers at Warwick with 12 OSRAMTM SFH 4235 infrared LEDs (peak wavelength 850 nm). Light from the LED ring light expands over the Fresnel lenses with approx. 1.4 x 1 m aperture and 1.2 m focal length, illuminating the test arena. The total recording volume captured is 2 × 2 × 1.4 m. The light was reflected to the cameras through the Fresnel lens via a custom designed Retro reflective screen (material; 3MTM ScotchliteTM High Gain 7610 tape) (Voloshin *et al*., 2020). The screen was placed 2m from the lenses measuring 210cm high and 240cm wide. The bed and test netting were positioned in the centre of the testing arena (Figure 4.1). The mosquito release cup was located 170cm above the floor and approximately 150cm from the edge of the bednet.

The system was operated from outside the testing room. Recordings were captured for both cameras on a single Windows PC (Intel® Xeon® Silver 4114 CPU 2.20 GHz, 24 Gigabytes RAM, Windows 10 Pro;

2 hard drives (24 Terabytes each), at 1 drive per camera) using StreamPix recording software (StreamPix V.7, Norpix, Montreal, Canada) at 50fps. All recordings were 5 TB per 2-hour experiment and were initially stored on the internal hard drives. Video files were transferred to a Seagate 5TB (Seagate, Thailand) external hard drive following analysis.

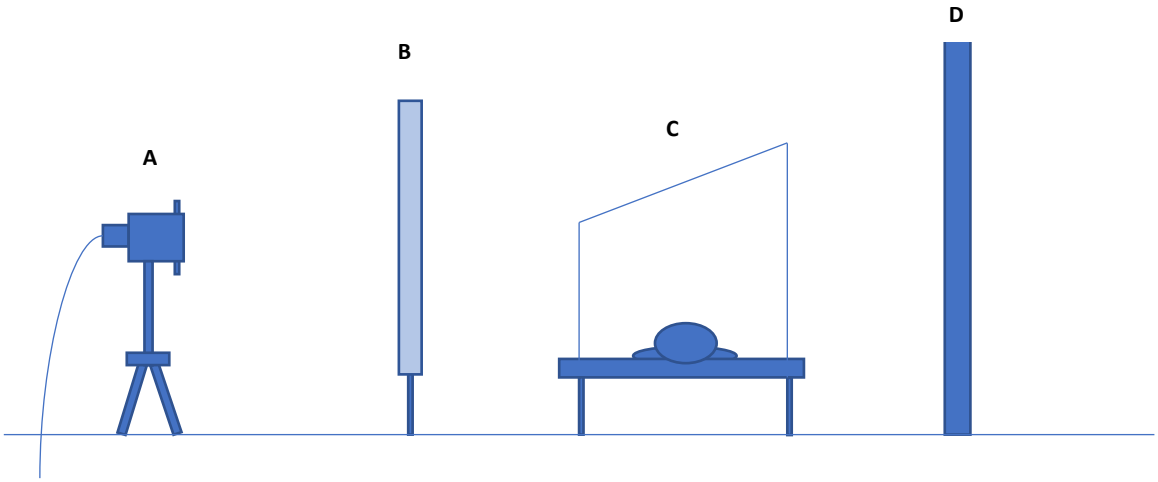


Figure 4.1: Simplified diagram showing the infrared video recording system for tracking mosquitoes at a human baited LLIN. A: Camera, lens and LED ring light. B: Mounted Fresnel lenses. C: Bed and Human baited bednet with tilted roof. D: Retro reflective screen.

4.2.6 Experimental procedure

The experimental procedure was broadly divided into four sections: preparation, acclimatisation, recording and collection.

4.2.6.1 Preparation

One-hour before testing mosquitoes were placed in the holding cup. In order for the mosquitoes to be released remotely into the tracking arena, the cup was attached to a cord. Once the mosquitoes were in place the recording system was switched on and the test net placed tightly around the bed frame.

4.2.6.2 Acclimatisation

Fifteen-minutes before testing, the volunteer entered the net. The net was tucked under the bed and the room was sealed shut for acclimatisation.

4.2.6.3 Recording

After the fifteen-minute acclimatisation period, the release cord was pulled from outside the room, removing the net cover and overturning the cup, freeing 25 mosquitoes into the test room. Mosquito activity was recorded for two hours.

4.2.6.4 Collection

Following the two-hour recording, the operator carefully entered the room and collected free flying and dead mosquitoes using a HEPA filter mouth aspirator (John.W.Hock company, USA) to avoid damaging the mosquitoes.

Two assays were recorded each day, as not all mosquitoes were always collected post-test, this resulted in some assays having more than 25 mosquitoes present during the test (Table 4.3).

After testing all mosquitoes were placed in cups and knock down was scored at 1-hour and mortality at 24-hours. All test mosquitoes were then monitored for sublethal effects following the sublethal pipeline detailed in Chapter 5.

4.2.6.5 Room decontamination

Treatments were alternated approximately every three weeks. Decontamination was required between each treatment. All recording equipment was unplugged and removed if necessary. The walls, floor and ceiling were washed with 5% Decon90 (Decon laboratories, Conway Street, Hove UK) solution followed by a water wash and then 70% ethanol. Following washing the room was aired with a fan in the doorway.

WHO cone tests (WHO 2006) were performed on the walls 24-hours after decontamination as a quality control procedure. Cones were attached randomly to the 4 walls of the insectary and the floor. To perform the assay, fifty, 3-5 day old female susceptible *An. gambiae* were removed from stock cages and transferred to 118ml cups, each cup containing 10 mosquitoes. Using an aspirator, 10 mosquitoes were introduced into each cone and the holes plugged by pieces of cotton wool. After thirty minutes, mosquitoes were transferred back to holding cups, stored in insectary conditions and provided with 10% glucose solution. Mortality was recorded at 24-hours.

During the decontamination process between nets, no WHO cone assay resulted in > 20% mortality and therefore all cleaning procedures were considered to pass the QC process. Although 24-hour mortality scoring is considered adequate for the cone test it is important to note that this may be a limitation of this procedure as results from Chapter 5 suggest the importance of also assessing longevity and fecundity effects post insecticide exposure. For the purpose of cleaning the room

however, 24-hour mortality assessments were used for ease of the method and then untreated controls used as a comparison to ITNs for any baseline longevity and fecundity effects.

4.2.7 Video analysis

Segmentation was completed on original Seq files prior to compression. All video files were manually reviewed and cleaned to remove false tracks, noise and human movement using Seq file processing a bespoke software written in Matlab (Mathworks) by collaborators at Warwick university (Angarita-Jaimes *et al.,* 2016). To extract the data, including trajectory duration, track velocity, distance travelled, tortuosity of tracks, and the duration and number of contacts made with the net surface, tracking algorithms were also developed using bespoke software and written in Matlab (Mathworks) by collaborators at Warwick University (Angarita-Jaimes *et al*., 2016).

Post-processing software also developed by collaborators at Warwick (Angarita-Jaimes *et al.,* 2016), was used for additional track joining, and the deletion of false tracks created from volunteer movement and camera noise. In post-processing, activity was categorised into regions and behavioural modes to assess the mosquitos' positions around the baited net. Time before first contact was described as the time lag from the release of the mosquitoes into the filming arena and the first time a mosquito contacted the net. Using previously reported quantification algorithms (Parker *et al*., 2015, Angarita-Jaimes *et al.,* 2016), mosquito activity around the net was categorized into four behavioural modes as previously described by Parker *et al* (2015);

- **Visiting:** tracks where long periods of flight were interspersed with infrequent contacts with the bednet. Contacts were characterized as sharp 80° turns or more, and when multiple contacts occurred with the net, the minimum interval between each contact was 0.4 seconds.
- **Swooping:** flight tracks without net contact.
- **Bouncing:** tracks where the mosquito made multiple contacts at intervals of less than 0.4 seconds with the bednet surface; including tracks with short flights between the contacts, or tracks maintaining contact with the bednet surface but not static. This includes 'walking' or 'probing' the net with gaps in movement lasting less than 0.75 seconds.
- **Resting:** tracks where the mosquitoes were static for at least 0.75 seconds on the net surface, or where the velocity of mosquito movement was less than 1.33 mm/s. Dead mosquitoes were excluded by limiting resting periods to a maximum of 300 seconds, however, no dead mosquitoes were found on nets at the end of each test.

To assess the localisation of contact at the bednet interface, the field of view was divided into regions, splitting the net into 10 regions, 6 on the roof, two on the sides and two at the front (Figure 4.4). Mosquito tracks were assigned to regions 1–10 when contact with that region was detected by the system. Total duration of net contact with each area was analysed without scaling for area size.

4.2.8 Data analysis

For behavioural analysis, mosquito activity was classified into the four behavioural modes as described in section 4.2.7 and Parker *et al* (2015). Results for each behavioural mode are shown in Table 4.2, Figures 4.2 and 4.5. Location of activity and total net contact was calculated based on regions described in Parker *et al* (2015). The results are displayed in Table 4.3. The location of activity was assessed, splitting the net into 10 regions as described in Parker *et al* (2015). The average time spent in contact with each region is shown in Figure 4.3 for each strain exposed to untreated and Olyset net.

Time to first contact was analysed using a Kruskal Wallis test, due to the non-normal distribution of the data, in SPSS (IBM SPSS Corp. 2017. Version 25.0). Stacked bar charts were created for proportion of activities in GraphPad Prism version 8.4.3 for Windows (GraphPad Prism version 8.4.3 2020). As the Time spent in each behavioural mode was normally distributed (tested in SPSS; IBM SPSS Corp. 2017. Version 25.0 using Shapiro-Wilks test), the data was analysed using generalised linear models with gaussian distribution in R (R Core Team 2019) with Tukey adjustment for multiple comparisons using packages LSmeans (Length 2016). Due to the variation of data exceeding the mean, number of contacts were analysed using negative binomial regression in R (R Core Team 2019) package MASS (Venables & Ripley 2002). Activity over time was plotted for 5-minute time intervals for both mean activity and split into behavioural modes. These analyses were done in R (R Core Team 2019) package ggplot2 (Wickham, 2016).

Exponential decay modelling was considered for the analysis of activity over time, as reported by Parker *et al.* (2015) but many of the test replicates violated the models' constraints. Instead, activity over time was analysed using the alternative method described in Parker *et al* (2017). The total activity recorded in the first 5-minute time interval was subtracted from total activity in the final interval, a negative value indicated that activity decayed over time and a positive value represents an increase in activity between the start and end point of the test. This was compared using a linear model in R (R Core Team, 2019).

4.3 Results

A total of 1,050 mosquitoes were used in 40 tests. Although 25 mosquitoes were released into each test replicate, not all mosquitoes were recovered during the collection at the end of every assay. The mean number recovered per test was 23 (20 - 25). Two tests were completed each day between July and December 2019.

Due to time constraints and technical issues (e.g. data lost in network failure), the total number of test replicates completed were; Olyset: six for all strains (except VK7 where 5 were completed) and untreated: five for Kisumu, and four for N'gousso, Banfora and VK7. This meant that for some of the treatments and strains the test was underpowered. This could have resulted in bias from volunteers as for some strains/treatments there were not enough different volunteers, therefore more replicates using more volunteers would be required to ensure that there is no bias introduced by differing responses to each.

During the test, although volunteers were protected by the net, some mosquitoes successfully blood fed, presumably by entering the net in areas not properly tucked in, or through the net where the volunteer's skin was in contact with the net. In total, 11 mosquitoes were found blood fed at the end of the untreated and 1 at the end of the Olyset assays respectively.

4.3.1 Behaviour of *An. gambiae s.l* at an untreated bednet

Both resistant and susceptible strains showed almost identical behaviour around an untreated bednet. Between 94-97% of all activity involved contact with the bednet interface, in the behaviour modes, bouncing, visiting or resting; the remining 3-6% was swooping (Figure 4.2 and 4.3). Bouncing is the most common behaviour seen across all four strains, accounting for 64%-80% of overall activity observed (Table 4.1; Figure 4.2).

There were no significant differences between any of the strains or modes with one exception: Banfora spent more time bouncing than VK7 at a human baited net (Table 4.1; P = 0.0013). All four strains spent time resting on the untreated net, with the proportion of resting accounting for between 4%-5% of the overall contact time (Figure 4.2).

4.3.2 Responses of mosquitoes to ITNs

Figure 4.3 shows a snapshot image of one replicate, representing each strain and net tested using the video tracking. Images reveal a spatial preference for the area above the head/torso (which was alternated between tests to avoid bias), particularly evident with all strains when exposed to an

untreated net. Images also show the dramatic impact exposure to Olyset net has on activity level for both susceptible strains, and a lesser extent to both resistant strains; this is evident by the observed disappearance of mosquito tracks, shown in Figure 4.3. With the susceptible strains this disappearance occurs midway through the assay, showing mainly blue mosquito tracks on the image below. For resistant strains the images show less tracks observed throughout the assay compared with the untreated control. However, unlike that observed with the susceptible strains, some tracks still occur at the end, shown as red tracks in the figure below (Figure 3.4).

The duration of all behavioural modes was significantly shorter when all four strains were exposed to treated net compared with the untreated control (Table 4.1, Appendix 2.4; Table A.2.3; $P<0.05$ generalised linear model).

For example, all four strains spent significantly less time Bouncing (Banfora $P<0.001$; VK7 $P<0.001$; Kisumu $P<0.001$; N'gousso $P<0.001$), Resting (Banfora $P=0.000588$; VK7 $P= 0.000628$; Kisumu $P<0.001$; N'gousso $P<0.001$), Visiting (Banfora $P=0.00177$; VK7 $P=0.000248$; Kisumu $P<0.001$; N'gousso $P<0.001$) and Swooping (Banfora $P=0.00745$; VK7 $P=0.00122$; Kisumu $P=0.000178$; N'gousso $P= 0.000175$) when exposed to Olyset net compared to untreated.

Moreover, not only did the duration of all events decrease when exposed to an ITN, but the proportion of overall activity time spent in each mode changed when exposed to the ITN. (Figure 4.2). The proportion of overall activity time spent visiting increased when exposed to Olyset net, but for most strains bouncing remained the most common behaviour observed when exposed to the ITN (54-56%). N'gousso however, shifted from mainly bouncing when exposed to untreated net, to when exposed to Olyset (45%).

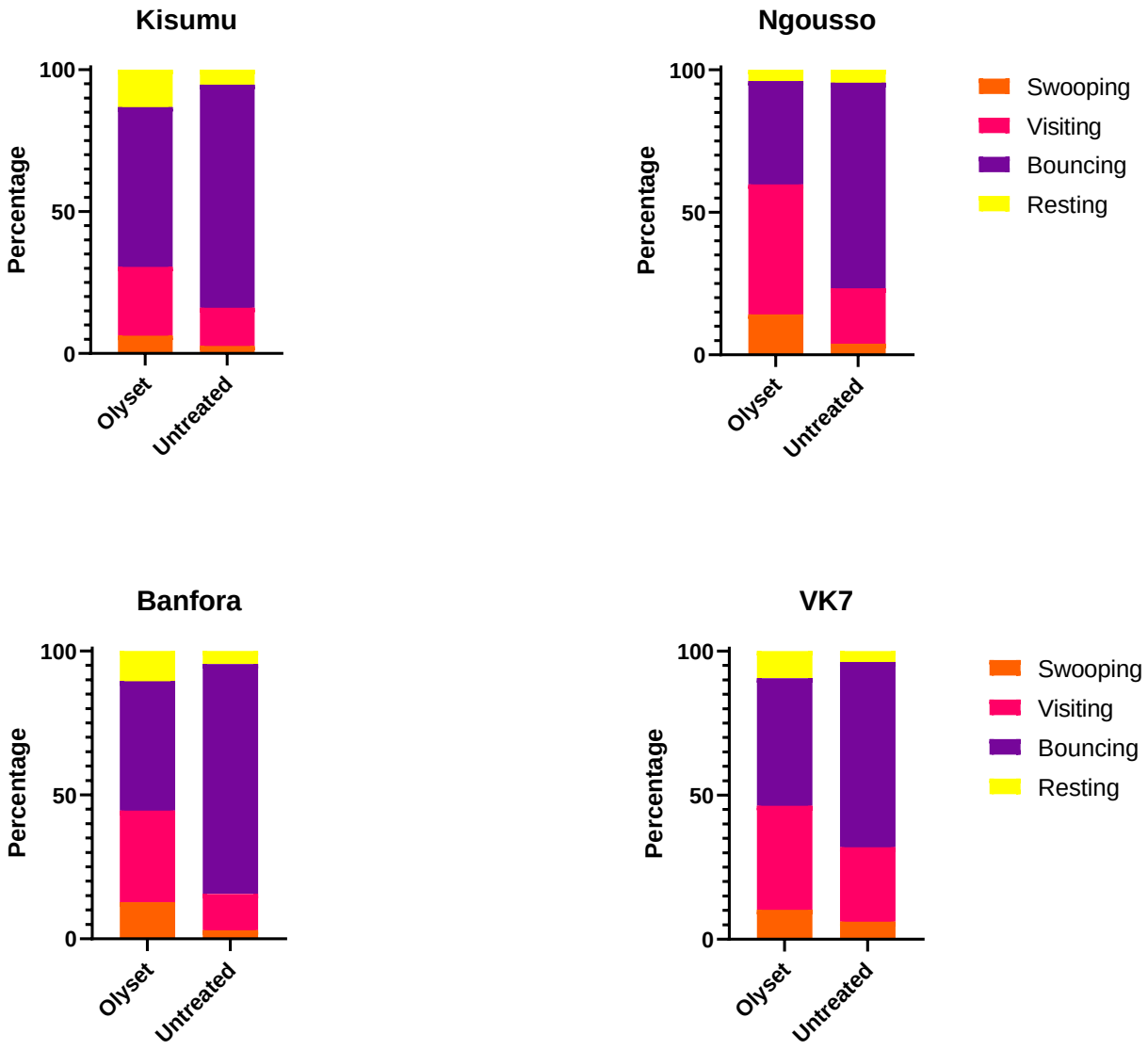
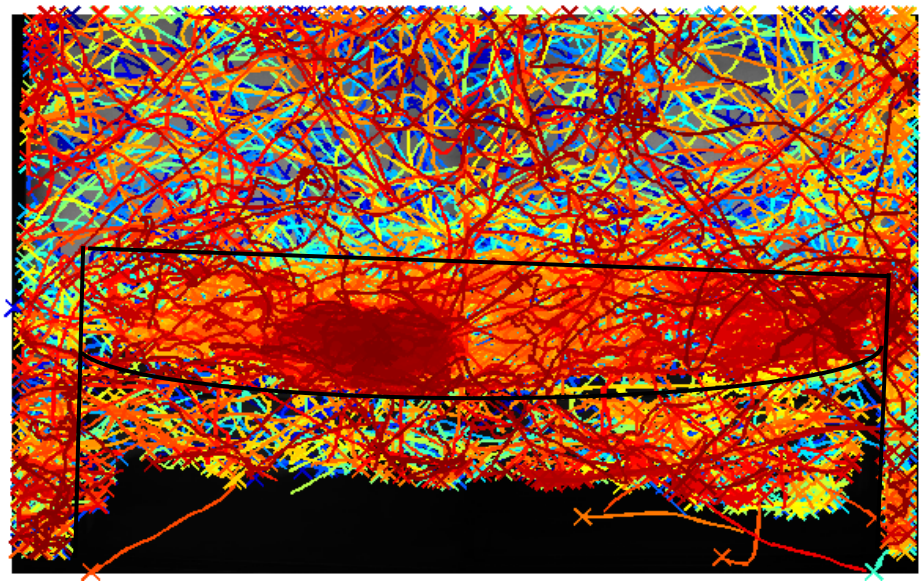


Figure 4.2: Activity of two pyrethroid resistant *Anopheles gambiae s.l.* strains at a human baited net. Activity of *Anopheles gambiae s.l* susceptible strain at a human baited net. Top left: Proportion of activity time Kisumu spent in each behavioural mode when exposed to a baited Olyset and Untreated net (N; Untreated 5, Olyset 6). Top right: Proportion of activity time N'gousso spent in each behavioural mode when exposed to a baited Olyset and Untreated net (N; Untreated 4, Olyset 6). Bottom left: Proportion of activity time Banfora spent in each behavioural mode when exposed to a baited Olyset and Untreated net (N; Untreated 4, Olyset 6). Bottom right: Proportion of activity time VK7 spent in each behavioural mode when exposed to a baited Olyset and Untreated net (N; Untreated 4, Olyset 5).

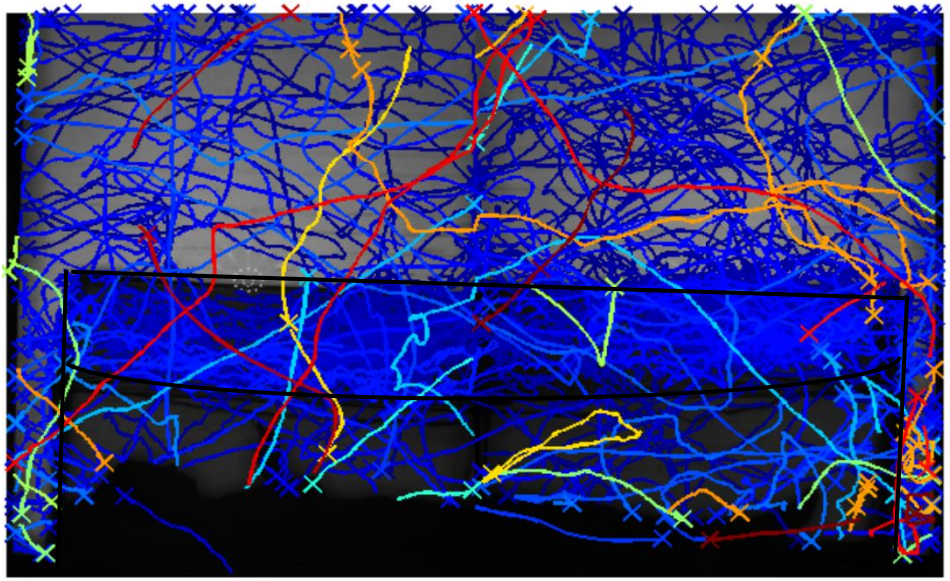
Table 4.1: Behaviour of *Anopheles gambiae s.l* at a human baited Olyset net. Total activity time (seconds) of *Anopheles gambiae s.l* recorded in each behavioural mode. Duration of all tracks classed in each behaviour mode over the 2-hour recording (Mean and 95% confidence interval, seconds). As multiple mosquitoes were active simultaneously in the field of view, the total activity time could exceed the 2-hours. Duration of each behaviour was significantly less for all strains when exposed to Olyset net compared to untreated ($p < 0.05$, generalised linear model) refer to the main text for further statistical details.

Treatment	Strain	N	Swooping	Visiting	Bouncing	Resting
Untreated	Banfora	4	1318 (491.7-2145)	5599 (1930-9269)	35316 (24524-46108)	1985 (1008-2962)
	VK7	4	1547 (790.6-2304)	6564 (3848-9279)	16360 (10905-21814)	944.7 (574.1-1315)
	Kisumu	5	918.8 (549.2-1288)	4708 (3459-5957)	27471 (21218-33725)	1840 (1366-2313)
	N'gousso	4	1291 (755.6-1826)	6485 (4431-8539)	24047 (15483-32611)	1501 (721.5-2280)
Olyset	Banfora	6	483.3 (249.0-717.7)	1203 (603.1-1803)	1698 (615.3-2781)	397.6 (72.19-723.0)
	VK7	5	368.1 (159.3-576.8)	1304 863.7-1745)	1598 (1058-2139)	338.3 (297.7-378.9)
	Kisumu	6	158.3 (82.14-234.5)	613.7 (379.5-847.8)	1423 (549.2-2296)	334.2 (111.7-556.7)
	N'gousso	6	360.3 (259.8-460.8)	1162 (985.7-1338)	924.5 (590.3-1259)	100.3 (71.21-129.5)

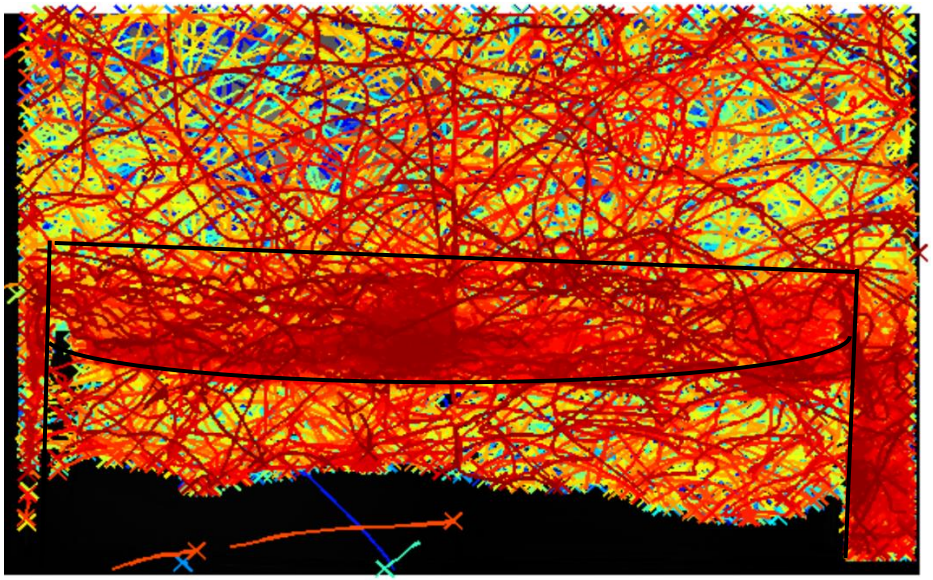
Kisumu Untreated



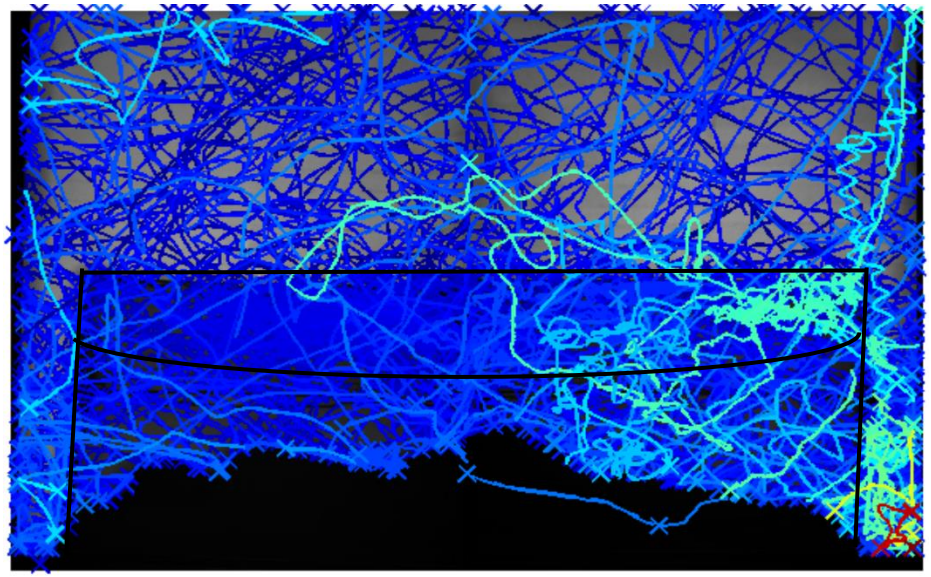
Kisumu Olyset

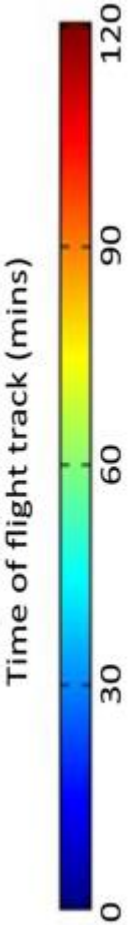


N'gousso Untreated



N'gousso Olyset





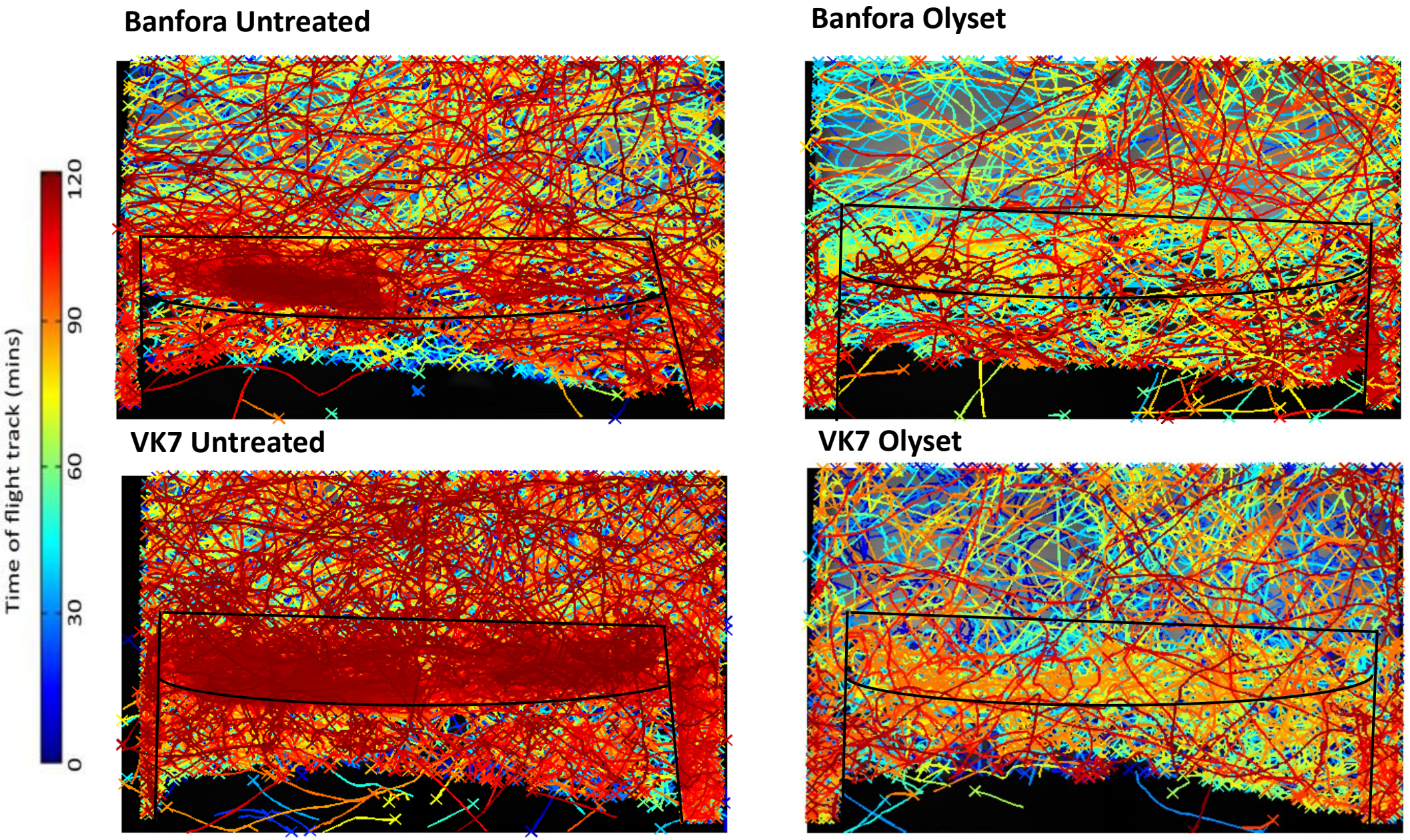


Figure 4.3: Examples of a two-hour recording of a test showing flight tracks of four strains of *Anopheles gambiae s.l* at an untreated and Olyset baited bednet. Twenty-five mosquitoes were released in all tests and activity was recorded for two-hours. Each coloured track is the path of a single mosquito flight event. Tracks are colour-coded according to time they first appeared in the field of view as shown in the key: blue tracks at the start through to red at the end of the test. Outline of the net was added in black.

4.3.3 Duration of net contact

No repellency effects were observed when all strains were exposed to Olyset net. No significant difference was observed in the amount of time between the start of test and the time taken for the first mosquito to contact each net type for all strains (Table 4.2; P>0.05 Kruskall Wallis). However, the 'time to first contact' observation was only recorded once per rep, therefore the sample size for this data set was small (between 4-6 time points). In addition, the lag time between release of the mosquitoes and the first recorded contact time with the net varied greatly between each replicate resulting in negative CI and therefore and suggesting the sample size for this is too small and data too variable for any further analysis (Table 4.2).

Table 4.2: Lag time from release of *Anopheles gambiae s.l* until first contact with a human baited Olyset or untreated control net, in video tracking tests. Mean and 95% confidence intervals. There were no significant differences in the time to first contact measured at untreated nets and Olyset net for all strains (Table 4.2; P>0.05 Kruskall Wallis).

Treatment	Strain	N	Time to first contact (Seconds)
Untreated	Banfora	4	49.97 (-26.65-126)
	VK7	4	40.57 (-15.69-98.83)
	Kisumu	5	17.42 (-4.792-39.62)
	N'gousso	4	23.56 (-15.41-62.52)
Olyset	Banfora	6	586.8 (-199.2-1373)
	VK7	5	619.7 (-458.7-1698)
	Kisumu	6	121.1 (-45.22-287.4)
	N'gousso	6	26.22 (-4.886-57.32)

The average contact time per mosquito is shown in Table 4.3. Determining the total number of mosquitoes responding throughout each test was not feasible, therefore mean minimum and maximum contact time values were determined. As described in Parker *et al* (2015), the range of estimates for the total contact time per single mosquito, were calculated using the maximum number of mosquitoes simultaneously active in the field of view, alongside an average for 25 respondents. Results are shown in Table 4.3.

The longest contact time recorded for a single track was 363 seconds which was for N'gousso exposed to untreated net. For Banfora the longest contact time with the untreated net was 282 seconds and 248 seconds for Kisumu. For VK7 when exposed to untreated net, the longest contact time recorded was 137 seconds. When exposed to Olyset net, the longest contact time recorded for a single track

was 159 seconds for Banfora but 88 for VK7, and 105 seconds for Kisumu but only 47 seconds for N'gousso.

The range of estimates for the total contact time for a single mosquito is shown for each strain (Table 4.3).

The duration of contact observed with the Olyset net surface was significantly reduced in both susceptible strains compared to an untreated net (Table 4.3; P<0.0001). Kisumu spent an average an average of between 9-23 seconds in contact with the Olyset net and N'gousso spent even less time in contact with the treated net, with each mosquito only spending 5-19 seconds in contact with this net type. As shown in Figure 4.3, most activity occurred at the start of the assay and so this reduced contact could be caused by insecticide induced mortality.

Exposure to the ITN significantly reduced the amount of contact with the bednet surface for both resistant strains (Table 4.3; P<0.0001). For the Banfora strain, this was an average contact time of just 11-25 seconds per mosquito, and 9-36 seconds per mosquito for the VK7 strain. Banfora spent significantly more time in contact with the nets than VK7 (Table 4.3; P= 0.0019). The majority of all visible activity time, between 85% and 93% was spent in behavioural modes that involved net contact (Bouncing, visiting or resting) for all strains (Figure 4.2), compared to between 94-97% when exposed to an untreated net. Figure 4.3 shows that although activity reduced for both strains when exposed to the ITN, activity still occurred towards the end of the assay, unlike both susceptible strains.

The average number of contacts made on the net surface by all strains was significantly less when exposed to Olyset compared to untreated net (Table 4.3; P<0.001; Appendix 2.5; Table 2.4). N'gousso made an average of 4,214 contacts with the Olyset reduction of 93% when compared to untreated net. The number of contacts made by Kisumu and N'gousso on Olyset net was not significantly different (P=0.8383). The VK7 strain, on average, made the least amount of contact with the untreated net (40,365 contacts) than all four strains (Table 4.3).

Table 4.3: Duration of *Anopheles gambiae s.l* contact with a human baited Olyset net. Table shows net contact duration as calculated for the mean total time of all contacts observed (all mosquitoes); the minimum mean contact time per mosquito, assuming all 25 mosquitoes responded, and calculated using the maximum mean contact time per mosquito, based on the maximum number of individual mosquitoes observed in each test. Mean total number of contacts made per test was also recorded. Values shown are means with 95% confidence intervals. Mean total contact time (seconds) was significantly higher at untreated nets than the Olyset net ($p < 0.05$, generalised linear model). Mean total number of contacts (seconds) was significantly higher at untreated nets than the Olyset net ($p < 0.05$, negative binomial regression model).

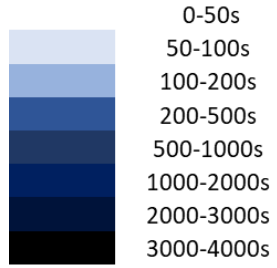
Strain	Treatment	Max. no. of mosquitoes in field of view	Mean Total Net Contact Duration	Mean duration/mosquito (25 mosquitoes)	Mean duration/mosquito (observed max number)	Average number contacts
Banfora	Untreated	17	11135 (8020-14250)	330.8 (211.3-450.4)	606.9 (404.1-809.7)	79110 (42569-115651)
	Olyset	16	794.2 (258.8-1330)	10.58 (-1.217- 22.38)	24.63 (4.589- 44.67)	7115 (3445-10785)
VK7	Untreated	14	5783 (3590-7977)	81.17 (9.946-152.4)	172.7 (68.04-277.3)	40365 (30762-49967)
	Olyset	9	707.9 (655.1-760.7)	8.741 (5.334-12.15)	36.27 (18.76-53.79)	7340 (3807-10874)
Kisumu	Untreated	29	9044 (7717-10372)	88.77 (-15.41-193)	131.0 (63.05-199)	72480 (51230-93729)
	Olyset	18	636.9 (249.8-1024)	8.819 (-0.09034-17.73)	23.02 (3.350-42.68)	6043 (2399-9686)
N'gousso	Untreated	17	8348 (5038-11658)	98.89 (59.88-137.9)	240.0 (62.08-417.9)	60646 (38770-82522)
	Olyset	10	346.0 (265.3-426.7)	5.408 (2.617-8.2)	18.46 (6.524-30.39)	4214 (2790-5638)

4.3.4 Location of activity at the bednet interface

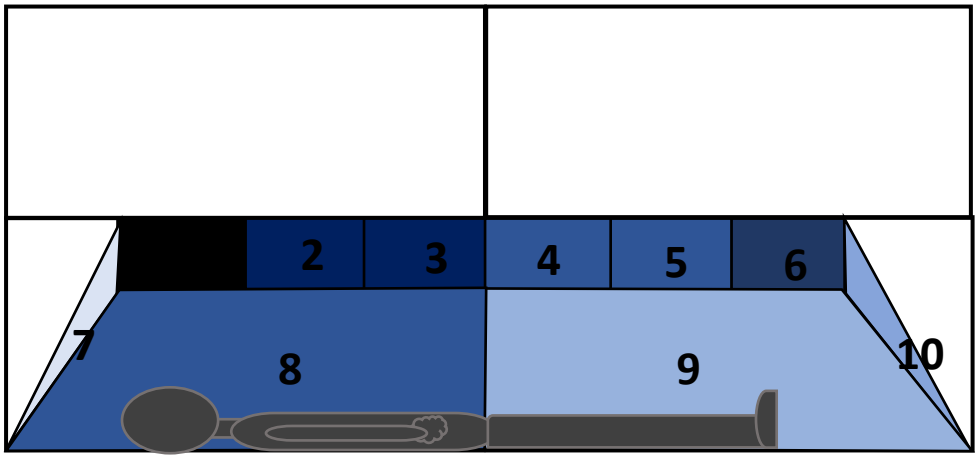
Figure 4.4 shows the average contact made at each net region in all assays. The figure shows that the majority of contact was made on the roof of the untreated net (between 79% to 92%), mainly above the head region (Regions 1-3) for all strains (Figure 4.4). As shown in Figure 4.4 the distribution of net contact was very similar for both the susceptible strains when exposed to untreated net (Figure 4.4). The duration of contact overall, was highest in region 1 for Kisumu, N'gousso and VK7 when exposed to untreated interface, and region 2 for the Banfora strain.

As discussed previously the amount of contact was significantly reduced for all strains when exposed to Olyset net, however the location of this activity remained mainly on the roof for the Kisumu and N'gousso strain (Figure 4.4). As with the susceptible strains, the majority of activity remained on the roof of the net for both resistant strains when exposed to Olyset net. However, as shown on Figure 4.4 contact with the net was also observed on the side of the treated net (region 8), in contrast to both susceptible strains, where contact in this region was completely lost when exposed to this net type.

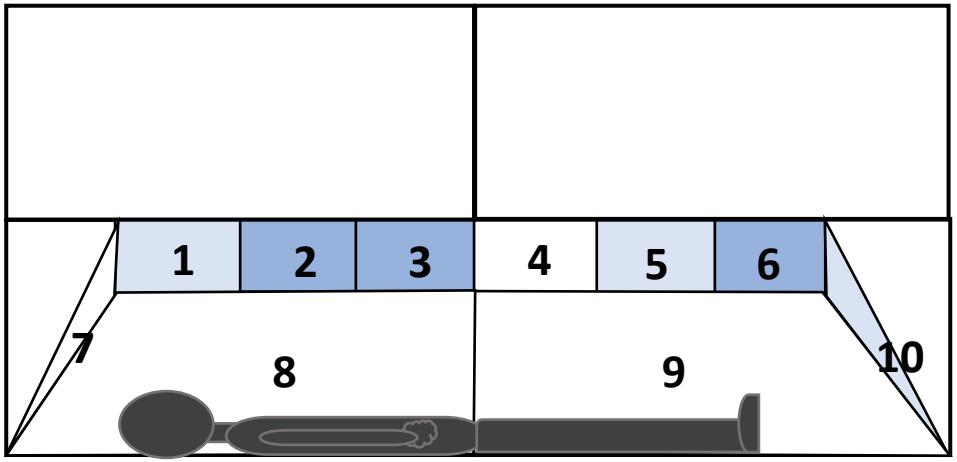
*Charts only include regions relevant to each behavioural category



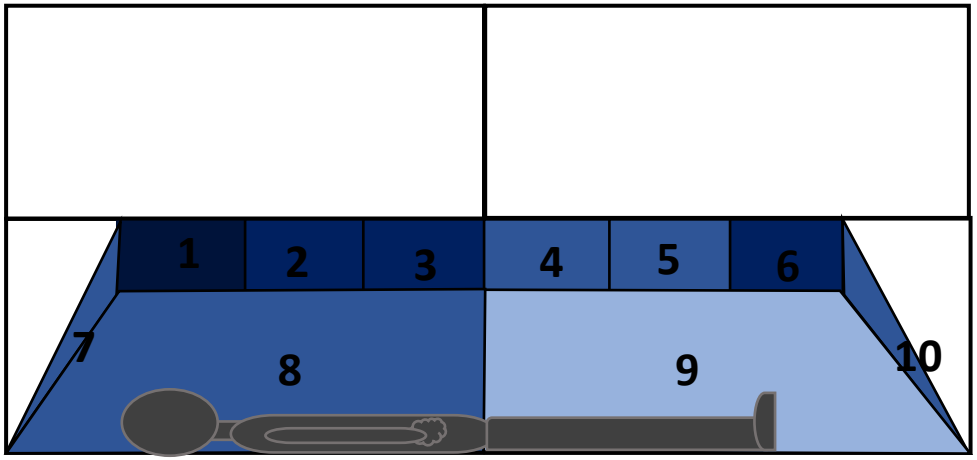
Kisumu Untreated



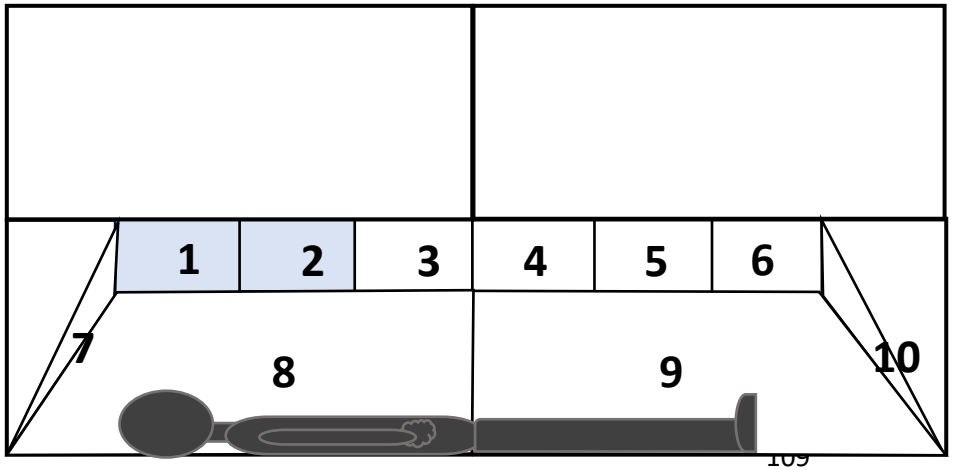
Kisumu Olyset



N'gousso Untreated



N'gousso Olyset



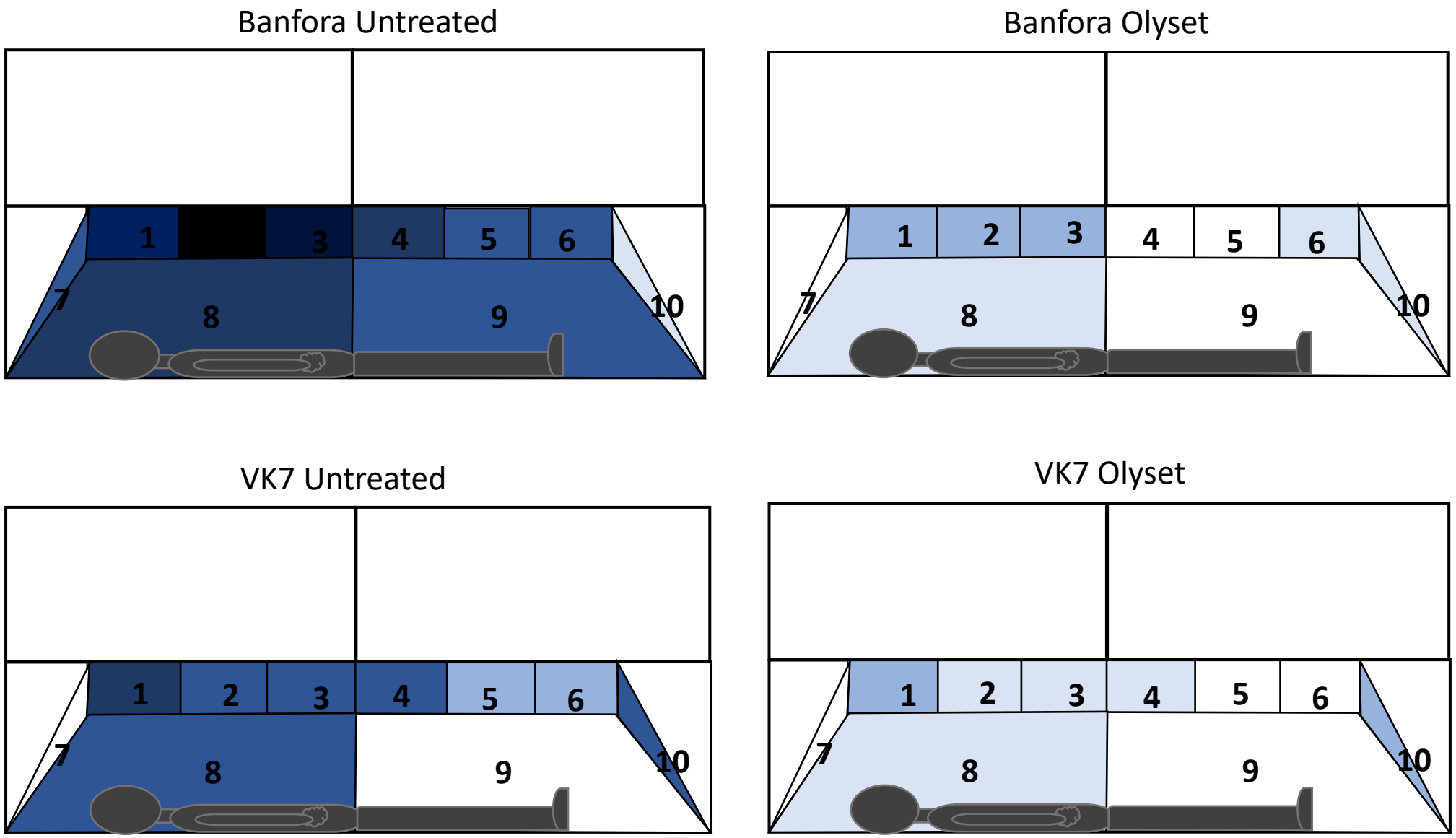


Figure 4.4: Distribution of *Anopheles gambiae s.l* net contact at different regions on the bednet. The average duration (seconds) of all contacts (includes contacts made during visiting, bouncing and resting behaviour) by all mosquitoes on each of the 10 regions of the bednet surface over the 2-hour test (average duration of contact for 4 replicates on untreated for all strains except Kisumu where there are 5 replicates on untreated, 6 replicates for Olyset net bar VK7 where there were only 5 replicates on this net type).

4.3.5 Temporal variation in responses throughout the assay

All strains showed similar profile of activity during the 120-minute assays. In both susceptible strains, when exposed to untreated net this general profile comprised a sustained high level over the two-hour assay with the highest levels between 10-20 minutes (Figure 4.5).

The profile of activity for Banfora shows a similar trend of activity during the 120-minute assays as both susceptible strains. Generally, activity remained high throughout the test for this strain, with the activity level in the last time interval similar to the first (Figure 4.5).

When exposed to untreated net, the profile of activity observed by VK7, appears different to all other strains (Figure 4.5). Activity for this strain peaked at the end of the assay, with a gradual incline in activity over the whole two-hour time-period.

Over the two-hour time-period, activity around an Olyset net decreased rapidly (Figure 4.5), and there was a significantly greater decrease in activity when exposed to Olyset compared to untreated for both N'gousso ($P=0.0002$) and Kisumu ($P<.0001$).

For the Kisumu strain, the profile of activity observed within the first time-interval was similar to an untreated net, before rapidly reducing to almost no activity within 30 minutes (Figure 4.4). This observed trend was the same for N'gousso when exposed to the ITN, (Figure 4.4) however, activity never reached the same levels as that observed during untreated net exposure. The level of activity peaked for N'gousso at 10-minutes with a rapid decrease over the first 30-minutes of the assay. As with Kisumu, the N'gousso activity almost ceases to occur after 30-minutes (Figure 4.5).

For both resistant strains, the profile of activity around an Olyset net remained at a constant level over the two-hour assay. For the VK7 strain activity levels appear to peak between 30-minutes to 1.5 hours, whereas the Banfora strain, reduces activity slightly towards the end of the assay after a gradual increase at the start (Figure 4.5). in conclusion, results show a distinct difference in the behaviour of each strain around the ITN depending on resistance status.

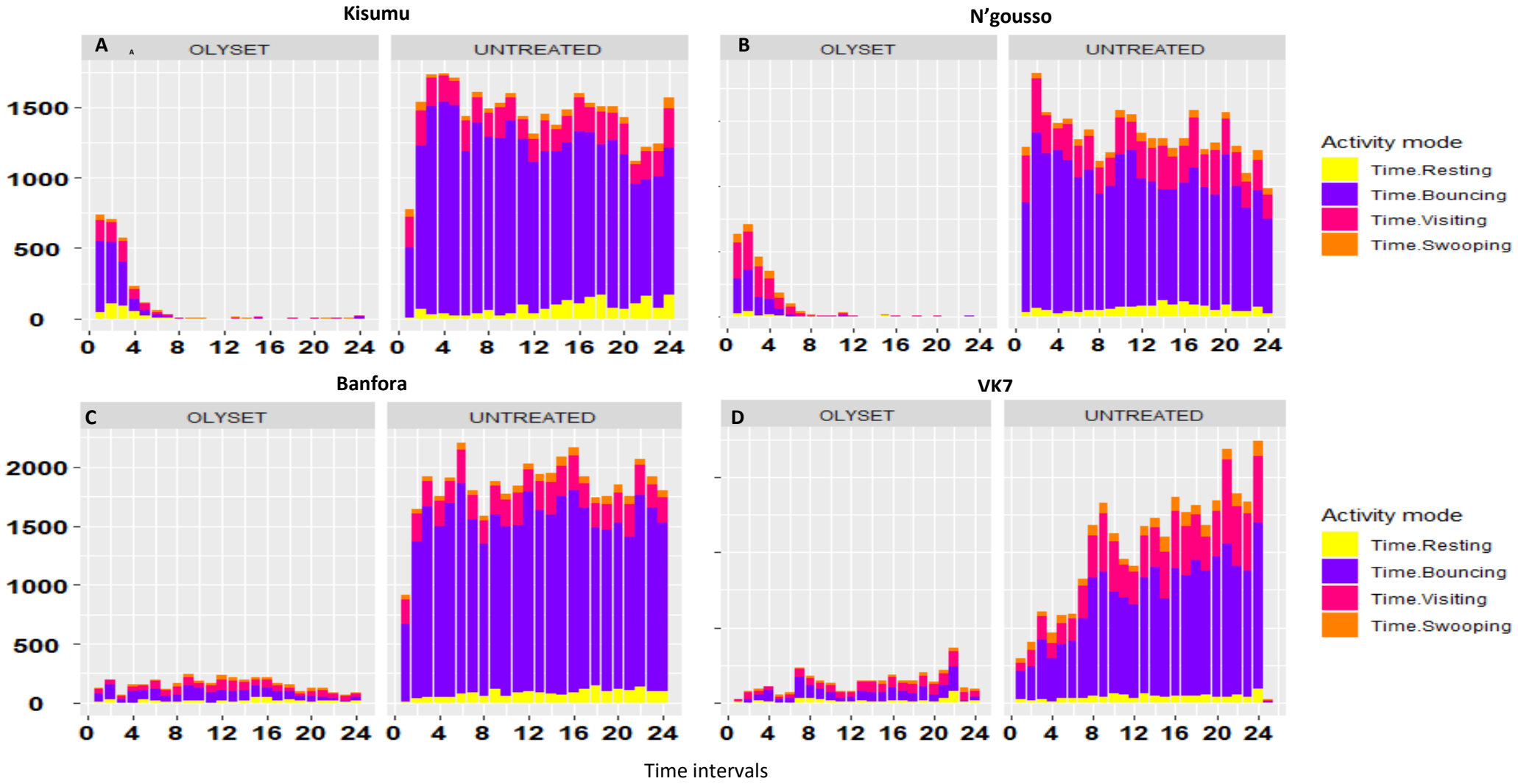


Figure 4.5: Temporal activity profiles of *Anopheles s.l gambiae* pyrethroid susceptible and resistant strains showing the mean proportion of time recorded in each behaviour modes at 5-minute intervals over the two-hour assay. (A) Kisumu (N; Untreated 5, Olyset 6). (B) N'gousso (N; Untreated 4, Olyset 6) (C) Banfora mosquito activity separated by behavioural activity, at an Olyset and untreated baited net (N; Untreated 4, Olyset 6). (D) VK7 mosquito activity separated by behavioural activity, at an Olyset and untreated baited net (N; Untreated 4, Olyset 5).

4.4 Discussion

The results of this chapter provide detailed insight into the behaviour of four different strains of *An. gambiae s.l* at the baited ITN interface. The study uses the improved diffuse retro-reflective imaging system (Voloshin *et al*., 2020) to assess the detailed behaviour of these strains around an Olyset net compared to an untreated control. The results from the current study highlight how currently the WHO methods such as the cone test, although a basic method for assessing the efficacy of nets, are missing vital information such as contact duration, persistence of host seeking and the correlation between these contact times and the subsequent effects on behaviour and delayed effects post exposure. All these factors were investigated using the video tracking system and discussed in more details below.

4.4.1 Behaviour of *An. gambiae s.l* at an untreated and baited ITN

Behaviour at an untreated net was similar in both susceptible strains. At the untreated net surface, bouncing (a behaviour involving longer contact duration) was the most common behaviour, with 72% of the overall activity time spent bouncing for the N'gousso strain and 79% for the Kisumu, with resting only accounting for 5% of the overall contact time for both strains. When exposed to the Olyset net Kisumu and N'gousso spent significantly less time in all behavioural modes compared with the untreated control (Table 4.2; P>0.05 generalised linear model), the proportion of time spent in each mode changed depending on exposure. Bouncing was still the most common behaviour observed in the Kisumu strain, when exposed to Olyset net, however the proportion of time spent visiting increased compared to untreated net (Figure 4.2). The same trend was observed for the N'gousso strain (Figure 4.2). Results strongly suggests the existence of contact irritancy to Olyset net, with a shift from long contact behaviours (Bouncing) on untreated nets, to more short infrequent contacts (visiting) on the Olyset net. Contact irritancy to insecticides has been described previously by many others, (Grieco *et al*., 2000; Lindsay *et al*., 1991; Maia *et al*., 2013; Maia *et al.,* 2016; Spitzen *et al.,* 2017; Roberts *et al*., 1997) and is a major concern for bednets as a control tool; this effect can mean mosquitoes having minimal contact with the ITN allowing the mosquito to leave a house without fatal exposure to the insecticide (Killeen *et al.,* 2016).

So far discussions have focused on pyrethroid susceptible mosquito strains, however, with the previously discussed findings in mind, the success of bednets is currently threatened by the widespread insecticide resistance, and the overall effect of resistance on these control tools is not fully understood. The current study expanded our knowledge of mosquito behaviour further by exposing two pyrethroid resistant mosquito strains to the Olyset net to assess the impact of resistance

on the overall effectiveness of the bednet and evaluate any changes in behaviour due to resistance status.

As with Kisumu and N'gousso strain, bouncing was observed more frequently for both resistant strains, when exposed to an untreated net and comparably, the least amount of contact time was spent resting on the net. Similarities between the susceptible and resistant strains around an untreated net were also noted in baited box assays (Chapters 2, 3 and Hughes *et al.,* 2020). When exposed to Olyset net, Banfora and VK7 spent significantly less time in all behavioural modes compared with the untreated net (Table 4.2). When analysing the behavioural modes, for both resistant strains, the proportion of overall activity time spent bouncing reduced with an increase in visiting when exposed to Olyset net (Figure 4.5), again probably due to contact irritancy effects of the Olyset net on these strains. However, regardless of this shift, between 87% and 90% of all activity, for both Banfora and the VK7 strain, was still spent in behavioural modes that involved contact with the treated net. Results demonstrate how the presence of a host beneath the treated net attracts the mosquitoes to the net, overriding the irritancy effects of the insecticide (Sungvornyothin *et al.,* 2001).

When comparing the strains, Banfora spent more time bouncing than VK7 at a human baited net (Table; P = 0.0013) but there was no difference between this behaviour for all other strains. As discussed previously, strain differences have been identified in other tests (Chapter 2 and 3) most notably the VK7 strain appearing to be less active around the net than other strains such as Banfora. Perhaps due to resistance profile. Previous research has also identified these differences in activity level between strains (Chareonviriyaphap *et al.,* 2004; Sutcliffe *et al*., 2014) indicating the importance of a more flexible approach to malaria control, targeting different strategies to different mosquito populations.

Overall, all strains increased visiting behaviour when in the presence of a treated net, regardless of their resistance profile, indicating the presence of contact irritancy to Olyset net, something previously described in many studies (Chareonviriyaphap *et al.,* 1997; Chandre *et al.,* 2000; Hauser *et al.,* 2019; Hougard *et al.,* 2003; Thiévent *et al.,* 2019). The results from this study show how resistant mosquitoes interact with a whole baited Olyset net, something not investigated previously. Results suggest regardless of resistance status permethrin treated nets cause a shift in behaviour, ultimately reducing the amount of time spent in contact with the net. This is an important factor to consider when developing new, combination nets treated with permethrin plus other novel chemistries to target resistant mosquitoes, ensuring exposure to these nets is enough for the mosquito to pick up a lethal dose.

4.4.2 Duration of net contact

The average amount of time spent in contact with the untreated net accounted for just 2%-3% of overall test time. This is a similar result to that found by Parker *et al*. (2015), with the average amount of time spent in contact with the untreated net accounting for between 2%-9% of overall the overall assay (Parker *et al.,* 2015). VK7 spent an average of 173 seconds per mosquito in contact with net, just 2% of the overall experiment time. Banfora however, spent 607 seconds a total of 8% of the total test time and an increase compared to all other strains. But the majority of all activity recorded during the assay involved behaviours where net contact occurred for all strains (bouncing, visiting and resting). For example, between 96-97% of all the activity time observed was spent in one of the three behavioural modes for both susceptible strains, similar to the 93.7% observed by Parker *et al* (2015).

Impairment to host seeking responses by exposure to insecticide has been described previously (Cohnstaedt *et al*., 2011; Hauser *et al*., 2019; Hougard *et al.,* 2003; Siegert *et al*., 2009; Strode *et al.,* 2014) but studies using baited PermaNet have shown that short to mid-range repellency effects were not observed when susceptible *Anopheles* were exposed to the net (Hughes *et al.,* 2020; Parker *et al.,* 2015; Spitzen *et al.,* 2017). As studies have suggested that permethrin treated nets are more repellent than deltamethrin (Asidi *et al.,* 2004; Siegert *et al.,* 2009), this thesis looked at how exposure to Olyset net effects the host seeking behaviour of both resistant and susceptible mosquitoes.

In the current study no initial repellency effects were observed when exposed to Olyset net for all strains. Time to first contact was not significant between the two treatments (Table 4.1; $P>0.05$ Kruskall Wallis), however, in order to confirm the results seen in this study, further replicates are needed (Table 4.1). Other factors may also need to be accounted for, like the attractiveness of the volunteer, as other studies using a human bait have observed different response rates between operators (Hughes *et al*., 2020). This will be investigated as part of the larger essentials project to assess the number of operators/volunteers and appropriate sample sizes required for each test when used as a screening tool.

When exposed to Olyset net, the amount of time spent in contact with the net significantly reduced, with both susceptible stains spending an average of just 19 and 23 seconds on the treated net surface. This is lower than that observed when exposed to PermaNet 2.0 (Parker *et al*., 2015, Parker *et al*., 2017). Similar to the susceptible strains, exposure to the ITN significantly reduced the amount of contact observed with the bednet surface for both strains ($P<0.0001$). For these strains, contact reduced to an average of just 25 and 36 seconds per mosquito (Table 4.3). Referring to Chapter 2 and 3, this result might be expected as we see lower contact times in the bench top assays to Olyset

compared with PermaNet. Other studies have also shown that mosquitoes will disengage and take off quicker when exposed to Olyset net than PermaNet 2.0 (Hodjati *et al*., 2003; Hougard *et al*., 2003; Siegert *et al*., 2009). Results from smaller bench top assays are therefore in agreement with the video tracking tests, and overall reveal mosquitoes spent less time in contact with Olyset net compared to PermaNet and untreated net.

When directly comparing the strains, Banfora spent significantly more time in contact with the nets than VK7 (P= 0.0019) but there was no difference between any of the other strains in the amount of contact time. All strains, regardless of their resistance profile reduced the amount of contact they had with the net when exposed to treated nets.

For all strains although activity time is reduced at the treated net surface, the majority of all activity was still spent in behavioural modes that involve contact with the net, as mosquitoes are attracted to the bait even in the presence of insecticides (Kongmee *et al.,* 2012; Parker *et al*., 2015; Siegert *et al.,* 2009), emphasizing how effective a bed net is as a human-baited lethal mosquito trap (Gillies *et al*., 1968; Killeen *et al.,* 2006; Snow, 1970).

Previous work has suggested exposure to ITNs modifies the mosquito's behaviour, either resulting in avoidance (Gatton *et al.,* 2013), changes in biting time (Russell *et al.,* 2011) or biting location, increasing exophilic behaviour (Githinji *et al*., 2020; Govella *et al.,* 2010; Kreppel *et al*., 2020; Mbogo *et al*., 1996; Molineaux & Gramiccia, 1980; Perugini *et al*., 2020; Reddy *et al.,* 2011; Russell *et al.,* 2010; Russell *et al*., 2011; Sanou 2020; Tirados *et al.,* 2006). Although tracking experiments did not reveal any repellent effects of Olyset net, irritancy of the net may be leading to behavioural resistance in the field.

It is also important to note that the amount of exposure to the ITN in video tracking is considerably lower than the amount of time exposed in a standard WHO cone test, something remarked on previously by other studies (Parker *et al.,* 2015, Parker *et al.,* 2017, Spitzen *et al.,* 2014). Results from this study provide a much more realistic picture of doses a mosquito receives in the field. Although the standard WHO tests are widely used for measuring resistance and net efficacy in the field, many studies have highlighted that these basic assays do not explain the full effects of resistance on malaria epidemiology and success of control tools (Bradley *et al.,* 2017; Oxborough *et al*., 2015; Ranson & Lissenden, 2016; WHO 2016). Results suggest video tracking to be a more realistic test of net efficacy as used in the field.

4.4.3 Location of activity at the bednet interface

From basic laboratory exploration of behaviour around a baited net (Lynd *et al.,* 2013; Sutcliffe *et al.,* 2015), to more complex video tracking and modelling experiments, previous research has shown the majority of mosquito activity occurs on the roof of the bednet (Jones *et al.,* 2021; Lynd *et al.,* 2013, Murray *et al.,* 2021; Parker *et al.,* 2015, Parker *et al.,* 2017). In agreement with this research, total activity, was highest on the net roof, accounting for 89% and 79% of all net contact for both the Banfora and VK7 strain and most roof contact occurred above the head/torso region for all stains. Orientation of the volunteer was alternated between tests to avoid any bias from the entry point.

When exposed to the Olyset, this activity remained mainly on the roof of the treated net for Kisumu (81%) and N'gousso (66%) supporting previous findings emphasising the importance of the bednet roof regardless of the net treatment (Lynd *et al*., 2013; Murray *et al*., 2020; Parker *et al.,* 2015; Parker *et al*., 2017; Sutcliffe *et al.,* 2014; Sutcliffe *et al.,* 2017). When exposed to Olyset net the majority of activity also remained on the roof for both resistant strains. Results show that regardless of resistance status, mosquitoes are drawn to the roof of the net by the plume of heat and moisture created by the human bait beneath (Guillet *et al.,* 2001; Parker *et al.,* 2015; Sutcliffe *et al.,* 2014; Sutcliffe *et al.,* 2017; Sutcliffe & Yin, 2014). For the VK7 strain although the majority of contact was still on the roof, the proportion of activity in this area reduced to just 59% of when exposed to the treated net, something also observed in wild populations of *An. arabiensis* previously (Parker *et al*., 2017), suggesting some differences in location preference between the strains.

Overall, the knowledge that most mosquito activity is focused on the top of a bednet has already facilitated the development of newer nets such as the PermaNet 3.0 the ability to assess localisation of activity with the tracking system has proven a valuable tool when evaluating all new net types, even facilitating new innovative designs such as the barrier bednet (Murray *et al.,* 2020).

4.4.4 Temporal variation in responses throughout the assay

Similar to the results observed in Chapter 3, both susceptible strains remained active at the untreated net surface for the entire assay (Chapter 3, Figure 3.5). Over the whole 2-hour assay, the general profile of activity around the untreated net remained stable with an initial peak in activity at 10-20 minutes. For both resistant strains, when split into time intervals the activity remained high around an untreated net, with Banfora displaying a similar trend in activity to both susceptible strains, most notably the Kisumu strain (Figure 4.3 and 4.5). However, for the VK7 strain, as observed in previous chapters (Chapter 3, Figure 3.5), the trend in activity looks very different over time to all the other strains.

As with the bench top assays (Chapter 3), results suggest a subtle difference in the way both resistant strains behave around the net interface, whether this difference is a result of the different resistance profiles each strain has, remains undetermined. Research shows a link with different genetic backgrounds and a difference in feeding/resting behaviours (Guelbeogo *et al*., 2014) therefore suggesting that the subtle differences in host seeking around the net interface could be linked to difference in genotype.as discussed in previous chapters some research has suggested differences in responses to ITNs associated with the presence of the Kdr mutation, again suggesting that a difference in resistance mechanism could cause differences in host seeking activity (Porciani *et al.,* 2017). Additionally, previous research that some species are more active than others especially during host-seeking and perhaps the differences in behaviour are more innate (Chareonviriyaphap *et al*., 2004; Cooperband *et al.,* 2009; Parker *et al.,* 2017; Sutcliffe *et al.,* 2014).

When behaviour was analysed over time, activity around the Olyset net decreased rapidly, as expected for both susceptible strains (Parker *et al.,* 2015; Parker *et al.,* 2017). For both strains, activity almost ceases after the first 30-minutes. For both susceptible strains, these results demonstrate how use of ITNs reduces host seeking behaviour rapidly (Cohnstaedt *et al*., 2011; Glunt *et al*., 2017; Parker *et al.,* 2015), and the small amount of contact made with the net (19-23 seconds), is enough to cause significant mortality effects on these strains (Discussed in Chapter 5). However, in contrast to observations made for both susceptible strains, but also in agreement with findings of wild *An. arabiensis* populations (Parker *et al.,* 2017), there was no evidence of a drastic activity decay for both resistant strains exposed to the ITN (Figure 4.5). Activity remained low and constant for both Banfora and VK7 throughout the two-hour assay at the treated net. Previous studies have only assessed activity decay around a baited net using susceptible strains or populations with low levels of resistance. Therefore, the decay in activity observed previously is likely to be an effect of insecticide induced KD/mortality. This is confirmed by the lack of activity decay observed when both resistant strains are exposed to Olyset net.

In the current study the most notable difference between susceptible and resistant strains is the lack of activity decay resistant strains exhibit in the presence of treated nets. However, the activity of the resistant strains never reached the levels observed at the untreated nets, host seeking activity around the ITNs remained at relatively low levels throughout the test. No impairment to host seeking was therefore observed over time on these strains but further work on the range of possible effects toxic or sensory impairment experienced during ITN contact, may explain the overall reduction of activity at this net surface. Compared to the susceptible strains, the resistant strains remain active at the net interface throughout the assay. Previous work by Kefi *et al.* 2021 suggested that detoxification of the insecticide by the resistant strains is likely occurring in legs. The research indicated that short term

insecticide-induced tolerance is suggested to be linked to the overexpression of GPCRs, ABC transporters, odorant-binding proteins and salivary gland proteins (Kefi *et al.,* 2021). This perhaps suggests why the resistant mosquitoes are able to withstand the sustained activity at the ITN interface. The sublethal or delayed effects of prolonged ITN contact on mosquito's life history traits will be considered in Chapter 5.

4.5 Conclusion

Although a more complex method than the standard WHO tests for evaluating net effectiveness, the video tracking system can be used alongside more basic bench top assays (Chapter 2 and 3), to gather a complete understanding of mosquito-net interactions and overall bednet efficacy. With an increase in shorter contact behaviours (visiting) and an overall reduction in the amount of time spent on the net surface for all strains, the Olyset net evidently is a contact irritant to mosquitoes, a result that confirms what was observed in the smaller bench top assays discussed in Chapters 2 and 3. For resistant strains although the amount of contact reduces around the treated net, the activity levels remain constant and mosquitoes continue host-seeking behaviour throughout the assay, something the susceptible strains do not, most likely due to insecticide induced KD or mortality. Despite the observed reduced contact with the Olyset net in video tracking experiments, for both resistant strains contact with the ITN induced average mortality rates of 20% (VK7) and 48% (Banfora) at 24-hours post exposure. As this was an increase compared to the mortality rates observed post ITN exposure in the bench top assays (Chapter 2 and 3) it displays the importance of using more field relevant tests to evaluate overall efficacy of ITNs. All delayed and sublethal effects post ITN exposure will be discussed in more detail in Chapter 5.

Chapter 5: Delayed or sublethal effects of insecticidal bednet exposure on insecticide resistant *An. gambiae s.l*

5.1 Introduction

The previous chapters (Chapters 2-4) focused on the behaviour of *Anopheles gambiae s.l.* at the treated net interface. In the current chapter the delayed or long-term consequences of Olyset and PermaNet exposure are investigated in order to complete our understanding of the effectiveness of ITNs and possible impacts on transmission.

The emergence of insecticide resistance is one of the greatest threats to the control of malaria and mosquito transmitted diseases (Hemingway *et al.,* 2016). However, the overall impact of resistance on bednet effectiveness is unclear. Research suggests that standard insecticide treated nets still provide personal protection in areas of pyrethroid resistance. (Kleinschmidt *et al.,* 2018). Although numerous influences might be involved, sublethal effects that do not become apparent until after standard test follow-up periods might be contributing to the success of ITNs as a control tool (Ferguson *et al*., 2012; Glunt *et al.,* 2018; Hughes 2018; Hill 2003; Hughes *et al*., 2020; Tchakounte *et al*., 2019; Viana *et al*., 2016).

Standard WHO tests typically use a pre-defined exposure time to either a discriminating dose of insecticide or the formulated product, assessing KD and mortality at 24-hours post exposure (WHO, 2016; WHO, 2013). The standard test for evaluating the effectiveness of a bednet is the WHO cone test. The criteria for a net passing the WHO cone test are either knockdown >95% or mortality at 24 hours greater than 80% (WHO 2013). Although these tests provide valuable data, their use for predicting malaria epidemiology following control interventions is unclear. These standardised bioassays do not replicate the exposure of wild mosquitoes to ITNs or IRS. Specifically, as shown in the previous chapters (Chapters 2,3 and 4) existing pre-determined durations of exposure that force the mosquito to contact the insecticide are not representative of exposure the mosquito encounters in the field.

Reducing the mosquito's lifespan directly impacts the parasites' ability to complete its extrinsic incubation period, which may explain in part why ITNs remain an effective control tool despite the emergence of insecticide resistance (Ferguson *et al*., 2012). For this reason, previous studies have of introduced extended monitoring procedures to the standard test methods to measure the effects of

insecticide exposure on life history traits (Hauser *et al*., 2019; Tchakounte *et al*., 2019; Viana *et al*., 2016). By monitoring long term survival after exposure to PermaNet in cone tests some studies have shown a reduction in the longevity of pyrethroid resistant mosquitoes in comparison to those exposed to untreated controls (Tchakounte *et al.,* 2019; Viana *et al.,* 2016). However, reduced longevity has not been observed after exposure to all pyrethroid nets. Hauser *et al* (2019) showed no effect of exposure to Olyset plus on long term survival of *Anopheles*. Not only is it important to look at the effects of exposure on longevity of the mosquito when assessing the impact of resistance on pyrethroid nets but also necessary for evaluating the second generation of bednets treated with slow-acting or novel chemistries.

Additionally, standard tests do not assess sublethal effects such as impaired feeding, post insecticide exposure, which could impact on the mosquitoes' vectorial capacity and subsequently affect malaria transmission. ITN exposure has been shown to not only affect longevity of the adult mosquito but also inhibit their host-seeking behaviour for up to several days (Liu *et al*., 1986; Takken *et al*., 2001; Thiévent *et al*., 2019). Exposure to sublethal doses of pyrethroids has been shown to result in mosquitoes being less responsive to attractants 24-hours to 48-hours after insecticide exposure and to also affect flight orientation towards a host (Cohnstaedt *et al*., 2011; Thiévent *et al*., 2019). However, Glunt *et al* (2018) showed that the effects of ITN exposure on host seeking only lasted between 2 and 7-hours post exposure of resistant *Anopheles*. It has also been suggested that neurotoxic pyrethroids cause long term nerve damage to sensory organs or to nerves responsible for the activation of flight, coordination, and orientation to a host, ultimately reducing the chances of bloodfeeding activity (Cohnstaedt *et al*., 2011).

Bloodfeeding plays a part in malaria transmission in two ways: directly, as when bloodfeeding the *Plasmodium* parasite is transmitted to the host or picked up by the mosquito, and indirectly, as the *Anopheles* mosquito requires the blood meal for reproduction, thereby influencing the mosquito population density and ultimately, transmission. Although a ITN can prevent a mosquito taking a successful blood meal, it is unknown whether exposure has any effect on the volume of blood ingested and what effect this consequently has on malaria transmission. Churcher *et al.* (2017) showed that mosquitoes with a higher number of sporozoites after blood-feeding were more likely to cause malaria infection suggesting larger blood meals may enhance the chances of transmission.

Bloodfeeding inhibition has been measured using a WHO tunnel test, where feeding on an animal host such as rabbit or guinea pig is assessed overnight following ITN exposure (WHO, 2005). A systematic

review concluded that when mosquitoes were exposed to insecticide treated nets in these tests, there was a lower risk of bloodfeeding compared with untreated nets, regardless of resistance status (Strode *et al*., 2014). Although both tunnel tests and experimental hut trials provide useful information on net efficacy, tunnel tests use an unnatural host and both tests give little indication of when (after or before exposure) feeding occurred. Similarly with experimental huts, they do not give an accurate representation of how bloodfeeding occurred (through the net/before hut entry) but the source of the bloodmeal can be elucidated using ELISA assays or similar and inferences made from these data. Other studies have used a variety of laboratory bioassays to conclude also that ITN exposure inhibits bloodfeeding (Glunt *et al*., 2018; Hauser *et al.,* 2019; Hougard *et al*., 2003; Mulatier *et al*., 2019; Thiévent & Koella, 2019). However, standardisation of the test method is needed to assess the overall effects ITN exposure has on bloodfeeding behaviour (Mulatier *et al*., 2019).

Whilst ITN exposure has shown to have detrimental effects to both susceptible and resistant mosquitoes, bloodfeeding through the net may also have some immediate protective effects. Hauser *et al* (2019) found increased survival than expected in mosquitoes that fed directly through an Olyset plus net. They hypothesize that when a blood meal is taken simultaneously with permethrin exposure, it increases the concentration of reactive oxygen species (ROS), increasing antioxidants in the midgut, thus helping the mosquito to reduce the detrimental effects of the insecticide. A second theory is that as the mosquito's temperature rises during bloodfeeding and pyrethroids become less effective at high temperatures (Narahashi, 1971; Suh *et al.,* 2019), therefore the insecticide becomes less toxic during feeding (Hauser *et al.,* 2019). It is therefore important to assess ITN efficacy during the different scenarios of exposure that the mosquito may encounter in the field.

Standard ITNs may still be effective against insecticide resistant mosquitoes by reducing the density of mosquito populations through a reduction of their fertility and fecundity. Exposure to pyrethroids, has shown to reduce egg laying and hatching in *Aedes* mosquitoes (Bibbs *et al*., 2018) Reproductive output post- exposure to first generation nets was not previously characterised using standard WHO tests (Mulatier *et al*., 2019). However as newer net types containing reproductive modulators such as pyriproxyfen are developed, the WHO guidelines do state that it may be necessary to modify test procedures for these net types (Koffi *et al*., 2015; Ngufor *et al*., 2014; Toé *et al*., 2019; WHO 2013).

Ultimately, standard tests are missing vital information on how interventions impact malaria transmission. Therefore, when assessing the overall effectiveness of ITNs, the consequences to all life

history traits post insecticide exposure that could potentially reduce their disease transmission capability, should be considered.

The present study investigated sublethal effects or delayed effects of insecticide exposure. The sublethal effects or delayed effects measured in this chapter were defined as occurring over 24-hours post-ITN exposure. Immediate mortality effects were also recorded in order to capture overall lethality of ITNs. The aim of this chapter was to determine if exposure to a treated net had any detrimental effect on pyrethroid susceptible and resistant mosquitoes' ability to blood-feed at 1-hour and 24-hours post exposure, their reproductive output, and lifespan.

The specific objectives of these experiments were to explore:

1) Immediate mortality: Assess KD and mortality effects 1-hour and 24-hours post exposure to standard pyrethroid nets.
2) Willingness to feed: Determine if mosquito ability to take a blood-meal was impaired following exposure to ITNs, using the baited box feeding prevented version of the assay and video tracking assays.
3) Blood meal volume: For those mosquitoes that successfully fed, determine if feeding during (Chapter 2) or post-ITN exposure (Chapter 3 and 4) reduces the volume of blood meal ingested.
4) Fertility and Fecundity: Determine if mosquito fertility and fecundity is reduced after exposure to standard ITNs in both baited box and video tracking assays.
5) Longevity: Determine if mosquito life span is reduced post-ITN exposure in both baited box and video tracking assays compared to those exposed to untreated net.

2.2 Materials and Methods:

5.2.1 Mosquito colonies

In all experiments, two susceptible (Kisumu and N'gousso) and two pyrethroid resistant strains (Banfora and VK7) of *An. gambiae s.l.* were exposed to untreated nets, PermaNet 2.0 (subsequently referred to as PermaNet) or Olyset ITNs. All details were unchanged from those described in Chapter 2 (Section 2.2). Total number of responding mosquitoes for each assay is displayed in Appendix 1.1 (Table A1.1).

5.2.2 Experimental procedures

All surviving mosquitoes post-ITN exposure were taken through the sub-lethal effects monitoring pipeline (Figure 5.1). Delayed and sublethal effects were recorded after exposure to two ITNs and control netting detailed in Chapter 2 (Section 2.2.2). Mosquitoes were exposed to either all three nets for up to 20-minutes in baited box experiments (Chapters 2 and 3) or two nets (untreated and Olyset net) for 2-hours in video tracking experiments (Chapter 4). Survivors were maintained and followed up in an LSTM testing room under standard insectary conditions (as per Chapter 2).

The sub-lethal pipeline comprised of six parameters: immediate KD and 24-hour mortality, willingness to feed post exposure, blood meal volume, fecundity and fertility, and longevity. All mosquitoes were put through the sublethal pipeline regardless of bloodfeeding activity to ensure all mosquitoes underwent the same level of handling. To achieve this all mosquitoes were moved into tubes the same number of times and scored for the different outcomes each day.

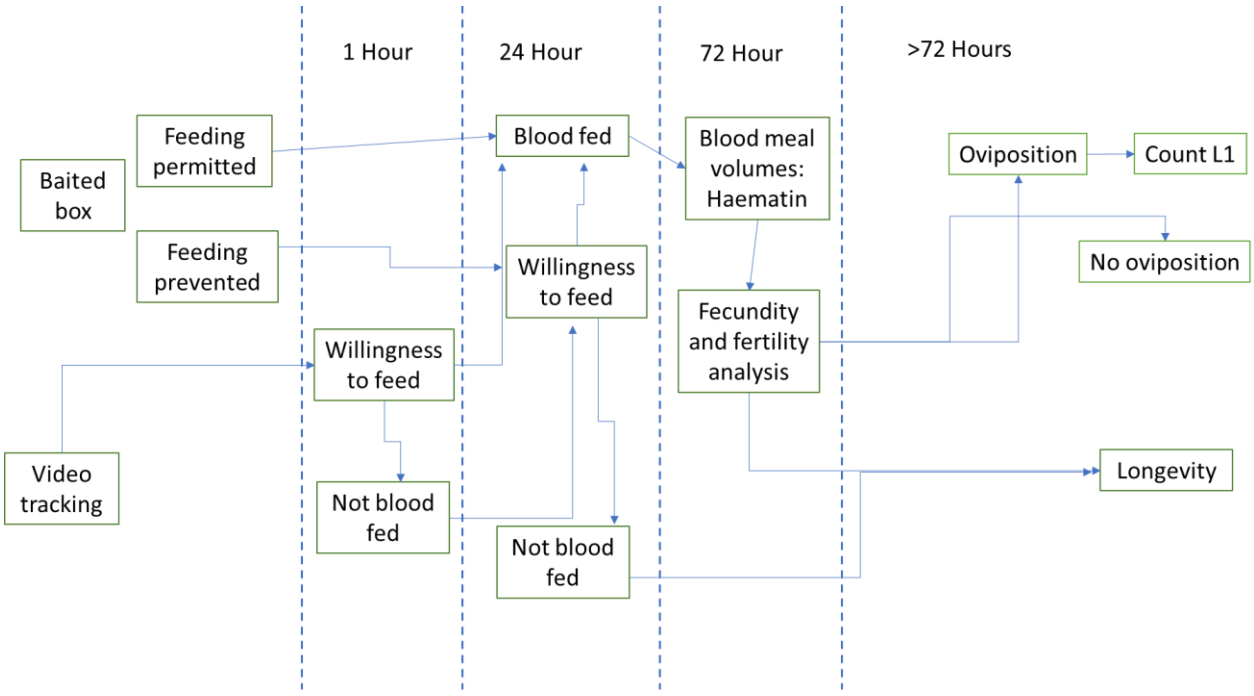


Figure 5.1: Sublethal effects monitoring pipeline. All mosquitoes exposed to all net types in each assay were put through the sublethal effects monitoring pipeline. Each stage of the pipeline is explained in more detail below.

5.2.2.1 Immediate mortality (1-hour KD and 24-hour mortality)

Knockdown rate at one hour was scored for cohorts of 25 mosquitoes exposed to each net type in the video tracking experiments (Chapter 4). Due to the logistics of the test, it was not possible to measure

one-hour KD for baited box assays (Chapter 2 and 3). Mortality was recorded 24-hours post exposure for all mosquitoes exposed in all tests (Chapters 2, 3 and 4).

Due to insecticide induced mortality susceptible populations could not be assessed for any further changes in life history traits (steps 5.2.2.2- 5.2.2.5).

5.2.2.2 Willingness to feed post-ITN exposure.

In order to determine if the mosquito's ability to take a blood meal was impaired following exposure to ITNs in the baited box and video tracking assays compared to those exposed to an untreated net, the number of blood fed females was analysed at 1-hour and 24-hours post exposure. Willingness to feed was defined as the number of females that visibly fed when offered a human arm post ITN exposure.

For the analysis, all knocked down or dead mosquitoes were removed from the sample size when assessing willingness to feed, to ensure the results represented the proportion of mosquitoes that fed out of those that were still alive after exposure. The process for monitoring feeding post –ITN exposure differed between the two testing procedures: baited box where feeding was prevented (Chapter 3) and video tracking assays (Chapter 4):

Prevented feeding baited box assays (Chapter 3)

Mosquitoes exposed in the feeding prevented version of the baited box (Chapter 3) were offered a human arm for 20 minutes at 24-hours post exposure. Successful bloodfeeding was recorded, and the mosquito moved to tubes with access to an egg pot for fecundity/fertility monitoring with access to 10% sugar solution. Mosquitoes remained in the tube for longevity monitoring.

Video tracking assays (Chapter 4)

Mosquitoes exposed in video tracking experiments, therefore not fed during the test (Chapter 4), were offered a human arm for 20 minutes at 1-hour and 24-hours post exposure. Following all video tracking experiments performed in Chapter 4 mosquitoes that fed at 1-hour were removed from their holding cup and placed into individual falcon tubes with net lids. All remaining mosquitoes were transferred after the second feed at 24-hours. Successful bloodfeeding was recorded, and the mosquito moved to tubes with access to an egg pot for fecundity/fertility monitoring with access to 10% sugar solution. Mosquitoes remained in the tube for longevity monitoring.

5.2.2.3 Ingested blood meal volume (µl)

In order to determine if feeding during (Chapter 2) or after ITN exposure (Chapter 3 and 4) reduces the amount of blood meal ingested, the volume of blood volume ingested was determined by measuring the quantity of haematin excreted as a proxy for the volume of blood ingested. Haematin is a digestive waste product excreted after the blood meal and permits estimation of bloodmeal size without damaging the mosquito (Briegel *et al.,* 1979).

Mosquitoes that bloodfed were stored individually in falcon tubes for 72-hours, then transferred into a separate tube for oviposition (5.2.2.4). Following the transfer of test mosquitoes, empty falcon tubes were stored at 4°C and excreted haematin from bloodfeeding was measured within one week. Haematin measurements were performed by the addition of 1ml of 1% lithium carbonate to dissolve the excreted blood meal product. The optical density of triplicate samples (200µl) at 397nm was read. Absorbance at 397nm was converted to µg/ml measurements using a stored standard curve with a range of 1.76 µg/ml to 30 µg/ml. The µg/ml measurements were converted to ingested blood meal volume in microlitres using the midpoint of the haemoglobin reference range for adult women (as all mosquitoes for this study were fed by myself) in the UK (135g/L).

To calculate the volume of ingested blood the amount of haematin in µg/ml was first calculated using a haematin standard curve. Total haematin was then converted from µg to g and the number of moles of haematin were calculated by dividing the mass by the molecular weight of haematin (651.94 moles). To calculate the haemoglobin content, moles of haematin were divided by 4, as 4 moles of haematin is equal to one mole of haemoglobin. The concentration of haemoglobin was converted into mass by multiplying it by the molecular weight of haemoglobin (65,458 moles) and converted to micrograms of haemoglobin by multiplying by $1x10^6$. To convert this to µl of blood meal ingested the value was then divided by the average male or female haemoglobin concentration in blood (120g/l – 150g/l for female). A complete haematin measurement was only taken if the mosquito survived for 72-hours post-test to digest the full blood meal.

5.2.2.4 Fecundity and fertility

After 72-hours post exposure in all tests (Chapter 2, 3 and 4) all mosquitoes were transferred into a separate tube with access to wet filter paper in the bottom of the tube, to allow egg laying. Eggs were collected, counted under a dissection microscope and floated in 50ml of distilled water. Emergence of first instar larval stages were counted for 5 days. Each day L1 larva were counted and removed from the cohort to avoid counting errors and cannibalism which may have affected the final count. After 5 days all unhatched eggs were discarded.

All mosquitoes then remained in the falcon tubes for longevity monitoring (second 5.2.2.5).

5.2.2.5 Longevity

All mosquitoes exposed in all assays (Chapters 2, 3 and 4) were monitored in the individual falcon tubes, with access to 10% sugar solution, refreshed daily, until their natural death.

5.2.2.6 Wingspan measurements

All mosquitoes' wingspans were measured as a proxy for body size to account for this factor during statistical analysis. After death all mosquitoes from all experiments (Chapter 2, 3 and 4) were stored in Eppendorf tubes in the freezer (-18°C to -25°C). One wing was removed and measured under a dissection microscope. The wing measurements were recorded using GXCAM software (GXCAM version 6.7), taking two-point linear measurements of one wing from each mosquito. The wing was removed and secured onto a slide, and its length measured from the distal end of the alula to the tip of the wing, excluding the fringe scales, with an ocular micrometre (Briegel, 1990).

5.2.3 Data analysis

Immediate mortality rates and feeding rates post net exposure were analysed using Chi squared test in SPSS (IBM SPSS Corp. 2017. Version 25.0). Box plots were created for blood meal volumes in GraphPad Prism version 8.4.3 for Windows (GraphPad Prism version 8.4.3 2020). The mean volume (µl) of blood was calculated with 95% CI for each strain exposed to each net type. Blood meal volumes were analysed for resistant strains only using a generalized linear model in SAS adjusting for body size and contact time in the model (Version 9.4 of the SAS System for Unix 2002-2012). Survival analysis was performed using Cox regression in R (R Core Team 2019) survival package (Therneau, 2015). Survival curves were generated in GraphPad Prism version 8.4.3 for windows (GraphPad Prism version 8.4.3 2020). Fecundity was measured by counting the number of eggs laid by each female and fertility measured by number of l1 that hatched. Due to the variance of data exceeding the mean, egg counts were analysed using Zero inflated negative binomial regression and larval counts using negative binomial regression in R (R Core Team 2019) package glmmTMB (Brooks *et al.,* 2017).

5.3 Results

Three different tests (baited box feeding permitted and prevented and video tracking) were performed to assess the delayed or sublethal effects of insecticide exposure on two insecticide susceptible and two pyrethroid resistant strains.

Results are shown for all three experiments split into the subcategories of the sublethal effects monitoring pipeline (Figure 5.1): immediate 24-hour mortality, bloodfeeding rates, blood meal volume, fecundity and fertility, and longevity. The sample size for each test was selected to allow assessment of behaviour of the ITN interface, this resulted in differences in numbers between treatment groups and small sample sizes for some groups when analysing the sublethal and delayed effects.

5.3.1 Immediate mortality (1-hour KD and 24-hour mortality)

Regardless of the method of exposure, mortality rates of untreated controls did not exceed 20%. Exposure to both Olyset and PermaNet ITNs in the baited box feeding permitted assay resulted in significantly higher 24-hour mortality in both susceptible populations compared to untreated nets (Table 5.1; $P<0.0001$ for each comparison). When exposed to PermaNet both Kisumu and N'gousso had higher 24-hour mortality (Kisumu and N'gousso, 86% and 71%, respectively) than when exposed to Olyset net (Kisumu and N'gousso, 57% and 63%, respectively). When Banfora and VK7, were exposed to PermaNet (Table 5.1; $P=0.224$, $P= 0.123$) and Olyset (Table 5.1; $P = 0.09$, $P = 0.123$) there was no significant difference observed in 24-hour mortality compared to untreated nets.

When feeding was prevented in the baited box (Chapter 3) the immediate mortality rates were equal to or higher than the feeding permitted version (Chapter 2), with the exception of the resistant strains after exposure to Olyset.

As expected, and in agreement with the feeding permitted assays (Chapter 2), there were significantly higher 24-hour mortality rates in both susceptible strains when exposed to both net types compared with the untreated control (Table 5.1; $P<0.0001$). Again, there was no significant difference observed in immediate 24-hour mortality when both resistant strains (Banfora and VK7) were exposed to PermaNet (Table 5.1; $P= 0.171$, $P= 0.188$) and Olyset (Table 5.1; $P = 0.929$, $P = 1.0$).

When both susceptible strains were exposed to Olyset net in video tracking experiments (Chapter 4) knockdown and 24-hour mortality rates were high for both strains (Table 5.1; Kisumu 99% and N'gousso 98%). 24-hour mortality for all strains increased when exposed to the nets in video tracking assays (Chapter 4) compared with results post exposure in the baited box assay (Chapter 2 and 3). There was significantly higher knockdown observed for both Banfora and VK7 strain when exposed to Olyset net in video tracking experiments (Chapter 4) compared with untreated control (Table 5.1;

Banfora P<0.0001, VK7 P<0.0001) and 24-hour mortality was higher for both resistant strains in the treated net assays (Table 5.1; Banfora P<0.0001, VK7 P<0.0001).

Table 5.1: 24-hour mortality post exposure of four *An. gambiae s.l.* strains to two ITNs and control netting. Mosquitoes were exposed to PermaNet 2.0, Olyset or untreated net in baited box (Chapter 2 and 3) and video tracking assays (Chapter 4) on day 1 and their mortality recorded the following day. For video tracking experiments there was no exposure to PermaNet and 1-hour knockdown was also recorded post exposure to untreated and Olyset net.

24-hour mortality (%) Number of mosquitoes dead at 24 hours post exposure/ no. tested					
		Feeding Permitted	Feeding Prevented	Tracking	
		24hr	24hr	1hr KD	24hr
Kisumu	Untreated	3% (1/29)	9% (2/23)	20% (23/116)	11% (11/105)
	PermaNet	86%* (18/21)	100%* (20/20)	-	-
	Olyset	57%* (12/21)	64%* (16/25)	100%* (145/145)	99%* (143/145)
N'gousso	Untreated	4% (1/26)	9% (2/22)	10% (12/119)	18% (21/116)
	PermaNet	71%* (15/21)	89%* (16/18)	-	-
	Olyset	63%* (15/24)	95%* (19/20)	99%* (139/140)	98%* (137/140)
Banfora	Untreated	0% (0/29)	5% (1/22)	0% (0/89)	5% (4/86)
	PermaNet	5% (1/20)	16% (4/25)	-	-
	Olyset	10% (2/21)	4% (1/26)	56%* (71/127)	48%* (59/123)
VK7	Untreated	0% (0/25)	0% (0/19)	2% (2/92)	3% (3/90)
	PermaNet	9% (2/22)	9% (2/23)	-	-
	Olyset	9% (2/22)	0% (0/25)	43%* (59/137)	20%* (26/132)

**- denotes significance when compared to untreated net within the same test.*

Summary of immediate mortality effects post ITN exposure:

- Exposure to ITNs induced significantly higher mortality rates compared to an untreated control for both susceptible strains when exposed in all assays.
- No difference in immediate mortality was detected for both the resistant strains after exposure to ITNs and an untreated control in both baited box assays.
- Exposure to Olyset net in video tracking experiments induced significantly higher mortality rates for both VK7 and Banfora compared to an untreated control.

5.3.2 Willingness to feed post ITN-exposure.

Mosquitoes exposed in both the baited box feeding prevented assays (Chapter 3) and video tracking assays (Chapter 4), were offered a bloodmeal 24-hours post-exposure. The number of mosquitoes that took a blood meal and that did not feed 24-hours post exposure to the three net types were recorded (Table 5.2; Figure 5.2). For video tracking experiments feeding rates were recorded at 1-hour and 24-hours post or untreated net (Figure 5.3).

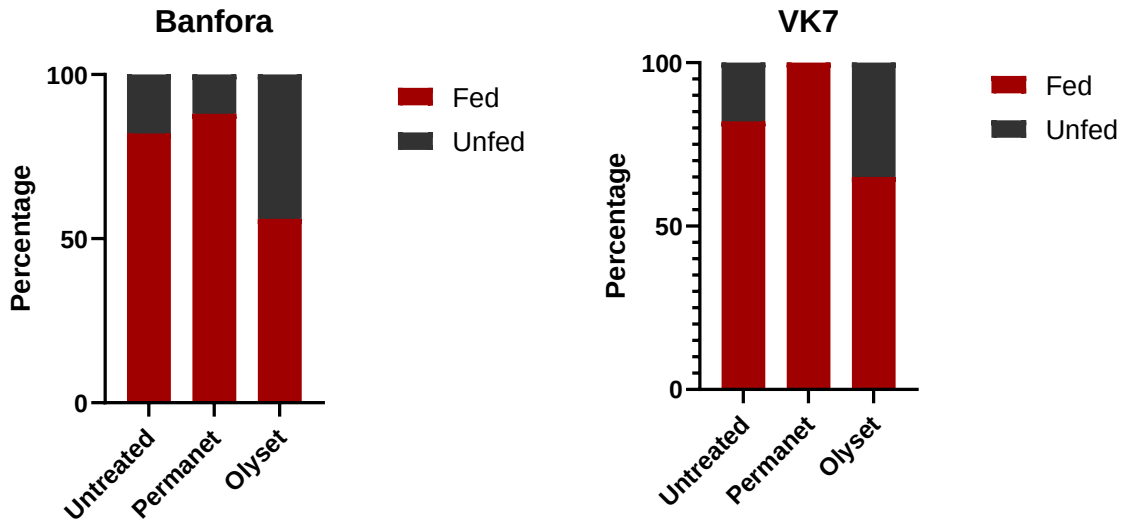


Figure 5.2: Feeding rates of two resistant *An. gambiae s.l.* strains, 24-hours post exposure to two treated nets and control netting in baited box prevented feeding assays (Chapter 3). **Left**: Banfora, **Right:** VK7. Number of fed mosquitoes; Red, Unfed; Grey. Banfora exposed to Olyset N= 14, PN2 N=23 and Untreated N=23. VK7 exposed to Olyset N= 17, PN2 N=23 and Untreated N= 18.

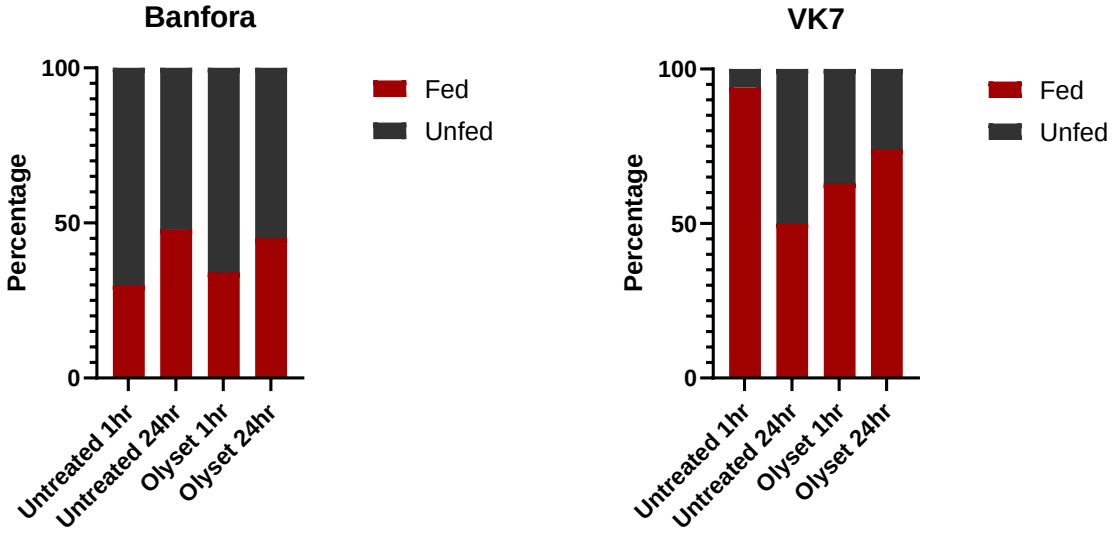


Figure 5.3: Feeding rates of two resistant *An. gambiae strains*, 1-hour and 24-hours post exposure to Olyset net and control netting in video tracking experiments (Chapter 4)**. Left**: Banfora, **Right**: VK7. Number of fed mosquitoes, Red; Unfed, Grey.

When exposed to an untreated net in baited box assays (Chapter 3), feeding rates were high for all strains (81%-100%). After exposure in video tracking experiments (Chapter 4) feeding rates were high when exposed to untreated net for both N'gousso and VK7 with 48% (48/101) and 94% (81/86) at one-hour and between 89% (41/46) and 100% (7/7) feeding at 24-hours post-test. The Kisumu strain had lower rates of feeding than N'gousso and VK7 at 1-hour post exposure (Table 5.3; 33%) but this increased to 95% (69/73) at 24-hours. Banfora however, had lower feeding rates than all other strains

with 30% (27/89) of mosquitoes taking a blood meal at 1-hour post exposure and 48% (28/58) at 24-hours.

As the 24-hour mortality of both susceptible stains was high (Olyset 64%-95%; PermaNet 89%-100) post ITN exposure in both assays (refer to Table 5.1), no further statistical analysis was completed on these strains. Of those susceptible mosquitoes that survived, eight bloodfed after exposure to the treated net.

For the analysis post exposure in video tracking experiments total sample size varied between 1-hour and 24-hours due to knockdown, mortality and the removal of those mosquitoes that previously fed.

Following exposure to PermaNet in the baited box assay (Chapter 3), significantly more VK7 bloodfed compared to an untreated net (Table 5.3; P= 0.032) although there was no significant difference in the number that fed post exposure to Olyset net compared to untreated (Table 5.2; P= 0.202). Conversely, significantly fewer Banfora bloodfed after exposure to Olyset compared with an untreated net (Table 5.2; P= 0.038) when exposed during baited box assays, with no difference in the feeding rates after exposure to PermaNet (Table 5.2; P= 0.514). Results show no evidence of feeding impairment 24-hours after exposure for the VK7 strain. Banfora exhibited feeding inhibition up to 24-hours but only after exposure to Olyset net.

Table 5.2: Feeding rates (%) of four *An. gambiae s.l.* strains 24-hours post exposure to two treated nets and control netting. Total number and percentage fed calculated 24-hours post exposure to PermaNet 2.0, Olyset and untreated control netting in the baited box feeding prevented assay.

Test	Strain	Treatment	Number responded	Total number fed	Feeding rates
					Number fed at 24hrs/Total alive %
Baited box: Prevented feeding. (Chapter 3)	**Kisumu**	**Untreated**	25	23	**100%** (23/23)
		PermaNet	20	0	0%
		Olyset	17	3	**33%** (3/9)
	N'gousso	**Untreated**	24	17	**81%** (17/21)
		PermaNet	20	2	**100%** (2/2)
		Olyset	21	1	**100%** (1/1)
	Banfora	**Untreated**	29	23	**82%** (23/28)
		PermaNet	29	23	**85%** (23/26)
		Olyset	26	14	**56%** (14/25)
	Vk7	**Untreated**	22	18	**82%** (18/22)
		PermaNet	27	23	**100%** (23/23)
		Olyset	26	17	**65%** (17/26)

In the video tracking experiments, although the host was protected by a bednet, some mosquitoes were able to feed through the net during the test. These mosquitoes were removed from the total sample.

In contrast to the baited box assay (Chapter 3), when exposed to Olyset net in video tracking experiments, there was no significant difference in the number of Banfora that fed at both 1-hour (Table 5.3; P= 0.651) and 24-hours (Table 5.3; P = 0.727) net compared to untreated, showing no impairment to bloodfeeding at both time points for this strain. Significantly less VK7 fed at 1-hour after exposure to Olyset net compared to untreated net Table 5.3; (P<0.0001). At 24-hours post exposure to untreated net only 7 mosquitoes were remaining in the total sample to offer a blood meal and therefore no further analysis was performed on this dataset. Results show that exposure to Olyset caused bloodfeeding inhibition at 1-hour post exposure for the VK7 strain, but this effect was not observed at 24-hours due to low sample sizes in the control arm.

Overall bloodfeeding rates where lower after exposure in the video tracking experiments after exposure to untreated net compared to the bench top assay, suggesting the assay itself has an impact on bloodfeeding behaviour.

Table 5.3: Feeding rates (%) of four *An. gambiae s.l.* strains, two susceptible and two resistant to pyrethroids, 1-hour and 24-hours post exposure to two treated nets and control netting. Total number and percentage fed calculated 24-hours post exposure to PermaNet 2.0, Olyset and untreated control netting.

Test	Strain	Treatment	No. Exposed In test	Total number fed	Feeding rates	
					Number fed at 1hr/Total alive %	Number fed at 24hrs/Total alive %
Video Tracking (Chapter 4)	**Kisumu**	**Untreated**	116	99	**33%** (30/91)	**95%** (69/73)
		Olyset	145	2	**0%** (0/0)	**100%** (2/2)
	N'gousso	**Untreated**	119	89	**48%** (48/101)	**89%** (41/46)
		Olyset	140	1	-	**50%** (1/2)
	VK7	**Untreated**	92	88	**94%** (81/86)	**100%** (7/7)
		Olyset	137	95	**63%** (49/78)	**74%** (46/62)
	Banfora	**Untreated**	89	55	**30%** (27/89)	**48%** (28/58)
		Olyset	127	41	**34%** (19/56)	**45%** (22/49)

Summary of willingness to feed post ITN exposure:

- When offered a blood meal after exposure in the baited box assay significantly more VK7 fed after exposure to PermaNet compared to untreated net, but there was no difference in the number of mosquitoes that fed after exposure to Olyset compared to the control netting.
- When offered a blood meal after exposure in the baited box assay significantly fewer Banfora fed after exposure to Olyset compared to untreated net, and there was no difference in the number that fed after exposure to PermaNet compared to the control.
- In contrast to the baited box results when exposed to Olyset in video tracking experiments significantly less VK7 fed at 1-hour post exposure and no difference was detected in the number of Banfora that fed post exposure to both net types.

5.3.3 Ingested blood meal volume (µl)

Due to the low number of susceptible individuals surviving to completely digest the blood meal when exposed to treated netting in all assays (between 1%-16% survival for both strains) further analysis of

blood meal volumes were only performed for the all the resistant strains that survived 72-hours to digest a full blood meal.

When feeding was permitted through the net (Chapter 2) the average blood meal volume ingested was calculated for both resistant strains. When VK7 fed through both treated net the average blood meal volume was 2.77µl 95% (CI 2.31- 3.24, VK7 exposed to PermaNet) and 1.96µl (95% CI 1.42- 2.5, VK7 exposed to Olyset net). Both strains on average ingested a similar amount of blood through the untreated net (VK7; 2.33µl 95% CI 1.78 – 2.87, Banfora; 2.27µl 95% CI 1.59- 2.95). Banfora ingested 2.34µl (95% CI 1.74-2.95) on average when feeding through an Olyset net and 1.75 µl (95% CI 1.18-2.31) when feeding through PermaNet. The amount of blood ingested was not significantly different for either the Banfora or VK7 strain when feeding through an Olyset net (Figure 5.4; Banfora P=0.5449, VK7 P=0.1721; Appendix 2.6; Table 2.5) or PermaNet for the Banfora strain (Figure 5.4; Banfora P=0.6274; Appendix 2.6; Table 2.5) compared to an untreated control. The amount of blood ingested when feeding through PermaNet was significantly more than untreated for the VK7 strain (P=0.0276; Appendix 2.6; Table 2.5).

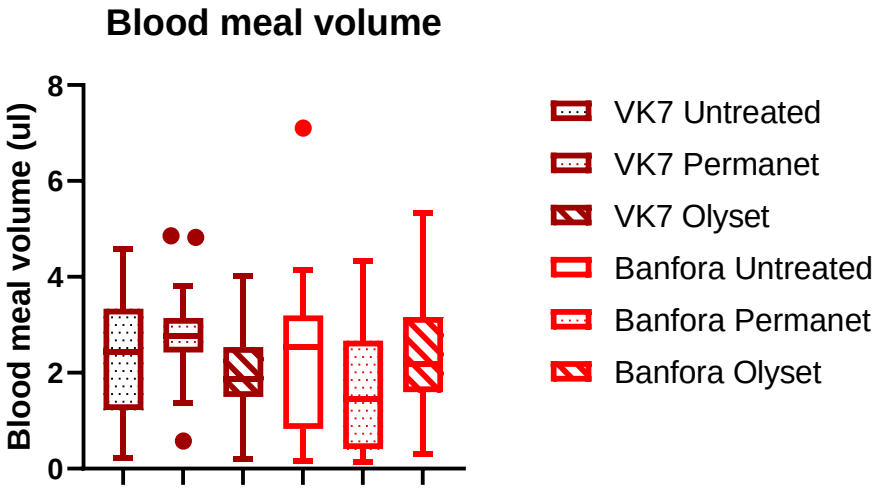


Figure 5.4: blood meal volume (µl) when feeding at a human baited net in baited box feeding permitted assays (Chapter 2), treated and untreated for two resistant strains of *An. gambiae* s.l. Mean blood meal volume: calculated for each resistant strain exposed to each net type. Outliers are displayed as points and are classed as 1.5x the inter quartile range. Banfora exposed to Olyset net N= 18, PN2 N= 22 and Untreated N= 25. VK7 exposed to Olyset net N= 16, PN2 N= 20 and Untreated N = 22.

For mosquitoes that had been exposed in feeding prevented assays (Chapter 3) and feeding permitted assays (Chapter 2) the average blood meal size was between 1- 3µl.

Both resistant strains ingested a similar amount of blood post exposure to untreated net when exposed in feeding prevented assays, with VK7 ingesting an average of 2.47µl (95% CI 1.44- 3.52) and

Banfora ingesting an average of 2.6µl (95% CI 1.58- 3.62). Post exposure to Olyset net the average blood meal was highest for VK7, ingesting on average 3.11 µl of blood (95% CI 2.18- 4.05). The lowest blood meal volume was observed post exposure to PermaNet for the VK7 strain (1.78µl 95% CI 1.04-2.51). For the Banfora strain, the average blood meal size was 2.89µl (95% CI 1.63- 4.13) after exposure to PermaNet and 2.16µl (95% CI 1.31-3) after exposure to Olyset net. There was no significant difference in the amount of blood ingested by either the Banfora or the VK7 strain post exposure to Olyset (Figure 5.5; Banfora P= 0.8725, VK7 P= 0.1182; Appendix 2.6; Table A2.5) or PermaNet net (Figure 5.5; Banfora P= 0.3571, VK7 P= 0.3557; Appendix 2.6; Table A2.5) compared with untreated net, in agreement with feeding permitted assays.

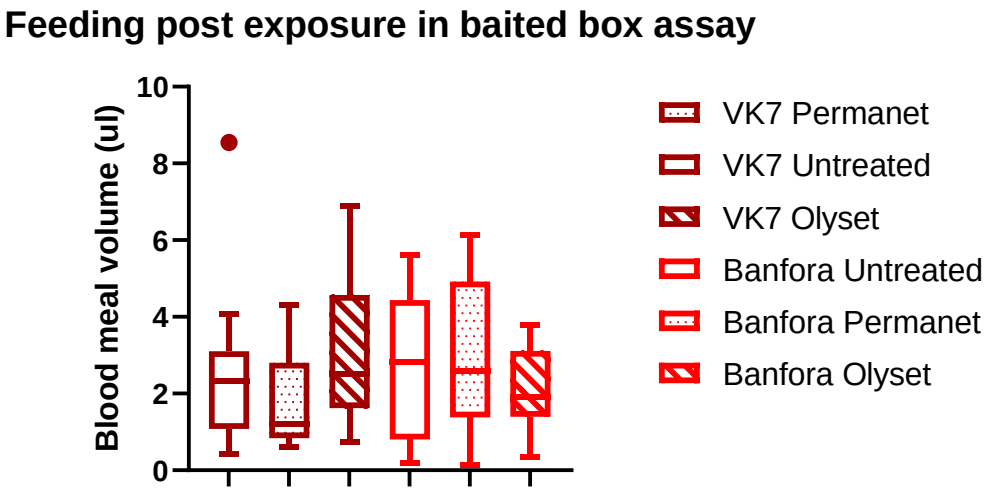


Figure 5.5: Blood meal volume (µl) when feeding 24-hours post exposure to a human baited net in baited box prevented feeding assays (Chapter 3), treated and untreated for two resistant strains of *An. gambiae* s.l. Mean blood meal volume fir each resistant strain exposed to each net type. Outliers are displayed as points and are classed as 1.5X the inter quartile range. Banfora exposed to Olyset net N= 9, PN2 N= 12 and Untreated N= 15. VK7 exposed to Olyset net N= 17, PN2 N= 13 and Untreated N = 16.

When exposed in video tracking experiments (Chapter 4) the average blood meal volume taken post exposure ranged from 1.8µl (95% CI 1.36-2.24) when Banfora fed post-exposure to Olyset net to 1.98µl (95% CI 1.67-2.29) post exposure to untreated net. VK7 ingested the largest blood meal on average after exposure to Olyset net (2.52µl 95% CI 2.24-2.79) and less after exposure to untreated net (1.81µl 95% CI 1.61-2.02). As with baited box feeding prevented assays, there was no significant difference in the amount of blood ingested by both the Banfora and the VK7 strain post exposure to Olyset net during these assays (Figure 5.6; Banfora P= 0.5651, VK7 P= 0.0763; Appendix 2.6; Table A2.5) compared with untreated net.

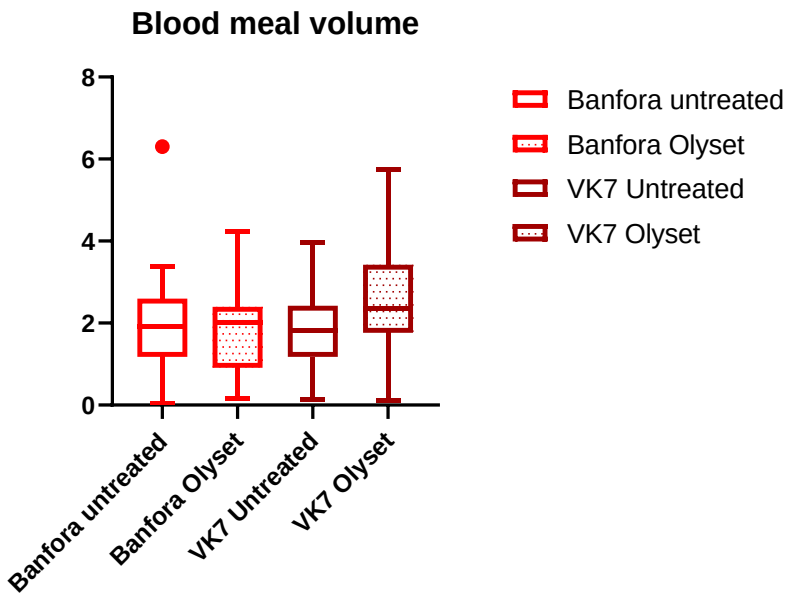


Figure 5.6: Blood meal volume (µl) when feeding after exposure to a whole human baited net, treated and untreated in video tracking experiments (Chapter 4), for two resistant strains of *An. gambiae s.l.* Mean blood meal volume for each resistant strain exposed to each net. Outliers are displayed as points and are classed as 1.5X the inter quartile range. Banfora exposed to Olyset N= 27, untreated N = 48. VK7 exposed to Olyset N= 72, untreated N= 72.

In all assays both strains ingested an average of between 1-3µl when exposed to all treatment types, however there was high variation in the data, with values ranging from 0.2ul up to 8.5µl.

Summary of Ingested blood meal volumes post net exposure:

- There was no significant difference in the amount of blood ingested by either the Banfora or the VK7 strain post exposure to PermaNet or Olyset net.

5.3.5 Fecundity and fertility

When exposed to untreated net egg production was variable between the different bioassays. Mosquitoes exposed in baited box feeding prevented assays had lower oviposition rates when exposed to untreated net than in feeding permitted version of the assay. In all baited box assays the oviposition rate was highest for the Kisumu strain (feeding permitted 70% and feeding prevented 61%). Banfora had the lowest oviposition rate when exposed to untreated net in both assays, with only 17% laying eggs when exposed in feeding prevented assays (Chapter 3) and 36% in feeding permitted assays (Chapter 2). N'gousso oviposition rate remained unchanged when exposed to untreated net in all assays ranging from 39%-44%. VK7 had the highest oviposition rate when feeding was permitted 55% compared with only 22% in feeding prevented assays. Although having the highest oviposition rates when exposed in both baited boxes, when exposed in video tracking experiments

(Chapter 4), Kisumu oviposition rate showed a trend towards reduction, reducing from between 61-70% to 55%.

Of those that laid; the mean number of eggs and larvae produced varied between strains and between treatment (Table 5.4, Appendix 1; Table A1.2 and Table A1.3). For all strains, when feeding was permitted during the assay, the rate of oviposition reduced post ITN exposure. As mortality of the susceptible strains was high following ITN exposure, the subsequent sample sizes were too small for further statistical analysis of fecundity and fertility effects. Although no statistical analysis could be performed, results show a trend of higher egg production in those susceptible mosquitoes that survive ITN exposure compared to untreated net.

When feeding was permitted through the net there was no significant difference in the number of eggs produced by VK7 post ITN exposure compared to an untreated control (Figure 5.7; Permanent 2.0 P= 0.449, Olyset P= 0.327; Appendix 2.10; Table A.2.9). Hatch rates were similar when VK7 was exposed to all three net types, ranging from between 30%-34% (Appendix 1; Table A1.2). Similarly, there was no significant difference in the number of eggs laid by Banfora post PermaNet exposure compared with untreated netting; however, the hatch rate dropped from 34%, when exposed to untreated netting, to 21% post-PermaNet exposure. Conversely, after exposure to Olyset net Banfora laid significantly fewer eggs (Appendix 1; Table A1.2, Figure 5.11; P= 0.005; Appendix 2.10; Table A.2.9), had a significantly lower hatch rate (3%) and therefore produced significantly less L1 larvae (Appendix 1; Table A1.2, Figure 5.7; P= 0.028; Appendix 2.10; Table A.2.9) compared with those that were exposed to untreated netting, indicating differential response to the two nets.

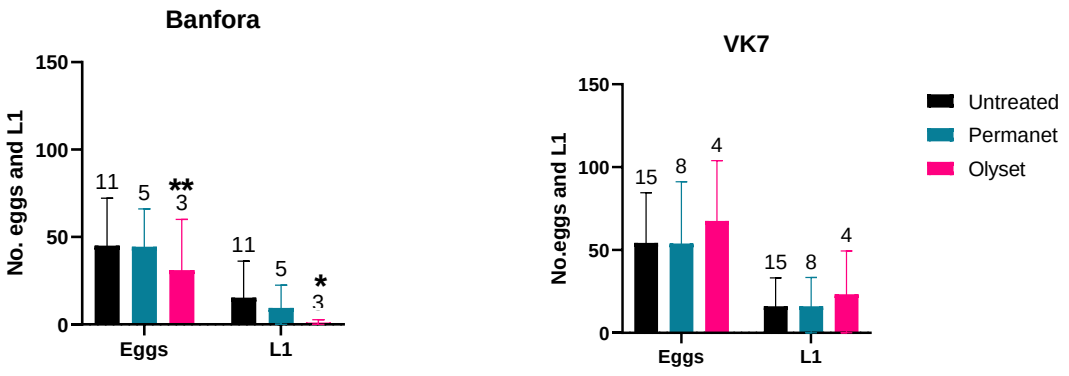


Figure 5.7: Fecundity and fertility of *An. gambiae s.l.* after exposure in baited box permitted feeding assays (Chapter 2) to PermaNet and Olyset net. Left: Resistant *An. coluzzii* Banfora strain. Right: resistant *An. coluzzii* VK7 strain. Mean number of eggs and L1 plotted with SD and N for each net type. Black: untreated, Green: PermaNet, Pink: Olyset.

For all strains, when feeding was prevented during the assay, the rate of oviposition was very low when exposed to all net types, again indicating a protective effect of a bloodmeal at the time of

exposure. For both resistant strains only four mosquitoes laid after exposure to the untreated control (Appendix 1; Table A1.3 and Figure 5.8). For this reason, no further statistical analysis was performed on this dataset.

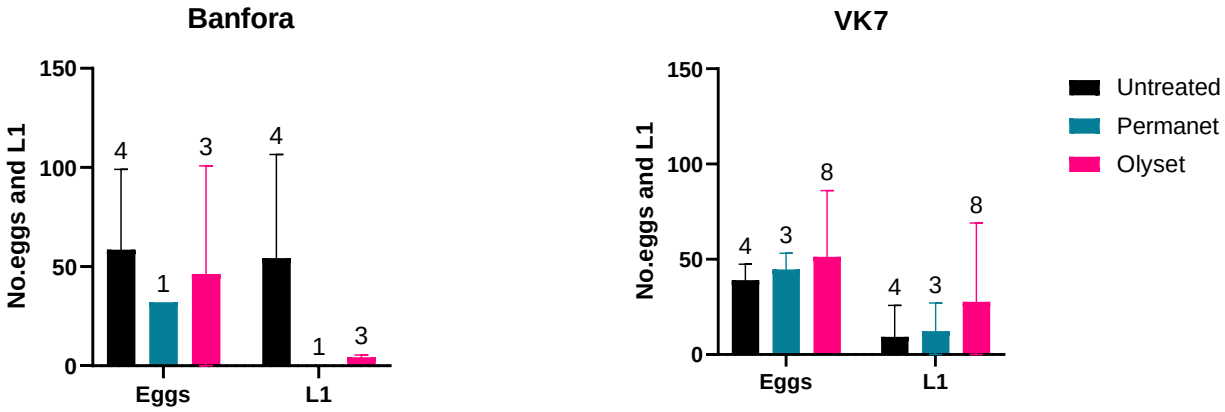


Figure 5.8: Fecundity and fertility of *An. gambiae s.l.* after exposure to Olyset net and PermaNet in baited box prevented feeding assays (Chapter 3). Left: Resistant *An. coluzzii* Banfora strain. Right: resistant *An. coluzzii* VK7 strain. Mean number of eggs and L1 plotted with SD and N for each net type. Black: untreated, Green: Permanent, Pink: Olyset.

For video tracking experiments (Chapter 4) there was no significant difference in the number of eggs produced by VK7 and Banfora post ITN exposure compared to an untreated control (Table 5.4, Figure 5.9; VK7 P= 0.351, Banfora P= 0.235; Appendix 2.11; Table A2.10). Therefore, there was no difference in oviposition rate for both resistant strains when exposed to untreated compared to treated net. On the other hand, significantly more larvae hatched post exposure to Olyset compared with untreated (Table 5.6, Figure 5.9; VK7 P= 0.015, Banfora P= 0.0056; Appendix 2.11; Table A2.10). Banfora and VK7 had between 19% and 28% more eggs hatch after exposure to Olyset compared to an untreated net. This is an interesting observation, suggesting that sublethal exposure to the Olyset net has an hormetic effect on these highly resistant strains.

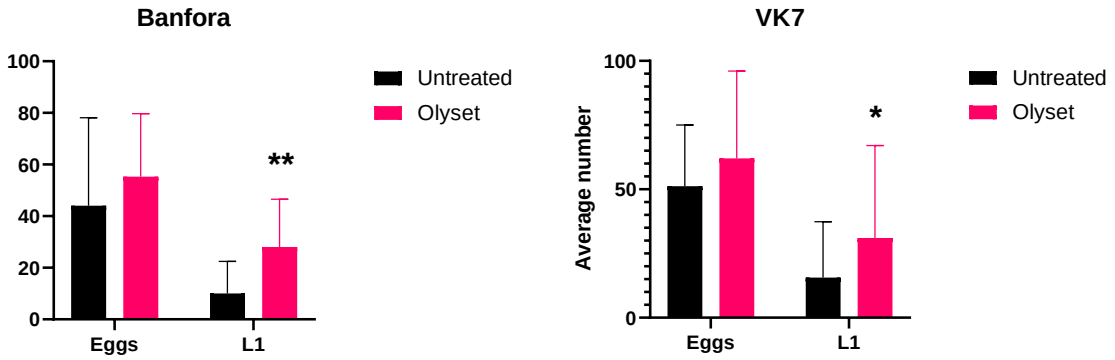


Figure 5.9: Fecundity and fertility of *An. gambiae s.l.* after exposure to Olyset net in video tracking experiments (Chapter 4). Left: resistant *An. coluzzii* Banfora strain. Right: resistant *An. coluzzii* VK7 strain. Average number of eggs and L1 plotted with SD for each net type. Black: untreated, Pink: Olyset.

Table 5.4: Summary table of sub-lethal effects data for all blood fed mosquitoes exposed in video tracking experiments (Chapter 4). N = number of mosquitoes that oviposited. Table shows fecundity; the total number of eggs, mean per female. Fertility; mean number of first instar larva 5 days post oviposition with SD, percentage hatch rate.

Strain	Treatment	No. fed	N	Fecundity		Fertility	
				Number eggs laid	Mean number of eggs/female (SD)	Mean number of first instar (SD)	Hatch rate (%)
Kisumu	Untreated	101	56	3442	62 (29.5)	42 (29.52)	68%
	Olyset	2	0	-	-	-	-
N'gousso	Untreated	95	37	1632	44 (24.81)	15 (15.52)	34%
	Olyset	2	1	88	88 (0)	40 (0)	46%
VK7	Untreated	91	38	1945	51 (23.88)	16 (21.77)	31%
	Olyset	95	36	2232	62 (34.04)	31 (35.96)	50%
Banfora	Untreated	55	22	969	44 (34.1)	10 (12.47)	23%
	Olyset	41	14	774	55 (24.38)	28 (18.47)	51%

Summary of fecundity and fertility results post ITN exposure:

- Although sample size was small, after exposure in the permitted feeding baited box assay (Chapter 2) Banfora laid significantly less eggs and had a significantly lower hatch rate (3%) producing significantly less L1 larvae compared with those that were exposed to untreated netting.

- When exposed in the baited box feeding prevented assay, the rate of oviposition was very low when resistant strains were exposed to all net types.
- After exposure in video tracking experiments significantly more larvae hatched post exposure to Olyset compared with untreated for both VK7 and Banfora strain.

5.3.4 Longevity

As exposure to a treated net significantly impacted the immediate survival of the susceptible strains in all assays, no further analysis was performed to assess the longevity impacts of ITN exposure on the Kisumu and N'gousso strains (Table 5.1).

When exposed to untreated net in feeding permitted assays (Chapter 2) the median survival of Banfora and VK7 was 12 and 11 days respectively. This was similar to that observed in feeding prevented assays (Chapter 3), where the median survival of Banfora was 14 days and 11 for VK7. When feeding was permitted there was no significant effect of treatment on the survival of Banfora and VK7 (Appendix 1; Table A1.4, Figure 5.10; Banfora P =0.35, VK7 P=0.2; Appendix 2.7; Table 2.6); in agreement with previous observations in highly resistant populations from Burkina Faso (Hughes *et al.,* 2020).

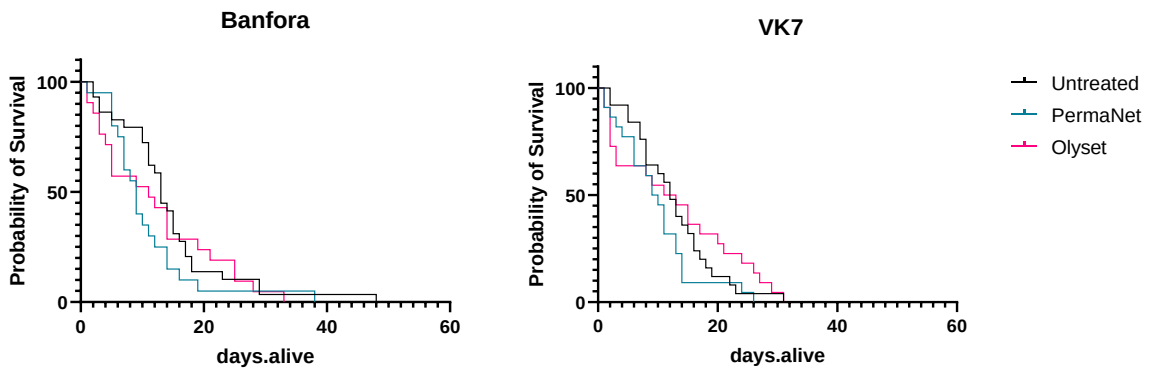


Figure 5.10: Effect of different net treatments on survival of two strains of *An. gambiae s.l.* following exposure in permitted feeding baited box assays (Chapter 2) to ITNs. Left: Resistant *An. gambiae s.l.* Banfora strain. Right: resistant *An. gambiae s.l.* VK7 strain. Percentage survival is plotted against number of days survival. All three net types are plotted for each mosquito strain. Black: untreated, Pink: Olyset, Green: PermaNet 2.

As in the feeding permitted assays, when feeding was prevented during exposure the resistant strains survived longer when exposed to treated netting than the susceptible strains (Chapter 3 and Appendix 1; Table A1.5). There was no significant effect of treatment on the overall survival of Banfora or VK7 that had both fed and not fed (Appendix 1; Tale 1.5; Banfora P =0.278, VK7 P=0.385). Comparative survival analysis was not possible when divided into those that fed and those that did not feed as the

sample size was too small in the non-fed arm to do any further analysis (Appendix 1; Table A1.1 and Table A1.5, Figure 5.8). Of those that fed at 24-hours there was no significant difference in the survival of those exposed to treated net compared with the control netting (Figure 5.11; VK7 P=0.558, Banfora P=0.156; Appendix 2.8; Table A2.7).

For mosquitoes that fed during the test in feeding permitted assays (Chapter 2) and fed at 24-hours after exposure in feeding prevented assays, the median survival was similar on all treatments for both strains. When exposed to untreated median survival for all four strains was between 10-14 days. When exposed to PermaNet median survival for the resistant strains in both assays decreased to 8 days on PermaNet with the exception of Banfora in feeding prevented assays. For Olyset net, median survival of both strains was between 10 and 11 days. Therefore, results suggest that there was no effect of feeding time on the average survival of mosquitoes, with similar survival rates between those that fed during exposure vs 24-hours post-exposure.

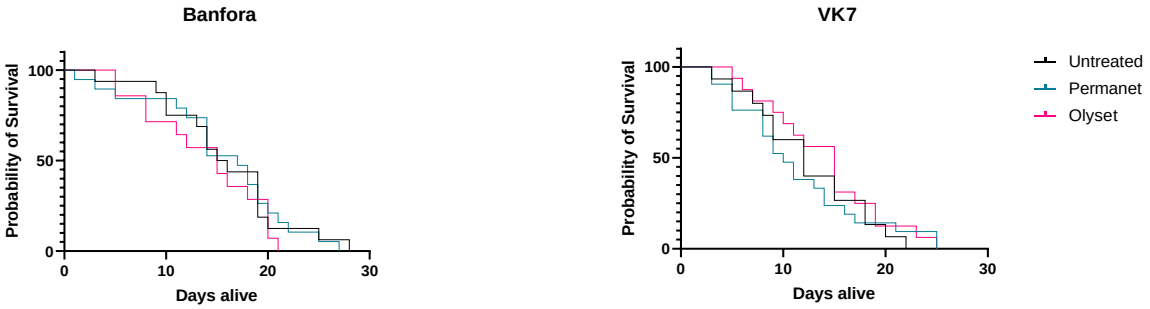


Figure 5.11: Effect of three net types on survival of two resistant strains of *An. gambiae s.l.* fed 24-hours following exposure to ITNs in feeding prevented baited box assays (Chapter 3). Left: resistant *An. gambiae s.l.* Banfora strain. Right: resistant *An. gambiae s.l.* VK7 strain. Percentage survival is plotted against number of days survival. All three net types are plotted for each mosquito strain. Black: untreated, Pink: Olyset, Green: PermaNet 2

For video tracking experiments there was a significant effect of treatment, reducing the average survival of Banfora and VK7, when feeding status was not accounted for (Appendix 1; Table A1.6; Banfora P =0.0015, VK7 P=0.0022). The difference in survival was compared for mosquitoes that had fed and those that had not fed post net exposure. There was no significant difference in survival of the resistant strains that fed at 1-hour and 24-hours post-exposure (P= 0.329). Therefore, all bloodfed mosquitoes were combined for analysis. Mosquitoes that fed post-exposure to Olyset net survived significantly longer than those that did not feed (P<0.0001), indicative of a protective effect of taking a bloodmeal. Those that didn't take a blood meal the average survival was 3-4 days, but those that fed, the average survival was between 8-12 days. Therefore, if mosquitoes do not feed during

exposure or 24-hours afterwards, this will reduce survival to below the extrinsic incubation period of the malaria parasite and ultimately reduce transmission.

Between 32%-69% percent of resistant mosquitoes took a blood meal after exposure to Olyset net and 62%-98% after exposure to untreated net. Of those that fed by 24-hours there was no significant difference in the survival of those exposed to treated net compared with the control netting (Appendix 1; Table A1.7; Figure 5.12; VK7 P= 0.332, Banfora P=0.0565; Appendix 2.9; Table A2.8). When survival analysis was divided into those that fed and those that did not feed all VK7 exposed to control netting fed post exposure, therefore no further analysis was possible on this dataset (Appendix 1; Table A1.7 and A1.8). Of the Banfora that did not take a blood meal, those exposed to Olyset net lived for significantly fewer days compared with those exposed to untreated net (Appendix 1; Table A1.8, Figure 5.13; P<0.0001; Appendix 2.9; Table A2.8).

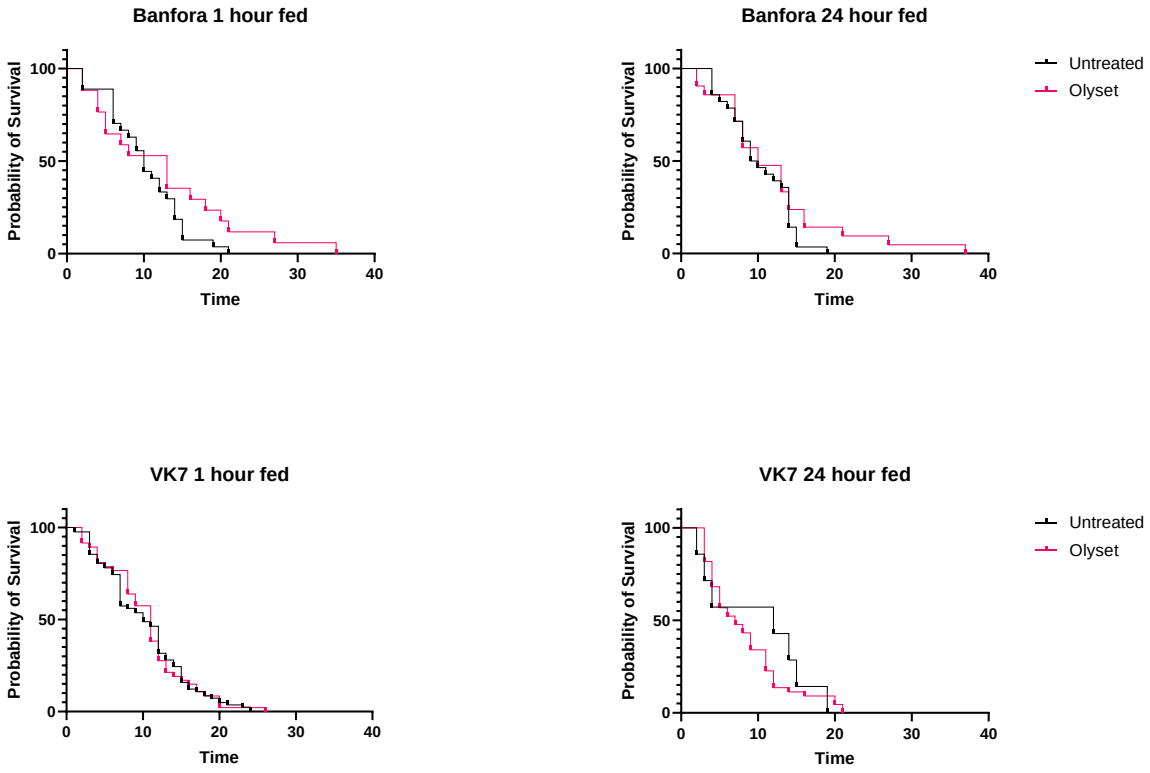


Figure 5.12: Effect of three net types on survival of two resistant strains of *An. gambiae s.l.* fed at 1-hour and 24-hours following exposure to ITNs, exposed in video tracking experiments (Chapter 4). Top: resistant *An. gambiae s.l.* Banfora strain. Bottom: resistant *An. gambiae s.l.* VK7 strain. Percentage survival is plotted against number of days survival. All three net types are plotted for each mosquito strain. Black: untreated, Pink: Olyset, Green: PermaNet

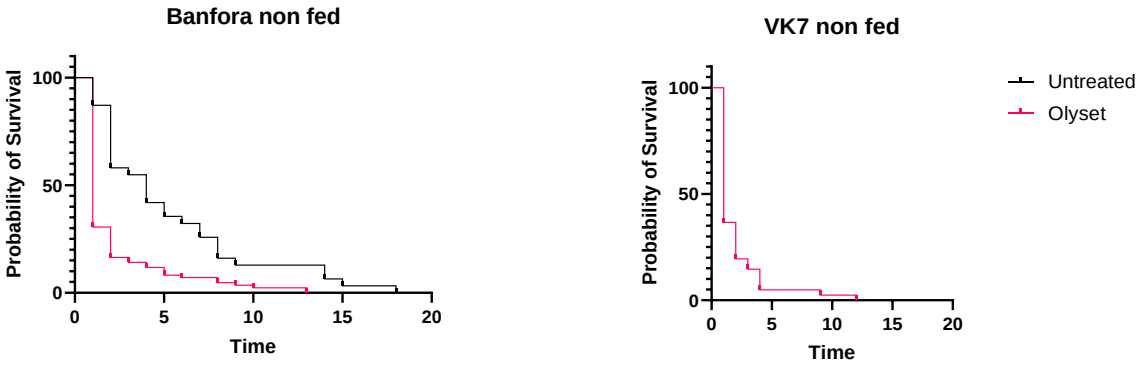


Figure 5.13: Effect of different net treatments on survival of four strains of *An. gambiae s.l.* which did not take a blood meal at 24-hours following exposure to ITNs, exposed in video tracking experiments (Chapter 4). Left: resistant *An. gambiae s.l.* Banfora strain. Right: resistant *An. gambiae s.l.* VK7 strain. Percentage survival is plotted against number of days survival. All three net types are plotted for each mosquito strain. Black: untreated, Pink: Olyset.

Summary of longevity results:

- When feeding was permitted during the baited box assay there was no significant effect of treatment on the subsequent survival of Banfora and VK7.
- When exposed in baited box feeding prevented assays there was no significant effect of treatment on the overall survival of Banfora or VK7.
- When exposed in video tracking experiments, analysis was split into those that had fed and not fed post ITN exposure.
    - For mosquitoes that had fed by 24-hours there was no significant difference in the survival of those exposed to treated net compared with the control netting
    - For mosquitoes that had not fed, analysis could not be performed for VK7 strain. Banfora that did not take a blood meal lived for significantly less days when exposed to Olyset compared to untreated net.

5.4 Discussion

This chapter has demonstrated the importance of measuring delayed and sublethal effects beyond 24-hours post exposure to insecticide treated nets, in order to assess a bednets overall effectiveness against malaria transmission. The present study showed how contact with sublethal doses of pyrethroids can impair the mosquito's ability to bloodfeed in some mosquito strains and decrease longevity in those that had not fed.

The standard pyrethroid-treated nets proved to be extremely effective against the susceptible *Anopheles* strains (Kisumu and N'gousso), and when exposed to Olyset in video tracking experiments the net induced significantly higher mortality rates for both resistant mosquito populations compared to untreated net. Exposure in the field occurs when feeding through a net (the host is against the net surface) or post exposure when unable to reach the host behind the net. To first evaluate the

differences observed during each exposure scenario we can compare the results after exposure to the control net. When exposed in all assays different control mortalities were observed in each test, between <5% in feeding permitted assays, <10% in feeding prevented assays and ≤20% in tracking assays for all strains. all assays had mortality levels below 20% which is the acceptable level of control mortality in standard WHO tests. Interestingly higher rates of mortality were observed in susceptible strains (Kisumu and N'gousso, 11% and 18%, respectively) than in resistant strains (Banfora and VK7, 5% and 3% respectively) after exposure in video tracking experiments, these effects may be attributed to greater levels of feeding after exposure in the resistant strains, as feeding at 1-hour may provide a fitness advantage. Previous studies have shown that the blood meal can increase the lifespan of a mosquito (Hughes *et al*., 2018; Nayar *et al*., 1975; Xue *et al*., 2018)

As expected, immediate mortality of both susceptible mosquito strains, Kisumu and N'gousso, was high post ITN exposure. Survival of both strains was higher when exposed to Olyset compared to PermaNet in both baited box assays, with the 24-hour mortality on treated nets ranging between 57%-100%. Immediate mortality in the baited box assay was lower than expected for the susceptible strains when exposed to Olyset net, and to some extent PermaNet. Survival was higher in the feeding permitted assays most likely due to the protective effect of feeding through the net opposed to feeding 24-hours after exposure. The current study showed lower mortality when exposed to treated net in baited box (Table 5.1, Chapter 5; 57%-95%) than exposure in WHO cone tests performed in the same group at the same time for both susceptible strains (results ranged between 94%-100% mortality Emery *et al*., 2019; Foster *et al*., unpublished). The results imply that exposure to nets in forced contact bioassays such as the cone test may enhance the lethal effects of ITNs, as it causes forced, uninterrupted periods of insecticide contact, that is not representative of exposure in field situations. The baited box assay allows the mosquito to make more 'natural' contact with the treated net. Immediate mortality was highest when all strains were exposed in the video tracking experiments. Mortality of both resistant strains rose from below 20% when exposed during baited box assays to between 20% and 48% when exposed to Olyset in video tracking experiments. Results are similar to that observed in experimental hut trials on Olyset net. The results demonstrate the importance of exposure to treated nets in a more 'natural' conditions to gain a full understanding of the how ITNS perform in the field (Koffi *et al*., 2015; N'Guessan, *et al*., 2008; Ngufor *et al*., 2014; Pennetier *et al*., 2013). Results support the use of the video tracking system for evaluation of the effectiveness of ITNs as a control tool.

Discussions so far have covered the results that could be expected from standard monitoring practices. However, this thesis looked at gaining an overall picture of ITN effectiveness, evaluating all delayed and sublethal effects post exposure that most standard tests miss, therefore all results

discussed from this point on would not be routinely captured by the current standard protocols. Some of the testing procedures discussed in this chapter used individual mosquitoes, which allowed determination of correlations and trends between duration of ITN contact and the subsequent impacts on longevity and fecundity of that exposure.

Bloodfeeding was assessed when directly in contact with an ITN, as might happen in the field if the host was resting against the side of the bednet, and post ITN exposure when entering through a hole of a damaged net or leaving the net to bite another unprotected host. When feeding was permitted through the net all strains regardless of their resistance status were able to take a blood meal. This has been shown previously when susceptible *An. gambiae s.l.* were exposed to Olyset Plus (Hauser *et al.,* 2019), however mortality effects post exposure to Olyset and PermaNet in this study exceed that of the previous study. Hauser *et al.,* (2019) suggest that the low mortality observed could with be due to the irritant effects of Olyset plus causing avoidance or they suggest that the blood meal itself may mitigate the toxicity of the insecticide. However there also some questions regarding the durability of PBO nets and perhaps this also contributed to the lower mortality levels (Gleave *et al*., 2018).

Despite the ability of the mosquito to feed through an ITN in baited box assays, video tracking experiments have shown ITNs are extremely effective in preventing mosquito bloodfeeding. Previous testing with the system displayed rapid decay of host seeking activity in susceptible *An. gambiae s.l.* within the first ten minutes of the assay (Parker *et al.,* 2015), similar to that observed in Chapter 4. However, this result was only observed for susceptible stains of *Anopheles*, and therefore may be the result of insecticide-induced knockdown or mortality.

Post exposure to ITNs the number of mosquitoes able to take a blood meal varied between strains, assays and time of feeding. Bloodfeeding inhibition after exposure to treated nets is something that has been observed previously (Agossa *et al*., 2014; Chandre *et al*., 2010; Glunt *et al*., 2018; Malima *et al*., 2008). Glunt *et al*. (2018) observed differences in feeding rates between strains that had different resistant mechanisms and intensities of resistance. In agreement with this, the current study observed differences in bloodfeeding inhibition post ITN exposure between the two resistant strains. In baited box assays there was a trend for less mosquitoes to take a blood meal after exposure to Olyset compared to PermaNet and untreated. A significant decrease in feeding rates was observed in the Banfora strain at 24-hours post exposure to Olyset net (P= 0.038). In video tracking experiments bloodfeeding inhibition was observed at 1-hour post exposure for the VK7 strain however this effect is lost over time with 74% of the mosquitoes feeding at 24-hours. The results of this study show how first-generation nets are affecting bloodfeeding ability immediately after exposure for some resistant strains. This is likely contributing to the ongoing effectiveness of standard pyrethroid nets even in

areas of resistance, as results suggest that exposure to sublethal doses of pyrethroids could reduce the chances of the mosquito being diverted and feeding on an unprotected host. However, results do show the effects of pyrethroid exposure on bloodfeeding behaviour is short lived, as observed by others previously (Glunt *et al*., 2018).

Although in the previous chapter (Chapter 2) overall duration of bloodfeeding was observed to be significantly reduced when feeding through a treated net, the amount of blood ingested was not significantly different for both resistant mosquito strains with the exception of VK7 feeding through PermaNet. When feeding through the PermaNet VK7 ingested a higher volume of blood compared to when feeding through untreated net. Although this has only been shown for the resistant strains, the findings strongly suggest the ability of a mosquito to feed at a faster rate when in the presence of an insecticide.

The reduction in feeding duration could have a subsequent effect on the spread of malaria, potentially affecting the number of sporozoites delivered to a host (Jin *et al.,* 2007). Despite this, some studies have suggested that sporozoites are released within the first few minutes of feeding (Frischknecht *et al.,* 2004) and therefore even though duration of feeding is shorter this would not directly affect disease transmission. Although not observed in the current experimental set up reduced feeding has also been associated with an increase in re-feeding on another host, suggesting a major impact on transmission potential (Edman *et al*., 1975; Jackson *et al*., 2012; Sugiharto *et al*., 2016). A deeper understanding of the factors that determine parasite transmission during mosquito feeding is required to enable us to understand in greater detail the effects this may have on subsequent disease spread. Unfortunately, the methods used in the current study were ineffective in measuring blood meal volumes within the susceptible population. This was due to low survival and therefore digestion rates of these strains post ITN exposure (1%-16% survival post ITN exposure).

Further studies may also identify if this is an adaptive response to the insecticide in only those mosquitos' resistant to pyrethroids. Conversely, Hauser *et al.* (2019) found that a reduction in blood meal duration resulted in a reduction in the amount ingested when susceptible strains were exposed to Olyset plus (Hauser *et al.,* 2019), suggesting there could be a difference in feeding behaviours between the susceptible and resistant strains.

Despite the efficacy of these treated nets against the susceptible strains, long-term survival of both pyrethroid resistant *An. gambiae* was generally not affected by ITN exposure. In disagreement, previous studies have shown contact with ITNs to reduce the immediate survival of moderately resistant *An. gambiae* but to further reduce overall life spans by one-half (Viana *et al.,* 2016). However more recently WHO cone tests, tube bioassays and experimental hut trials using mosquito strains the

same or with similar resistance status to those used in the current study, have shown the delayed effects on longevity from ITN exposure to be absent in those field populations highly resistant to pyrethroids (Hughes *et al.,* 2020). Hughes *et al.* (2020) exposed VK7 and Banfora with 24-hour post-exposure showing low levels of mortality. They observed no delayed mortality post ITN exposure for the VK7 strain but did observed delayed mortality in the Banfora strain. However, as cone tests do not mimic the natural exposure a mosquito has with a baited ITN the study also used experimental hut trials, concluding that when exposed in a more realistic setting to the ITN, no difference between the longevity of mosquitoes exposed to ITNs or control nets was observed.

In the current study no difference in longevity was observed for both resistant strains when exposed to ITNs in the baited box compared to untreated net. In the case of the video tracking experiments, immediate mortality increased by exposure to treated nets when both strains were exposed to Olyset. Of those that survived past 24-hours post exposure, a reduction in longevity was only observed in those mosquitoes that did not take a blood meal. Once the mosquito had bloodfed no insecticidal effects of Olyset on longevity were observed. In agreement to this Hughes *et al.* (2020) also concluded that feeding status had a significant effect on the longevity of mosquitoes.

The time of the blood meal after ITN exposure also had no effect on survival and there was no significant difference in survival of the resistant strains that fed at 1-hour and 24-hours post ITN exposure. Mosquitoes that fed post exposure to Olyset net did survive significantly longer than those that did not feed post exposure ($P<0.0001$), indicative of a protective effect of taking a bloodmeal, as suggested in previous studies (Hauser *et al*., 2019; Hughes *et al.,* 2020; Oliver *et al.,* 2014; Oliver *et al.,* 2016; Spillings *et al.,* 2008).

Of those mosquitoes that survived exposure and took a blood meal the effects of ITN exposure on fecundity and fertility were measured.

Overall oviposition rates for all *Anopheles* sp. ranged between 39% – 59% when exposed to untreated net in video tracking and 17% to 70% in baited box assays. Higher egg laying rates were observed in the *Anopheles gambiae* Kisumu strain that has been colonised in LSTM for the longest, suggesting that lower oviposition rates may have been caused by the artificial environment. As the Kisumu strain is *An. gambiae* and others *An. coluzzii*, results could also suggest that there are strain differences in oviposition rates. Further work using a larger range of strains would be needed to conclude these findings.

When exposed to ITNs in baited box assays the sample size of those that laid post-ITN exposure was low. Although egg production was low for both strains when feeding was prevented in the baited box,

there was a trend for oviposition rate to be higher when exposed to Olyset compared to untreated, a trend that was also apparent in the video tracking dataset. Higher oviposition rates were generally observed following ITN exposure in the feeding permitted assays, compared to when feeding occured post ITN exposure, again showing the importance of natural exposure on feeding behaviour and mosquito life history traits. Although results could be accounted for by the artificially restricted feeding time post net exposure in feeding prevented assays, feeding during exposure to the insecticide may have provided a protective effect, therefore the mosquito experiences less sublethal effects post ITN exposure.

Hatch rates also varied between tests and when exposed to Olyset in video tracking experiments both resistant strains had higher hatch rates than the untreated. When exposed in the baited box, Banfora's hatch rates reduced to between 3-9%, something not observed for the VK7 strain.

It is important to consider the difference between both exposure scenarios which may occur in endemic settings (feeding during and post ITN exposure) to gain a full understanding of how nets will perform as part of a control programme.

5.5 Conclusion

In conclusion, while the PermaNet and Olyset net remained extremely effective against both susceptible strains of *An. gambiae*, the results showed that the efficacy of both net types was much lower against both insecticide resistant strains. The insecticidal impacts did not have any lasting effect on biting rates 24-hours post exposure and blood meal engorgement was not affected, with resistant mosquitoes feeding at a faster rate through both treated nets. No long-term effects of the insecticide exposure were observed for both resistant strains with increased fecundity after exposure to Olyset net. Finally, taking a blood meal had protective effects on the mosquito's post-exposure to permethrin and deltamethrin treated nets with only those that did not feed suffering longevity impacts. Results point to bednets still having some immediate impacts in the field on resistant mosquito populations but no long-terms effects on life history traits.

Chapter 6: General Discussion

Insecticide treated nets are an extremely effective tool against malaria (Pryce *et al*., 2018) and their scale up across Africa is thought to have been the main driver in the dramatic reductions in malaria cases seen since the beginning of the century (Bhatt *et al.,* 2015). However, the drop in malaria cases has plateaued in recent years (WHO, 2020) and there is debate about the role of the emergence of insecticide resistance to pyrethroids used in ITNs in this slowdown (Lindsay *et al.,* 2021).

There is clear evidence from WHO tests and experimental hut studies that ITNs are losing efficacy in areas with high resistance (Asidi *et al*., 2012; Churcher *et al*., 2016; N'Guessan *et al*., 2007; N'Guessan, *et al*., 2007; Pennetier *et al*., 2013; Toé, 2015) Transmission models indicate that the higher levels of resistance, the greater the impact this will have on the number of clinical cases (Churcher *et al*., 2016). However, the extent to which pyrethroid resistance is already contributing to an increase in malaria transmission is still unknown (Lindsay *et al.,* 2021).

Some epidemiological evidence indicates that pyrethroid treated nets do still protect (Alout *et al*., 2017; Bradley *et al*., 2017; Lindblade *et al*., 2015; Ochomo *et al*., 2017) and continue to be an effective control tool against malaria vectors even in the presence of resistance. Experimental hut trials have shown that standard ITNs are still more effective against resistant mosquitoes than untreated nets (Pryce *et al*., 2018; Strode *et al*., 2014).

Insecticide resistance reduces the immediate efficacy of standard ITNs, however changes in behaviour, or delayed or sublethal effects post ITN exposure, may explain why standard nets are not entirely ineffective even when immediate mortality is lost. Glunt *et al* (2017) displayed how sublethal exposure to an insecticide treated net caused a reduction in bloodfeeding rates and host-seeking behaviour of resistant mosquitoes. Delayed mortality after ITN exposure has also been demonstrated using pyrethroid-resistant colonies (Viana *et al*., 2016), however this finding was not replicated using highly resistant populations from Burkina Faso (Hughes *et al*., 2020). Indeed, evidence is growing that standard ITNs are losing their efficacy in areas of high resistance prompting many countries to include 'next generation' nets in their recent national distribution programmes.

In order for countries and purchasers to make informed decisions about which class of net to use in a given setting, data on the full range of phenotypic effects induced by ITN exposure, and how these are impacted by insecticide resistance, is needed.

In addition to physiological resistance concerns have also been raised about behavioural resistance (Gatton *et al.,* 2013) with many studies have suggested behavioural changes are associated with

widespread bednet use (Gatton *et al.,* 2013; Govella *et al*., 2010; Killeen *et al*., 2016; Russell *et al.,* 2011) resulting in lower house entry (N'Guessan *et al*., 2001; Siegert *et al*., 2009; Sharma *et al*., 2009; Soleimani-Ahmadi *et al.,* 2012) and altered feeding patterns (Russell *et al*., 2011). Changes in host-preference (e.g. a switch to biting non-human animals) has also been hypothesised as a behavioural resistance mechanism, caused by the increased pressure of ITN use (Tirados *et al.,* 2006).

Currently there is a lack of evidence to assess whether these behavioural resistance traits are genetic or adaptive responses to these control tools (Gatton *et al*., 2013). Without this understanding it is harder to monitor changes in behavioural traits and subsequently implement surveillance programs. Ultimately this knowledge gap makes behavioural resistance very difficult to target and evaluate with the current control tools. There is ultimately a need to look at behavioural changes in response to ITN exposure.

To evaluate the effectiveness of ITNs the WHO has standard test systems. Although these were thought to be adequate when testing the fast knock down effect of pyrethroids (for which they were originally designed), the tests fail to measure beyond 24-hour mortality effects. Not only is this important to assess the overall impacts of standard ITNs but the insecticidal impacts of next generation nets may be underestimated using these tests due to more complex modes of action and more delayed mortality effects. To accelerate the search for new control tools using novel chemistries and to evaluate their entomological modes of action there is now a need for new bioassays that measure and monitor insecticide impacts where the current testing methods fall short.

This thesis investigated the effects of ITNs on insecticide susceptible and resistant mosquito host seeking and bloodfeeding behaviour. A new benchtop bioassay 'the baited box' (Chapters 2 and 3) was used to describe and quantify short range host-seeking and bloodfeeding behaviours of *Anopheles* at a baited ITN interface, using standard pyrethroid only Olyset and PermaNet 2.0 ITNs. The room scale video tracking system (Chapter 4) was used to analyse longer range host-seeking at whole human baited Olyset net. Delayed and sublethal impacts resulting from insecticide exposure in each assay were measured by following up mosquitoes surviving the first 24-hour post exposure (Chapter 5) to quantify impacts of natural insecticide exposure on longevity, blood-feeding and reproductive output. The suitability of these testing methods for evaluating the efficacy of ITNs and their potential as a future screening tool is discussed in the current chapter.

The impacts of pyrethroids on mosquito behaviour

Results from this thesis showed that the general profile of behaviour around a human baited ITN is similar for both pyrethroid resistant and susceptible mosquito strains. All strains responded to the

baited net and are able to take a blood meal through all net types. However, overall contact duration was reduced when exposed to ITNs compared to untreated nets.

Permethrin treated nets displayed varied levels of repellent and irritancy effects depending on the mosquito's resistance status, with this net having the lowest contact times in all assays compared to other net types. In agreement with other studies, exposure to the Olyset net (permethrin) resulted in higher levels of contact irritancy than PermaNet (deltamethrin) and untreated netting (Hodjati *et al*., 2003; Hougard *et al*., 2003; Siegert *et al*., 2009). Mosquitoes spent the least amount of time feeding through and in contact with the Olyset net, as well as being more likely to disengage from this net earlier than untreated and deltamethrin treated nets. Results indicated that deltamethrin-treated ITNs are not repellent to host seeking mosquitoes.

In this thesis both repellency and contact irritancy were distinguishable using the three assays combined. How ITNs might deter mosquitos was assessed as this was considered an important factor when evaluating overall performance of a bednet.

Although repellency of a net may reduce house entry rates, it also reduced the chances of a mosquito receiving a lethal dose of insecticide, therefore reducing the lethality of this control and potentially diverting the mosquito to an unprotected host, ultimately reducing the community protection from this intervention.

Many studies have suggested that exposure to pyrethroid coated ITNs cause spatial repellency effects prior to contact for both *Aedes* and *Anopheles* mosquitoes (Achee *et al*., 2009; Liu *et al*., 2021; N'Guessan *et al*., 2001; Soleimani-Ahmadi *et al.,* 2012). Lui *et al* (2021) suggested that olfactory receptors neurons in the antennae of the mosquito detect pyrethrum and hyper-activation of sodium channels enhances the repellency effect. However, in agreement with some previous work, results from all three bioassays used in this thesis showed no evidence to suggest spatial or close range repellency effects of deltamethrin and permethrin impregnated nets on behaviour (Hughes *et al.,* 2020; Parker *et al*., 2015; Parker *et al.,* 2017).

Although short-range repellency was not observed to both net types in all assays, all three tests revealed a reduction in contact duration when mosquitoes are exposed to pyrethroid coated ITNs. Differences in the irritancy effects of different pyrethroids has been described previously (Hodjati *et al*., 2003; Siegert *et al*., 2009) and was observed in tests using the baited box which revealed earlier disengagement or time-before take-off with Olyset nets compared to PermaNet 2.0. This is a major concern for the success of ITNs, as mosquito exhibiting avoidance behaviour before being exposed to a lethal dose of insecticide reduces the overall community protection provided by this control. The

current study reveals that when susceptible mosquitoes are exposed to pyrethroid nets in the baited box where they are able to avoid the nets, 24-hour mortality does not reach 100% as would be expected. Having bioassays that enable us to understand the magnitude of this avoidance behaviour is imperative for the development of future control tools.

The baited box revealed that when feeding through a treated net all mosquitoes regardless of their resistance status spent less time in contact with the ITN compared to untreated. The findings from Chapter 2 using the baited box assay, revealed surprisingly similar bloodfeeding behaviour through the ITN between all four strains. However, when unable to reach the host behind the net the amount of time spent in contact with ITNs did differ between the strains. In the feeding prevented assays, the basic sequence of events was similar for both susceptible strains and Banfora when exposed to an untreated net. Although VK7 exhibited little contact with the control netting. When exposed to the ITN in the feeding prevented assay both susceptible strains contact with the net reduces after the initial 8-10 minutes eventually reaching only ~1/3 level seen at untreated nets supporting the fact that bednets are a lethal trap with exposure to the treated net effecting host seeking ability within 10 minutes. As this strain is susceptible to insecticides this effect is most likely due to knockdown of the mosquitoes in the test. However, when the resistant strains were exposed to the treated net, there was no significant difference in the behaviour profile (VK7 exposed to PermaNet and Olyset and Banfora exposed to PermaNet) compared to exposure to an untreated net (with the exception of Banfora exposed to Olyset net). Results suggest that despite some variability, the nets do not have a drastic effect on reducing the host seeking ability of these strains compared with susceptible strains.

Whilst Banfora and VK7 are both resistant to pyrethroids, subtle differences were observed in their behaviours when exposed to the two pyrethroid nets in the baited box. In baited box experiments where feeding was prevented the VK7 response rate was highest when exposed to PermaNet compared to the other net types, with this strain responding more to the bait behind the treated nets than untreated. However, after the first contact was made with the net, the VK7 strain displayed little contact behaviour with all net types compared to the other strains.

There was no difference in the response rates for the Banfora strain when exposed to all net types in baited box experiments. When feeding was prevented in the baited box assay, Banfora displayed a similar sequence of behaviour when exposed to PermaNet and untreated net, but in the same assay displayed remarkably similar behaviour to the susceptible strains when exposed to Olyset net, with obvious irritancy effects of this net type (Chapter 3, Figure 3.5).

The differences between Banfora and VK7 may be attributed to differences in the underpinning resistance mechanisms. Pyrethroid resistance in VK7 appears to be largely conferred by target site

resistance and elevated pyrethroid metabolism whereas in the Banfora strain pyrethroid resistance is conferred by these two mechanisms plus also increased rates of respiration and possibly also a contribution from the microbiome (V Ingham, personal communication). During the course of the study, pyrethroid resistance in the Banfora strain was unstable, possibly due to fitness costs associated with higher respiration rates, meaning this can be lost without regular selection pressure. In addition, if the mosquito microbiome plays a role in this strain's resistance, changes in rearing or environment may affect the resistance profile of this strain. The VK7 strain in comparison was profiled every year and genotyped every 6 months at LSTM showing that the resistance in this strain remained stable throughout the study (Williams *et al.,* 2019)

In baited box assays both susceptible strains spent less time probing through a treated net compared to an untreated net. This behaviour was also observed in the Banfora strain but only when exposed to Olyset net. Reduced probing has been observed in previous studies (Hauser *et al.,* 2019). Reducing the amount of probing time through the net subsequently reduced the amount of overall contact the mosquito has with the net therefore reducing the chances of being exposed to a lethal dose. However, the effects of reduced probing on mosquito physiology, or indeed on malaria transmission are unknown. Some studies (Li *et al.,* 1992) suggest that probing times have no effect on the number of mosquitoes that take up the infection and subsequently deliver sporozoites, with the majority released at the start of a blood meal and consequently does not affect the mean number of sporozoites that are deposited during feeding (Li *et al.,* 1992).

This thesis characterised behaviour using the video tracking system based on previously defined behavioural modes (Parker *et al.,* 2015) to assess host-seeking flight of mosquitoes at a baited net. All behaviours (bouncing, visiting, resting, and swooping) were observed in response to the untreated and the Olyset net. In agreement, with previous work (Lynd & McCall, 2013, Parker *et al.,* 2015, Parker *et al.,* 2017) the majority of contact was on the roof of the net for both untreated and Olyset net. When exposed to the untreated net in video tracking experiments the majority of activity time was spent bouncing, as observed in Parker *et al* (2015). In contrast, when mosquitoes were exposed to Olyset there was a shift from the long contact behaviours (Bouncing) seen on untreated nets, to more short infrequent contacts (visiting) on the ITN. These results further support the existence of contact irritancy to this net type, something described previously in by many others, (Grieco *et al*., 2000; Lindsay *et al*., 1991; Maia *et al*., 2013; Maia *et al*., 2016; Roberts *et al*., 1997; Spitzen *et al*., 2017).

Together evidence from baited box and tracking assays show exposure to a baited ITN reduced the overall amount of time spent in contact with the net for all strains compared to an untreated net. The results reveal mosquitoes, regardless of resistance status, will respond to the baited net, with a rapid

decay in activity only observed in the susceptible strains. Resistant strains continue to host seek throughout exposure to a treated net, but the amount of time spent in contact with the Olyset net was still reduced compared with an untreated control. All assays revealed the irritant properties of pyrethroid nets to all *Anopheles* mosquitoes, more evident when all strains were exposed to permethrin coated Olyset net. Lui *et al* (2021) suggests that mosquitoes can detect pyrethroids by odour receptors on the antenna, and although the current study did not reveal any repellent effects of the nets prior to the first contact, mosquitoes were able to detect the effects of the insecticide and disengage earlier then untreated controls. Perhaps insecticide detection is due to this mechanism, alongside hyper-activation of the sodium channels (Lui *et al*., 2021).

These behavioural effects of exposure to the treated net are important in determining the community protection potentially offered different net types. The irritant properties of Olyset observed in all assays suggest early disengagement and reduced overall contact with this net and therefore suggest there is a reduced chance of the mosquitoes receiving a lethal dose. The current study showed lower mortality when exposed to treated net in baited box (Table 5.1, Chapter 5; 57%-95%) than exposure in WHO cone tests performed in the same group at the same time for both susceptible strains (Emery *et al*., 2019; Foster *et al*., unpublished; results ranged between 94%-100% mortality), suggesting that the ability to avoid the net does result in reduced mortality effects. For mosquitoes that were not killed immediately by the treated net, this thesis then investigated any sub-lethal impacts of this exposure to fully understand the impact exposure has on malaria transmission potential.

The sublethal impacts of ITN exposure.

Currently the standard WHO bioassay method for monitoring and evaluating the effects of ITNs on insecticide resistant populations involves the exposure of mosquitoes to the net for a 3-minute exposure time and recording knockdown and 24-hour mortality. However, even if mosquitoes survive for a day post exposure, long-term survival of mosquitos exposed to first generation pyrethroid nets is reduced in some populations (Hughes *et al.,* 2020; Viana *et al*., 2016). Therefore, the standard cone test used alone is not enough to gather vital information on how control interventions are affecting overall vectoral capacity. The absence of a host may also be impacting behaviour (Parker *et al.,* 2015; Sutcliffe *et al.,* 2014) hence in this thesis the impact of exposure of susceptible and resistant *Anopheles* populations to a baited net was investigated to determine the impacts of insecticide exposure on life history traits beyond immediate mortality.

As expected, immediate mortality of both susceptible mosquito strains was high post ITN exposure (Chapter 5) in video tracking assays, with 98%-99% mortality after exposure to Olyset net. Mortality of the susceptible strains ranged from between 57%-100% all in baited box experiments. Kisumu

mortality was between 86%-100% when exposed to PermaNet 2.0 and 57%-64% after exposure to Olyset nets. For the N'gousso strain mortality ranged from 89%-71% when exposed to PermaNet in baited box assays and 63%-95% after Olyset exposure. As discussed above, the contact irritancy induced by permethrin may have contributed to the lower exposure and hence mortality. 24-hour mortality of both resistant strains was below 20% when exposed in the smaller bench top assays (Chapter 2 and 3), but when exposed to Olyset in video tracking experiments this rose to between 20% and 48%. This observed increase could be due to higher activity levels in the larger testing arena, leading to quicker energy depletion or exhaustion and therefore increased insecticidal effects.

Although insecticide resistance causes a drop in the immediate mortality observed post ITN exposure, additional effects caused by the ITN may reduce the ability of mosquitoes to transmit malaria. Malaria transmission relies on the ability of the mosquito to take an infectious bloodmeal, survive long enough for the parasite to develop and then find another host available to feed on (Beier, 1998; Vaughan, 2007) This thesis investigated the effects of PermaNet and Olyset exposure on the longevity, bloodfeeding ability and fecundity.

Post exposure to the ITN in video tracking experiments, a reduction in longevity was only apparent in in those mosquitoes that did not take a blood meal, indicative of a protective effect of taking a bloodmeal. No reduction in pyrethroid resistant mosquito's longevity beyond 24-hours were observed following exposure to PermaNet 2.0 and Olyset net regardless of how they were exposed (baited box and video tracking) when the mosquito had taken a blood meal.

In this thesis bloodfeeding was observed both directly through the ITN, and post ITN exposure to evaluate alternative scenarios possible in the field setting. In all assay's total inhibition of bloodfeeding after exposure to treated nets was not observed. As observed in a previous study (Glunt *et al.,* 2018) differences in bloodfeeding rates post ITN exposure differed depending on resistance status. In baited box assays, more VK7 bloodfed after exposure to PermaNet, compared to untreated net (P= 0.032) but significantly less Banfora bloodfed when exposed to Olyset net compared to the untreated control (P= 0.038). However, these results were not observed when both resistant strains were exposed in video tracking experiments where 90% of all surviving mosquitoes fed at 24hrs post-exposure, reiterating the importance of a more field relevant assay when testing the overall efficacy of ITNs.

In agreement with previous studies (Hauser *et al.,* 2019, Hughes *et al.,* 2020) mosquitoes responded and were able to feed through all net types regardless of resistance status (Chapter 2; Table 2.3 and Figure 2.4). When bloodfeeding through a treated net the total duration of feeding was reduced compared to an untreated net, but in most cases the amount of blood ingested during feeding was not significantly different, the exception was VK7 which ingested more blood when feeding through

PermaNet compared to untreated netting. Although only measured for both the resistant strains these results suggest the ability of a mosquito to feed at a faster rate in the presence of an insecticide treated net. As discussed in Chapter 5 the effects of this on malaria transmission are unknown, with some studies suggesting reduced feeding times could potentially affect the number of sporozoites delivered to a host (Jin *et al.,* 2007) whilst others suggesting minimal impact on transmission due to the fact the majority of sporozoites are usually released within the first few minutes of feeding (Frischknecht *et al*., 2004). As previous studies have shown a reduction in blood meal duration and amount of blood ingested when susceptible strains were exposed to Olyset plus (Hauser et al., 2019) further work should be conducted on this. An alternative haemoglobin method could be used to overcome the issues experienced in this study due to insecticide induced mortality occurring before full digestion of a blood meal.

Exposure to ITNs in the video tracking experiments did not impact mosquito fertility or fecundity, with some evidence suggesting exposure to Olyset net increased hatch rates. Results are in agreement with previous studies which have shown exposure to pyrethroids does not affect reproductive output (Hauser *et al*., 2019; Mulatier *et al.,* 2019). Despite this, it is important to note that in the current study high levels of mortality and bloodfeeding inhibition resulted in small sample sizes when analysing fecundity and fertility effects post ITN exposure. The highest oviposition rates were observed in the *Anopheles* Kisumu strain that has been colonised in LSTM much longer than all other strains, suggesting that lower oviposition rates may be caused by the artificial laying environment. Results suggest further validation is required for this method.

Novel bioassays

In order to gain information regarding the host-seeking and bloodfeeding behaviour of mosquitoes at the ITN interface two novel bioassays, the baited box and video tracking assay, both of which have undergone improvements from previous studies (Angarita-Jaimes *et al.*, 2016, Parker *et al.,* 2015; Hughes *et al.,* 2020) to improve the operation and outputs gained from these tests. Voloshin *et al* (2020) discusses the improvements and capability of the RRS video tracking system while details of the baited box modifications are described in Chapter 2. Both assays proved extremely valuable for the overall analysis of mosquito ITN interactions.

This thesis found that when mosquitoes were free flying in the assays, all strains made contact with the baited net interface. In video tracking experiments contact times at the ITN interface were lower per mosquito for each strain compared with the baited box. Throughout all non-feeding assays contact with the ITN averaged below the 3-minute exposure used in the standard cone test. Mosquitoes exposed to Olyset net in video tracking experiments on average spent less than 1-minute in contact

with the net surface and between 1 and 2 minutes on average in the prevented feeding baited box assays. These results are in agreement with previous research (Parker *et al.,* 2015; Parker *et al.,* 2017) stating exposure times in the cone tests are unrepresentative of those observed in a field relevant setting, therefore overestimating the insecticidal effects of the net.

The current study found the highest mortality rates after exposure in video tracking assays where the mosquitoes host-seeking was the least restricted, showing that the different tests produce different results when assaying net efficacy. Despite this, the baited box is useful as it quantifies the duration of contact an individual mosquito has with the net surface allowing direct correlation of sublethal or delayed effects. The baited box also allows us to assess bloodfeeding behaviour through the net something other assays do not. Overall, the baited box methodology is a simple tool for measuring host seeking and feeding behaviour and a potential assay for the evaluation of ITNs.

In the video tracking assays mosquito behaviour around the net mainly involved multiple brief contacts, this avoidance behaviour would not be as easily measured in tests restricting exposure to a smaller test area such as the video cone tests or baited box assays. In a true field setting, mosquitoes would have the option to exit the home during avoidance of the net, something this thesis did not extend to. Permethrin and Olyset nets have been described as a deterrent in the past due to increased house exiting (Grieco *et al.,* 2000; Lindsay *et al.,* 1991; Maia *et al.,* 2013; Maia *et al.,* 2016; Roberts *et al*., 1997; Spitzen *et al.,* 2017) but, potentially due to the confined area of video tracking, this level of repellency was not observed in the current study. Although missing some information that would be gained from experimental hut trials the video tracking assay is extremely useful in providing insight into changes in mosquito behaviour at the ITN interface. The assay is adequate in measuring the duration of ITN contact and able to detect behavioural mode changes that may impact the bednets success in the field.

The importance of test conditions is discussed by others (Kouassi *et al*., 2020; Glunt *et al*., 2018; Hodjati et al., 2002; Maliszewska et al., 2017; Oxborough *et al*., 2015) when testing pyrethroids and novel compounds such as Chlorfenapyr. Glunt *et al* (2018) revealed exposure to deltamethrin at temperatures both higher and lower than the standard insectary conditions increased mortality for both susceptible and resistant *An. funestus* and *An. arabiensis*. When testing chlorfenapyr, a novel chemistry which is a pro-insecticide, using standard WHO tests overlooked the effects of this chemistry during the laboratory screening stage due to the simplicity of testing methods. Overnight tunnel test revealed that the raised metabolism by light activity at night enhanced the mortality effects of this chemistry. *Anopheles* host-seeking behaviour is under circadian control and therefore enhances activity and respiration at night. Circadian rhythm of the mosquito has also been shown to

effect resistance to pyrethroids (Balmert *et al.,* 2014; Ingham *et al*., 2021; Villanueva *et al*., 2016; Yang *et al.,* 2010). Therefore, in this thesis all assays were performed between 0 and 7 hours after the start of the scotophase with free flying mosquitoes and a human bait. This ensured test conditions represented how the control tool would be encountered in the field, giving a more realistic idea of how these tools perform.

Broader Application of Findings

This thesis aimed to gain a greater understanding of behaviour of the mosquito at the ITN interface, specifically investigating the impacts of insecticide resistance on this behaviour around a standard pyrethroid net. Although the emergence of insecticide resistance is well documented, the impact this has on the success of ITNs is lacking. The findings from this thesis show how behaviour of all strains changed in response to the treated net regardless of the resistance status, with some subtle differences between the strains. Irritancy of the treated nets reduced overall net contact times in most assays. Although this contact is still sufficient in killing the susceptible mosquito strains, low mortality effects were observed by the exposure on the resistant mosquitoes. The results highlight the need for novel chemistries and tools to increase efficacy against the resistant populations alongside more suitable assays to evaluate the overall effects of these control interventions.

As most next generation nets still involve the use of pyrethroids combined with novel chemistries, understanding how these pyrethroids effect mosquito behaviour aids in the development of these new combination nets. It is also important to investigate potential interactions between the two active ingredients on these next generation nets.

Chapter 4 focuses on mosquito host-seeking behaviour around a whole baited net. Information on location of activity has aided the design of new bednets (Murray *et al.,* 2020), which could be used to apply higher concentrations of insecticides, otherwise not suitable for use over the whole bednet surface, to combat pyrethroid resistant populations. The development of the barrier bednet shows how a simple modification to the net can increase efficacy and control potential (Murray *et al.,* 2020). Results emphasise how the understanding of mosquito-net interactions can help to exploit these behaviours to create much needed novel control tools.

Results from this thesis also highlight the importance of further validation of these more suitable and robust testing systems to evaluate malaria control interventions and the potential gains to investigating the effects of ITN exposure beyond immediate mortality. Existing tests, such as the standard WHO cone test, may provide sufficient information when testing fast-acting insecticides such as pyrethroids against largely susceptible populations. However, for insecticides such as chlorfenapyr,

a pro-insecticide and clothianidin, a slower acting chemistry, these tests may underestimate their potential impact in a more field relevant setting. This, alongside the gains discussed previously of incorporating the behavioural effects and sublethal impacts of ITN exposure into testing methods suggest a more in-depth testing cascade is required for future control tools.

This thesis did not investigate the level of house exiting in response to ITNs. The irritancy effects of PermaNet and to a much greater extent Olyset net suggest further work is required in assessing the level of community protection offered by these nets in areas with highly resistant populations. The behaviour of outdoor biting mosquitoes was also not considered as part of this research, is a continuing concern for malaria transmission and its control (Govella & Ferguson, 2012; Govella *et al.*, 2010 Killeen, 2014).

Future Work

This thesis investigated effects of ITN exposure on mosquito behaviour, previously missed by standard testing procedures. In the current study, colonised mosquitoes maintained for many years at LSTM were used; these methods are now being evaluated on natural field populations in studies in Benin, Burkina Faso and Malawi as part of the ESSENTIALs project. Room-scale video tracking tests are also being performed to investigate how mosquitoes respond to next generation nets, investigating the behavioural effects of insecticides with novel modes of action. But further questions remain on the response of mosquitoes; below is a list of some remaining gaps;

- Further investigation could assess whether differences in the molecular mechanisms of pyrethroid resistant strains relates to behavioural differences.
- Further studies to identify if feeding at a faster rate during exposure to an ITN is an adaptive response to insecticide observed only those mosquitoes resistant to pyrethroids.
- Room-scale video tracking tests could be utilised to investigate the effects of diversion to individuals not protected with a bednet.
- Further validation of the test protocols used for measuring reproductive output following ITN exposure.

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Appendix 1:

Additional tables and figures: Chapter 5

Appendix 1.1: Sample size in all assays

Table A1.1: Sample size. Total number of responding mosquitoes exposed during each test, baited box feeding permitted and prevented assays and video tracking bioassays.

		Sample size Total number of responding mosquitoes exposed during testing		
		Feeding Permitted	Feeding Prevented	Tracking
Kisumu	Untreated	30	25	116
	PermaNet	21	20	-
	Olyset	22	27	145
N'gousso	Untreated	27	24	119
	PermaNet	22	20	-
	Olyset	26	21	140
Banfora	Untreated	30	29	89
	PermaNet	24	29	-
	Olyset	22	26	127
VK7	Untreated	27	22	92
	PermaNet	23	27	-
	Olyset	22	26	137

Appendix 1.2: Fecundity and Fertility after exposure in baited box feeding permitted assays.

Table A1.2: Summary table of sub-lethal effects data for all bloodfeeding permitted assays. N = number of mosquitoes that oviposited. Table shows fecundity; the total number of eggs, mean per female. Fertility; mean number of first instar larva 5 days post oviposition with SD, percentage hatch rate.

Strain	Treatment	No.fed	N	Fecundity		Fertility	
				Number eggs laid	Mean number of eggs/female (SD)	Mean number of first instar (SD)	Hatch rate (%)
Kisumu	Untreated	30	21	1726	82 (23.22)	65 (29.23)	80%
	PermaNet	21	-	-	-	-	-
	Olyset	22	4	224	56 (16.45)	1 (1.258)	2%
N'gousso	Untreated	27	12	659	55 (29.20)	40 (33.13)	78%
	PermaNet	22	2	229	115 (4.95)	84 (39.60)	73%
	Olyset	26	1	55	55.00 -	19 -	35%
VK7	Untreated	27	15	815	54 (30.25)	16 (17.14)	30%
	PermaNet	23	8	431	53.88 (37.35)	16 (17.39)	30%
	Olyset	22	4	270	68 (36.49)	23 (26.17)	34%
Banfora	Untreated	30	11	496	45 (27.07)	15 (20.82)	34%
	PermaNet	24	5	222	44 (21.62)	9 (13.09)	21%
	Olyset	22	3	93	31 (29.1)	1 (1.732)	3%

Appendix 1.3: Fecundity and fertility after exposure in the baited box feeding prevented assays.

Table A1.3: Summary table of sub-lethal effects data for all bloodfeeding permitted assays. N = number of mosquitoes that oviposited. Table shows fecundity; the total number of eggs, mean per female. Fertility; mean number of first instar larva 5 days post oviposition with SD, percentage hatch rate.

Strain	Treatment	No.fed	N	Fecundity		Fertility	
				Number eggs laid	Mean number of eggs/female (SD)	Mean number of first instar (SD)	Hatch rate (%)
Kisumu	**Untreated**	23	14	901	64 (33.26)	41 (33.3)	64%
	PermaNet	0	-	-	-	-	-
	Olyset	3	3	241	80 (33.02)	0	0%
N'gousso	**Untreated**	17	7	500	71 (46.13)	37 (40.29)	52%
	PermaNet	2	-	-	-	-	-
	Olyset	1	-	-	-	-	-
Vk7	**Untreated**	18	4	156	39 (8.45)	9 (16.56)	23%
	PermaNet	23	3	134	45 (8.51)	12 (14.75)	27%
	Olyset	17	8	410	51 (34.87)	28 (41.41)	55%
Banfora	**Untreated**	23	4	234	59 (40.6)	54 (52.35)	92%
	PermaNet	23	1	32	32 (0)	0	0%
	Olyset	14	3	139	46 (54.42)	4 (1.16)	9%

Appendix 1.4: Longevity after exposure in baited box feeding permitted assays.

Table A1.4: Survival rates post exposure to treated and untreated for four strains *of An. gambiae.* Average number of days survival post exposure calculated for each strain exposed to each net type (N, mean (standard deviations).

Strain	Treatment	Days alive post test N, Mean (SD)		
Banfora	Olyset	21	11.1	(9.8)
Kisumu	Olyset	21	4.2	(8.8)
N'gousso	Olyset	24	3.4	(8.5)
VK7	Olyset	22	11.8	(10.3)
Banfora	PermaNet	20	9.6	(7.7)
Kisumu	PermaNet	21	0.2	(0.5)
Ngousso	PermaNet	21	1.4	(2.8)
VK7	PermaNet	22	8.8	(6.5)
Banfora	Untreated	29	13.2	(9.4)
Kisumu	Untreated	29	12.5	(5.8)
N'gousso	Untreated	26	10	(6.7)
VK7	Untreated	25	11.6	(6.9)

Appendix 1.5: Longevity after exposure in feeding prevented assays.

Table A1.5: Survival rates post exposure to treated and untreated for four strains of *An. gambiae*. Average number of days survival post exposure calculated for each strain exposed to each net type (N, mean (standard deviations).

Strain	Treatment	Days alive post test N, Mean (SD)		
Banfora	Olyset	26	13.1	(6.4)
Kisumu	Olyset	25	3.8	(5.8)
N'gousso	Olyset	20	2.3	(6.1)
VK7	Olyset	25	12.32	(6.5)
Banfora	PermaNet	25	13.4	(7.8)
Kisumu	PermaNet	20	0.6	(0.5)

Ngousso	PermaNet	18	0.7	(1)
VK7	PermaNet	23	10.52	(6.9)
Banfora	Untreated	22	15.1	(7.6)
Kisumu	Untreated	23	11.6	(5.8)
N'gousso	Untreated	22	13.1	(10.7)
VK7	Untreated	19	12.8	(6.3)

Table A1.6: Longevity after exposure in video tracking assays.

Table A1.6: Survival rates post exposure to a treated and untreated for four strains of *An. gambiae*. Average number of days survival post exposure calculated for each strain exposed to each net type (N, mean (standard deviations).

Strain	**Treatment**	**Days alive post test N, Mean (SD)**		
Banfora	Olyset	123	4.268	(7.067)
Kisumu	Olyset	145	0.2	(1.953)
N'gousso	Olyset	140	0.2	(1.85)
VK7	Olyset	132	6.144	(5.797)
Banfora	Untreated	86	7.43	(5.103)
Kisumu	Untreated	105	7.867	(6.28)
N'gousso	Untreated	116	9.9	(7.563)
VK7	Untreated	90	9.156	(5.736)

Appendix 1.7: Longevity of mosquitoes that fed post exposure in video tracking assays.

Table A1.7: Survival rates of *An. gambiae s.l.* that took a blood meal post exposure to a human baited treated and untreated net. Average number of days survival post exposure calculated for each strain exposed to each net type (N, mean (standard deviations).

Strain	Treatment	Time fed (hrs)	Days alive post test N, Mean (SD)		
Banfora	Olyset	1	17	11.53	(9.408)
Banfora	Olyset	24	21	12.19	(8.322)
Kisumu	Olyset	1	0	-	-
Kisumu	Olyset	24	2	17.5	(3.536)
N'gousso	Olyset	1	1	12	(0)
N'gousso	Olyset	24	1	19	(0)
VK7	Olyset	1	47	9.511	(5.429)
VK7	Olyset	24	44	8.318	(5.251)
Banfora	Untreated	1	27	9.148	(4.920)
Banfora	Untreated	24	28	9.214	(4.228)
Kisumu	Untreated	1	29	10.97	(6.472)
Kisumu	Untreated	24	62	8	(5.634)
N'gousso	Untreated	1	53	13.68	(7.01)
N'gousso	Untreated	24	39	10.72	(5.145)
VK7	Untreated	1	82	9.293	(5.629)
VK7	Untreated	24	7	8.857	(6.768)

Appendix 1.8: Longevity of mosquitoes unfed after exposure in video tracking assays

Table A1.8: Survival rates of *An .gambiae s.l.* that did not blood feed post exposure to a human baited treated and untreated net. Average number of days survival for those mosquitoes that did not feed but survived past 24 hours post exposure, calculated for each strain exposed to each net type (N, mean (standard deviations).

Strain	Treatment	Days alive post test N, Mean (SD)		
Banfora	Olyset	26	3.615	(3.477)
Kisumu	Olyset	0	-	-
N'gousso	Olyset	1	4	(0)
VK7	Olyset	15	2.8	(2.908)
Banfora	Untreated	27	4.963	(4.587)
Kisumu	Untreated	4	3	(0)
N'gousso	Untreated	5	1.6	(1.342)
VK7	Untreated	0	-	-

Appendix 2:

Appendix 2.1:

Table A2.1: Resting behaviour analysed using logistic regression in Statistical analysis software (SAS). Model outputs showing the Estimate, standard error, Chi squared value and Odds ratio.

Parameter	Level1	DF	Estimate	StdErr	LowerWaldCL	UpperWaldCL	ChiSq	ProbChiSq	OR	lower95CI	upper95CI	Est
Intercept1		1	0.094	0.2992	-0.4924	0.6803	0.1	0.7534	1.098556	0.611187	1.974559	1.0986
Intercept2		1	4.6969	0.4944	3.7278	5.6659	90.25	<.0001	109.6036	41.58921	288.848	109.6036
Treatment	Olyset	1	-3.0501	0.4498	-3.9316	-2.1686	45.99	<.0001	0.047355	0.019612	0.114341	0.0474
Treatment	PermaNet	1	-1.4316	0.3225	-2.0637	-0.7996	19.71	<.0001	0.23892	0.126983	0.44953	0.2389
Strain	Banfora	1	0.4441	0.3794	-0.2995	1.1878	1.37	0.2418	1.559163	0.741176	3.279908	1.5592
Strain	Ngousso	1	-0.2405	0.3847	-0.9946	0.5136	0.39	0.5319	0.786231	0.369882	1.671236	0.7862
Strain	VK7	1	0.2354	0.3804	-0.5103	0.981	0.38	0.5361	1.265395	0.60033	2.667238	1.2654

Appendix 2.2:

Table A2.2: Overall contact over a twenty-minute bioassay. GEE model output. Contact time divided into 5-minute time intervals for each strain and net type compared to untreated net analysed in Statistical analysis software (SAS). Model outputs showing the Estimate, standard error, and Odds ratio.

Effect	Strain	Net	Time Point	Strain	Net	Time Point	Est	Std Err	Probz	Alpha	Lower	Upper	Odds Ratio	Lower OR	Upper OR
Strain*Net*Time_Point	Ban	Oly	300	Ban	Unt	300	-0.9557	0.2181	<.0001	0.05	-1.3832	-0.5281	0.385	0.251	0.59
Strain*Net*Time_Point	Ban	Oly	600	Ban	Unt	600	-0.9387	0.1944	<.0001	0.05	-1.3197	-0.5577	0.391	0.267	0.573
Strain*Net*Time_Point	Ban	Oly	900	Ban	Unt	900	-1.7261	0.4475	0.0001	0.05	-2.6032	-0.849	0.178	0.074	0.428
Strain*Net*Time_Poin	Ban	Oly	1200	Ban	Unt	1200	-1.2196	0.3714	0.001	0.05	-1.9476	-0.4916	0.295	0.143	0.612
Strain*Net*Time_Poin	Ban	Perm	300	Ban	Unt	300	-0.4426	0.1533	0.0039	0.05	-0.7431	-0.1421	0.642	0.476	0.868
Strain*Net*Time_Poin	Ban	Perm	600	Ban	Unt	600	-0.0005	0.424	0.999	0.05	-0.8315	0.8305	0.999	0.435	2.294
Strain*Net*Time_Poin	Ban	Perm	900	Ban	Unt	900	-0.1544	0.5909	0.7939	0.05	-1.3126	1.0038	0.857	0.269	2.729
Strain*Net*Time_Poin	Ban	Perm	1200	Ban	Unt	1200	-0.0529	0.4911	0.9141	0.05	-1.0156	0.9096	0.948	0.362	2.483

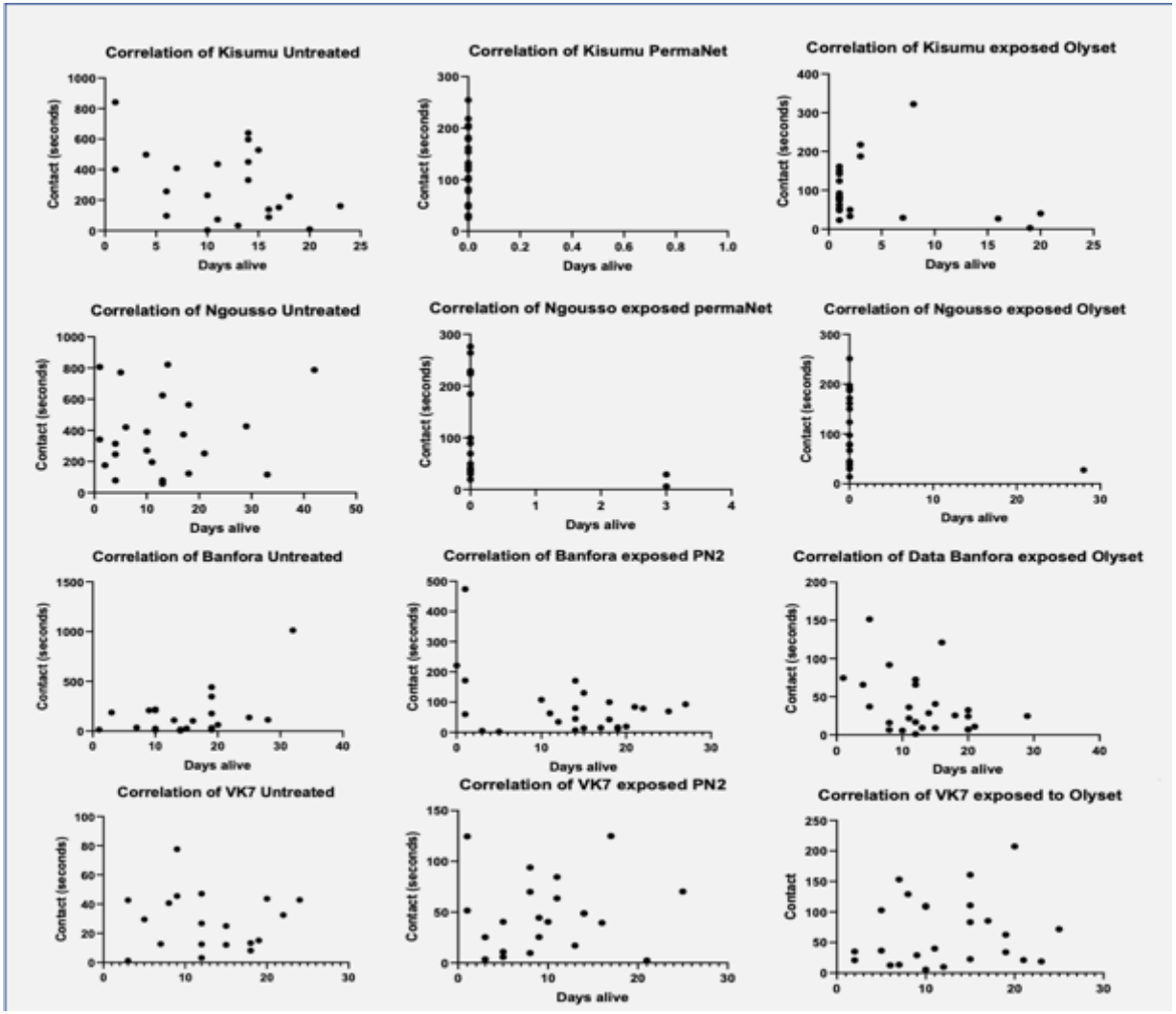
Strain*Net*Time_Poin	Kis	Oly	300	Kis	Unt	300	-0.9273	0.2986	0.0019	0.05	-1.5125	-0.3421	0.396	0.22	0.71
Strain*Net*Time_Poin	Kis	Oly	600	Kis	Unt	600	-1.013	0.9751	0.2989	0.05	-2.9241	0.8982	0.363	0.054	2.455
Strain*Net*Time_Poin	Kis	Oly	900	Kis	Unt	900	-1.0438	0.2454	<.0001	0.05	-1.5248	-0.5627	0.352	0.218	0.57
Strain*Net*Time_Poin	Kis	Oly	1200	Kis	Unt	1200	-0.8178	1.0235	0.4243	0.05	-2.8239	1.1883	0.441	0.059	3.281
Strain*Net*Time_Poin	Kis	Perm	300	Kis	Unt	300	-0.4162	0.1784	0.0196	0.05	-0.7659	-0.06662	0.66	0.465	0.936
Strain*Net*Time_Poin	Kis	Perm	600	Kis	Unt	600	-0.4244	0.3124	0.1743	0.05	-1.0367	0.1879	0.654	0.355	1.207
Strain*Net*Time_Poin	Kis	Perm	900	Kis	Unt	900	-0.5008	0.1512	0.0009	0.05	-0.7971	-0.2045	0.606	0.451	0.815
Strain*Net*Time_Poin	Kis	Perm	1200	Kis	Unt	1200	-1.2128	0.3158	0.0001	0.05	-1.8317	-0.5938	0.297	0.16	0.552
Strain*Net*Time_Poin	N'go	Oly	300	N'go	Unt	300	-0.8334	0.249	0.0008	0.05	-1.3215	-0.3454	0.435	0.267	0.708
Strain*Net*Time_Poin	N'go	Oly	600	N'go	Unt	600	-1.0232	0.8876	0.249	0.05	-2.7629	0.7165	0.359	0.063	2.047
Strain*Net*Time_Poin	N'go	Oly	900	N'go	Unt	900	-1.0297	0.2344	<.0001	0.05	-1.4892	-0.5703	0.357	0.226	0.565

Strain*Net*Time_Poin	N'go	Oly	1200	N'go	Unt	1200	-2.0972	0.7163	0.0034	0.05	-3.5012	-0.6932	0.123	0.03	0.5
Strain*Net*Time_Poin	N'go	Perm	300	N'go	Unt	300	-0.3777	0.2028	0.0625	0.05	-0.7751	0.01973	0.685	0.461	1.02
Strain*Net*Time_Poin	N'go	Perm	600	N'go	Unt	600	0.126	0.6421	0.8444	0.05	-1.1324	1.3844	1.134	0.322	3.993
Strain*Net*Time_Poin	N'go	Perm	900	N'go	Unt	900	-0.3896	0.2358	0.0984	0.05	-0.8517	0.07247	0.677	0.427	1.075
Strain*Net*Time_Poin	N'go	Perm	1200	N'go	Unt	1200	-1.621	0.5857	0.0056	0.05	-2.769	-0.473	0.198	0.063	0.623
Strain*Net*Time_Poin	VK7	Oly	300	VK7	Unt	300	-0.9254	0.2374	<.0001	0.05	-1.3907	-0.4602	0.396	0.249	0.631
Strain*Net*Time_Poin	VK7	Oly	600	VK7	Unt	600	-0.5806	0.2741	0.0341	0.05	-1.1177	-0.04343	0.56	0.327	0.957
Strain*Net*Time_Poin	VK7	Oly	900	VK7	Unt	900	0.2309	0.7424	0.7558	0.05	-1.2242	1.6859	1.26	0.294	5.397
Strain*Net*Time_Poin	VK7	Oly	1200	VK7	Unt	1200	0.1151	0.443	0.795	0.05	-0.7531	0.9833	1.122	0.471	2.673
Strain*Net*Time_Poin	VK7	Perm	300	VK7	Unt	300	-0.4296	0.143	0.0027	0.05	-0.71	-0.1493	0.651	0.492	0.861
Strain*Net*Time_Poin	VK7	Perm	600	VK7	Unt	600	-0.2304	0.1572	0.1426	0.05	-0.5385	0.07763	0.794	0.584	1.081

Strain*Net*Time_Poin	VK7	Perm	900	VK7	Unt	900	0.2531	0.2671	0.3433	0.05	-0.2704	0.7766	1.288	0.763	2.174
Strain*Net*Time_Poin	VK7	Perm	1200	VK7	Unt	1200	-0.1712	0.3348	0.6092	0.05	-0.8274	0.4851	0.843	0.437	1.624

Appendix 2.3:

Figure A2.1: Correlation graph of contact of and mortality when exposed in the baited box feeding prevented bioassay.



Appendix 2.4:

Table A2.3: Time spent in each behavioural mode. Analysed using generalised linear models with gaussian distribution in R (R Core Team 2019) with Tukey adjustment for multiple comparisons using packages LSmeans (Length 2016).

Strain	Behavioural mode	Estimate	Sd error	T value	P value
Kisumu	Visiting	4094.4	418.4	9.786	4.28e-06 ***
VK7	Visiting	5259.6	770.7	6.825	0.000248 ***
N'gousso	Visiting	5323.1	517.5	10.29	6.87e-06 ***
Banfora	Visiting	4396.3	957.1	4.593	0.00177 **
Kisumu	Resting	1505.5	181.3	8.302	1.64e-05 ***
Banfora	Resting	1587.4	289.7	5.48	0.000588 ***
N'gousso	Resting	1400.7	194.2	7.214	9.12e-05 ***
VK7	Resting	606.42	103.6	5.853	0.000628 ***
Kisumu	Swooping	760.47	124.56	6.105	0.000178 ***
Banfora	Swooping	835	234.8	3.555	0.00745 **
N'gousso	Swooping	930.6	141.7	6.568	0.000175 ***
VK7	Swooping	1179.2	225.6	5.228	0.00122 **
Kisumu	Bouncing	26049	2068	12.599	5.08e-07 ***
VK7	Bouncing	14761	1522	9.701	2.61e-05 ***
N'gousso	Bouncing	23122.5	2133.7	10.837	4.64e-06 ***
Banfora	Bouncing	33618	2732	12.305	1.77e-06 ***

Appendix 2.5:

Table A2.4: Binomial regression. Due to the variation of data exceeding the mean, number of contacts were analysed using negative binomial regression in R (R Core Team 2019) package

Strain	Estimate	Sd Error	T value	P value
Kisumu	2.4845	0.2677	9.28	<2e-16 ***
Banfora	2.4086	0.3815	6.314	2.72e-10 ***
VK7	1.7046	0.185	9.214	<2e-16 ***
N'gousso	2.6666	0.165	16.16	<2e-16 ***

Appendix 2.6:

Table A2.5: Blood meal volumes were analysed for resistant strains only using a generalized linear model in SAS adjusting for body size and contact time in the model (SAS). Model output showing estimate, standard error, t value and significance.

Strain	Treatment	Bioassay	Estimate	St error	t Value	P Value
Banfora	Olyset	Feeding permitted	-0.5773	0.6271	-0.61	0.5449
Banfora	PermaNet	Feeding permitted	-0.2221	0.456	-0.49	0.6274
VK7	Olyset	Feeding permitted	0.8253	0.5994	1.38	0.1721
VK7	PermaNet	Feeding permitted	0.9072	0.4049	2.24	0.0276
VK7	Olyset	Feeding prevented	0.5574	0.8016	1.59	0.1182
VK7	PermaNet	Feeding prevented	-0.716	0.7683	-0.93	0.3557
Banfora	Olyset	Feeding prevented	0.1527	0.9467	0.16	0.8725
Banfora	PermaNet	Feeding prevented	0.8437	0.908	0.93	0.3571
Banfora	Olyset	Tracking	-0.1808	0.3135	-0.58	0.5651
VK7	Olyset	Tracking	0.4487	0.2512	1.79	0.0763

Appendix 2.7:

Table A2.6: Survival analysis was performed using Cox regression in R (R Core Team 2019) survival package (Therneau, 2015) for feeding permitted bioassays. Table shows model output showing loglik, Chi Squared, DF and significance.

Ngousso				
	loglik	Chisq	Df	Pr(>|Chi|)
NULL	-184.53			
Treatment	-168.35	32.3661	2	9.371e-08 ***
contact.time	-168.34	0.0201	1	0.8871
wing.span	-168.07	0.5432	1	0.4611
Treatment:contact.time	-166.89	2.3614	2	0.3071
Treatment:wing.span	-166.19	1.4017	2	0.4962
contact.time:wing.span	-165.50	1.3668	1	0.2424
Treatment:contact.time:wing.span	-164.98	1.0494	2	0.5917

Kisumu				
	loglik	Chisq	Df	Pr(>|Chi|)
NULL	-213.53			
Treatment	-197.58	31.9102	2	1.177e-07 ***
contact.time	-197.37	0.4224	1	0.5157
wing.span	-196.09	2.5585	1	0.1097
Treatment:contact.time	-195.81	0.5462	2	0.7610
Treatment:wing.span	-194.14	3.3528	2	0.1870
contact.time:wing.span	-194.13	0.0078	1	0.9298
Treatment:contact.time:wing.span	-193.53	1.2037	2	0.5478

VK7				
	loglik	Chisq	Df	Pr(>|Chi|)
NULL	-192.74			
Treatment	-191.14	3.2022	2	0.20168
contact.time	-190.51	1.2496	1	0.26363
wing.span	-190.12	0.7800	1	0.37715
Treatment:contact.time	-189.92	0.4040	2	0.81710
Treatment:wing.span	-189.37	1.1019	2	0.57640
contact.time:wing.span	-187.96	2.8130	1	0.09351
Treatment:contact.time:wing.span	-187.31	1.3066	2	0.52032

Banfora				
	loglik	Chisq	Df	Pr(>|Chi|)
NULL	176.40			
Treatment	175.35	2.0999	2	0.3500
contact.time	175.32	0.0527	1	0.8184
wing.span	174.62	1.3891	1	0.2386
Treatment:contact.time	172.82	3.6140	2	0.1641
Treatment:wing.span	172.12	1.3883	2	0.4995
contact.time:wing.span	172.02	0.2157	1	0.6423
Treatment:contact.time:wing.span	171.61	0.8156	2	0.6651

Appendix 2.8:

Table A2.7: Survival analysis was performed using Cox regression in R (R Core Team 2019) survival package (Therneau, 2015) for feeding prevented bioassays. Table shows model output showing loglik, Chi Squared, DF and significance.

VK7: Blood fed				
	loglik	Chisq	Df	Pr(>|Chi|)
NULL	-114.03			
treatment	-113.45	1.1668	2	0.5579851
contact.time	-113.44	0.0152	1	0.9017562
wing.span	-113.11	0.6594	1	0.4167703
treatment:contact.time	-112.87	0.4860	2	0.7842641
treatment:wing.span	-111.43	2.8782	2	0.2371368
contact.time:wing.span	-105.83	11.2016	1	0.0008173 ***
treatment:contact.time:wing.span	-105.17	1.3161	2	0.5178691

Banfora: Blood fed				
	loglik	Chisq	Df	Pr(>|Chi|)
NULL	-81.558			
treatment	-79.702	3.7113	2	0.15635
contact.time	-78.131	3.1417	1	0.07631
wing.span	-77.339	1.5850	1	0.20804
treatment:contact.time	-73.055	8.5678	2	0.01379 *
treatment:wing.span	-70.589	4.9316	2	0.08494
contact.time:wing.span	-70.154	0.8703	1	0.35087
treatment:contact.time:wing.span	-65.588	9.1318	2	0.01040 *

Appendix 2.9:

Table A2.8: Survival analysis was performed using Cox regression in R (R Core Team 2019) survival package (Therneau, 2015) for video tracking bioassays. Table shows model output showing loglik, Chi Squared, DF and significance.

Banfora: Blood fed				
	loglik	Chisq	Df	Pr(>|Chi|)
NULL	-331.72			
Net	-329.90	3.6368	1	0.05652
wings	-312.38	35.0445	28	0.16858
Net:wings	-312.38	0.0000	0	1.00000

VK7:Blood fed				
	loglik	Chisq	Df	Pr(>|Chi|)
NULL	-758.25			
Net	-757.78	0.9407	1	0.3321
wings	-724.45	66.6597	56	0.1558
Net:wings	-724.45	0.0000	0	1.0000

Banfora: Not blood fed				
	loglik	Chisq	Df	Pr(>|Chi|)
NULL	-438.71			
Net	-427.90	21.635	1	3.299e-06 ***
wings	-419.58	16.622	17	0.4802
Net:wings	-419.58	0.000	0	1.0000

Appendix 2.10:

Table A2.9: Fecundity after exposure in feeding permitted assays was measured by counting the number of eggs laid by each female and fertility measured by number of l1 that hatched. Due to the variance of data exceeding the mean, egg counts were analysed using Zero inflated negative binomial regression and larval counts using negative binomial regression in R (R Core Team 2019) package glmmTMB (Brooks et al., 2017). Table shows model output showing Estimate, standard error, Z value and significance.

Strain	Treatment	Bioassay	Eggs/L1	Estimate	St error	Z Value	P Value
Banfora	Olyset	Feeding permitted	Eggs	42.37	15.37	2.758	0.00582
Banfora	PermaNet	Feeding permitted	Eggs	8.307	13.793	0.602	0.547
VK7	Olyset	Feeding permitted	Eggs	-17.57	17.93	-0.98	0.327
VK7	PermaNet	Feeding permitted	Eggs	-11.22	14.83	-0.756	0.449
Banfora	Olyset	Feeding permitted	L1	2.5089	1.1433	2.194	0.0282
Banfora	PermaNet	Feeding permitted	L1	8.307	13.793	0.602	0.547
VK7	Olyset	Feeding permitted	L1	-17.57	17.93	-0.98	0.327
VK7	PermaNet	Feeding permitted	L1	-0.02772	0.57216	-0.048	0.961

Appendix 2.11:

Table A2.10: Fecundity after exposure in video tracking bioassays was measured by counting the number of eggs laid by each female and fertility measured by number of l1 that hatched. Due to the variance of data exceeding the mean, egg counts were analysed using Zero inflated negative binomial regression and larval counts using negative binomial regression in R (R Core Team 2019) package glmmTMB (Brooks et al., 2017). Table shows model output showing Estimate, standard error, Z value and significance.

Strain	Treatment	Bioassay	Eggs/L1	Estimate	St error	Z Value	P Value
Banfora	Olyset	Video tracking	Eggs	-0.2355	0.1985	-1.187	0.235
VK7	Olyset	Video tracking	Eggs	-0.112	0.1202	-0.930	0.351
Banfora	Olyset	Video tracking	L1	-0.931	0.3359	-2.771	0.00558 **
VK7	Olyset	Video tracking	L1	-0.5962	0.2451	-2.433	0.015 *