The sub-lethal effects of pyrethroid exposure on *Anopheles gambiae* s.l. life-history traits, behaviour, and the efficacy of insecticidal bednets

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Abstract

Malaria control progress in Africa has stalled. Though the reasons for this will be multifaceted, increasing and intense resistance to pyrethroids in *Anopheles gambiae* s.l. is almost certainly a contributing factor. Standard methods to monitor insecticide resistance and evaluate vector control tools primarily focus on the immediate and lethal effects on the mosquito. These methods disregard other important delayed and sub-lethal effects, despite their implications for malaria transmission. In response to growing concerns over the sustained effectiveness of current control tools, next-generation products are being developed and evaluated. These aim to target insecticide-resistant mosquitoes or mosquitoes that contribute to residual malaria transmission. Adaptations to current standard efficacy tests are needed to evaluate the novel modes of action of such products.

The effect of insecticide exposure on the longevity, reproductive output and blood-feeding behaviour of a wild highly pyrethroid-resistant *Anopheles gambiae* s.l. population was evaluated. Mosquitoes were exposed to a range of insecticides and insecticidal bednets using laboratory tests and semi-field experimental hut trials. Benchtop video tests were evaluated for their feasibly in measuring the effectiveness of standard and next-generation nets. Subsequently, these tests were used to investigate the behaviour of field-populations of *An. gambiae* s.l. at the bednet interface in response to a human host.

Following exposure to both pyrethroid-only and next-generation nets, evidence of sub-lethal impacts were limited or non-existent. The mosquitoes exposed to insecticidal nets did not suffer from reduced lifespan or altered reproductive output. Evidence of delayed mortality was only recorded when mosquitoes were exposed to extremely high levels of pyrethroids in WHO tube bioassays. Some mosquitoes were inhibited from blood-feeding in experimental hut trials, however, lab tests suggest this effect is absent by 8-hour post net-exposure. The efficacy of next-generation nets on the field population was dependant on the product. Brief contact with PermaNet 3.0 roof (pyrethroid + PBO) caused rapid knock-down and 100% mortality in all tests. Exposure to all other insecticidal nets, including Interceptor G2 (pyrethroid + chlorfenapyr), resulted in low 24-hour mortality in both lab and semi-field experiments. Following adaptations for the field, video tests were able to collect behavioural data on mosquito responses to insecticidal nets such as flying, resting, and probing behaviour. Responses were similar between untreated and pyrethroid-

only netting. Extreme reductions in activity were observed following exposure to PermaNet 3.0, and Interceptor G2 showed signs of repellence.

The results suggest community protection offered by first-generation LLINs is extremely low in this setting, however, pyrethroid-PBO nets appear to be effective at controlling the highly pyrethroid-resistant population. This work highlights the need for additional studies of sub-lethal effects in other field populations, with lower insecticide resistance levels or differing mechanisms, to establish if such measurements should be incorporated into the evaluation of novel vector control tools.

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Table of contents

Table of figures

Table of tables

List of appendices

Chapter 1. Introduction and literature review

Malaria is rising in many high burden African countries, and worldwide progress is stalling (WHO, 2019b). As a result of increased funding, the scale-up of insecticidal net distribution and indoor residual spraying of insecticides (IRS) has led to reductions in malaria-associated morbidity and mortality since 2000 (Bhatt *et al.*, 2015). Yet in 2018, the World Health Organisation (WHO) estimated 405,000 malaria attributed deaths and 228 million malaria cases globally (WHO, 2019b). Most malaria cases (93%) and deaths (94%) occur in Africa.

The aim to reduce malaria cases and deaths by 40% by 2020, set out by 'The Global Technical Strategy for Malaria' (WHO, 2015a) is extremely unlikely to be met. The urgent need for an overhaul in the way malaria control was implemented led to the launch of the "high burden to high impact" (HBHI) initiative (WHO and RBM, 2018), which aims to reduce malaria in the highest-burden countries through targeted deployment of control tools using a country-led approach. This initiative is challenging as it relies on the availability of a vast amount of current high-quality data, and in many high burden countries limited infrastructure, political instability, and civil unrest, make the collection and compilation of such data difficult.

Burkina Faso, the study site for this thesis, is one of the eleven high-burden countries (ten in sub-Saharan Africa) where malaria is greatest. Despite high coverage of vector control tools, cases in the country have steadily increased since 2015, and in 2018 it was estimated to have had 7.8 million cases and 12,725 malaria attributed deaths (population at risk 19.7 million people) (WHO, 2019b). National long-lasting insecticidal net (LLIN) distribution campaigns, of mainly pyrethroid-only nets, were conducted in 2010, 2013, and 2016 (Tesfazghi *et al.*, 2016), and in 2019, Burkina Faso began to rollout and operationally monitor the use of next-generation insecticidal nets (pyrethroid nets additionally containing secondary insecticides or insecticide synergists) as part of the HBHI initiative (Unitaid, 2020).

In 2016, a Wellcome Trust Collaborative project was established to study Malaria in an Insecticide Resistant Africa (MIRA) (Ranson, 2016). The project primarily aimed to understand why, in a high burden setting such as Burkina Faso, malaria is persistently high despite widespread deployment of insecticidal nets. The research reported in this thesis

form part of the MIRA project, and focused specifically on the impacts of existing vector control tools on the local mosquito population, as well as testing new methods to evaluate next-generation nets in the field. The reasons for stalled malaria control in this setting will be multidimensional, and so a transdisciplinary approach was used to investigate other explanatory variables. Project MIRA also investigated risk factors associated with malaria, bednet usage, and treatment and prevention seeking behaviours in the local human population.

1.1 Malaria epidemiology

Human malaria is caused by six *Plasmodium* species (Calderaro *et al.*, 2013), with *P. falciparum* accounting for most severe disease in Africa (WHO, 2019b). The malaria parasite is acquired by female *Anopheles* mosquitoes when they ingest *Plasmodium* gametocytes and transmitted by them when they inject infective *Plasmodium* sporozoites into a host while obtaining a blood meal. It takes between 9-16 days for *Plasmodium* to develop within a mosquito into its infective form (termed: extrinsic incubation period) (Beier, 1998; Vaughan, 2007; Paaijmans *et al.*, 2010), therefore any intervention that reduces mosquito lifespan, or prevents them from acquiring the initial infection, will reduce their vectorial capacity, and disease transmission.

In humans, symptoms of the disease manifest between 7-10 days after the infectious mosquito bite (Phillips *et al.*, 2017), as a result of the synchronised rupturing of red blood cells, when *Plasmodium* merozoites are released into the bloodstream, causing fever, chills and anaemia. The severity of the disease is dependent on the *Plasmodium* species and host factors such as immunity. It disproportionally affects vulnerable groups, such as pregnant women and young children, and in 2018, 67% of malaria deaths occurred in children <5-years-old (WHO, 2019b).

Human malaria is transmitted by ~40 *Anopheles* species (Sinka, Bangs, *et al.*, 2010; Sinka, Rubio-Palis, *et al.*, 2010; Sinka *et al.*, 2011), which vary in their contribution to the disease burden depending on their vectorial capacity and behaviour. In Africa, members of the *An. gambiae* s.l. complex and *An. funestus* group are the most important vectors of malaria (Sinka, Bangs, *et al.*, 2010). These species complexes or groups are composed of morphologically indistinguishable sibling species, many of which differ genetically and

behaviourally. The *An. gambiae* complex is composed of eight sibling species, with *An. gambiae* Giles (historically Mopti "M-form"), *An. coluzzii* Coetzee & Wilkerson (historically Savannah "S-form", Coetzee *et al.*, 2013), and *An. arabiensis* Patton being the dominant vectors in sub-Saharan Africa (Gillies, 1968; Gillies and Coetzee, 1987). The *An*. *funestus* group is composed of eleven sibling species, of which *An. funestus* Giles is the most competent vector (Coetzee and Fontenille, 2004).

Anopheles females are anautogenous, *i.e.* they require a blood meal to develop their eggs and reproduce. In tropical climates, eggs are laid in water and hatch after 2-3 days, releasing first instar larvae (L1). Larvae are filter-feeders which moult several times (L1 through to L4) before developing into non-feeding pupae after ~ 7 days. Adult mosquitoes emerge from the pupal stage after 2-3 days (Service, 2012). After hatching, mosquitoes cannot survive desiccation (Service, 2012), and so are generally confined to the water bodies in which they are laid. As adults, mosquito species exhibit distinct feeding and post-feeding behaviours, such as a preference for feeding on humans (anthropophagic) or other non-human animals (zoophilic), feeding indoors/outdoors (endophagic/exophagic), and resting indoors or outdoors (endophilic/exophilic). The degree to which any population of a particular species exhibits these behaviours varies and determines their importance as a disease vector.

1.2 Malaria control

Vector control, prophylactic treatment of vulnerable groups, and chemotherapeutic treatment of infections with first-line drug therapies are key components of malaria control. Vector control interventions, particularly the distribution of LLINs and indoor residual spraying of insecticides (IRS) have dramatically reduced disease incidence over the past 20 years (Bhatt *et al.*, 2015; Hemingway, 2015). Other insecticide-based interventions, such as larviciding, space-spraying, and insecticide-treated materials (ITMs) are sometimes deployed as secondary mosquito control tools.

1.2.1 Insecticidal bednets

Insecticidal bednets have been extremely effective at reducing malaria morbidity and mortality (Gamble, Ekwaru and ter Kuile, 2006; Bhatt *et al.*, 2015; Pryce, Richardson and

Lengeler, 2018). They protect against malaria in two key ways. Firstly, the physical barrier they present reduces mosquito biting, offering personal protection to the users. Secondly, the insecticidal component kills susceptible mosquitoes. This reduces the vector density and average age of the population, which affects their vectoral capacity, and offers community protection which extends to non-net users (Hawley *et al.*, 2003). The insecticide, particularly on new nets, may additionally reduce mosquito biting though repellence or contact irritancy.

Prior to 2017, only pyrethroids were recommended by WHO for use in bednets and all nets distributed in Africa contained a single insecticide. Since 2017, bednets containing two active ingredients or an insecticide plus a synergist have been approved (referred to as 'prequalification' (PQ)) by WHO (Table 1.1 – 1.2, WHO, 2019a). However, all insecticidal nets currently in circulation contain pyrethroids. The pyrethroids target the mosquito's peripheral and central nervous systems, specifically, their voltage-gated sodium channel (VGSC) which affects nerve impulses (Bloomquist, 1996; Zlotkin, 1999; Soderlund *et al.*, 2002). The insecticide modifies the properties of the channel, causing the repetitive firing of neurons resulting in reduced movement, paralysis, and death (Davies *et al.*, 2007). The rapid metabolism of pyrethroids before they can reach the central nervous system in mammals means pyrethroids have low toxicity to mammals, including humans, but cause rapid 'knockdown' (characterised by impaired mosquito movement and paralysis) in susceptible mosquitoes (Zaim, Aitio and Nakashima, 2000; Davies *et al.*, 2007). The use and distribution of pyrethroid-only LLINs has increased markedly in recent years (Hemingway *et al.*, 2016). During the same period, a significant rise in pyrethroid resistance in malaria vectors has been documented (Ranson and Lissenden, 2016; WHO, 2018a). This has resulted in a push from the malaria control community to develop LLINs that are effective against pyrethroid-resistant mosquitoes.

Table 1.1. List of WHO prequalified insecticidal nets (WHO, 2019a) showing the manufacturer and level of active ingredient for each bednet.

Product name	Manufacturer	Active Ingredient	
Olyset Net	Sumitomo Chemical Co., Ltd	Permethrin (1000 mg/m^2)	
Olyset Plus	Sumitomo Chemical Co., Ltd	Permethrin (1000 mg/m^2) Piperonyl Butoxide (1%)	
Interceptor	BASF SE	Alpha-cypermethrin (200 mg/m^2)	
Interceptor G2	BASF SE	Alpha-cypermethrin (100 mg/m^2) Chlorfenaypr (200 mg/m^2)	
Royal Sentry	Disease Control Technology, LLC	Alpha-cypermethrin (261 mg/m^2)	
Royal Sentry 2.0	Disease Control Technology, LLC	Alpha-cypermethrin (203 mg/m^2)	
Royal Guard	Disease Control Technology, LLC	120 denier	Alpha-cypermethrin (5.5 g/kg) Pyriproxyfen (5.5 g/kg)
		150 denier	Alpha-cypermethrin (5.0 g/kg) Pyriproxyfen (5.0 g/kg)
PermaNet 2.0	Vestergaard S.A.	75 denier	Deltamethrin (1.8 g/kg)
		100 denier	Deltamethrin (1.4 g/kg)
PermaNet 3.0	Vestergaard S.A.	Roof	Deltamethrin (4 g/kg) Piperonyl Butoxide (25 g/kg)
		Sides 75 denier	Deltamethrin (2.8 g/kg)
		Sides 100 denier	Deltamethrin (2.1 g/kg)
Duranet LLIN	Shobikaa Impex Private Limited	Alpha-cypermethrin (261 mg/m^2)	
MiraNet	A to Z Textile Mills Ltd	Alpha-cypermethrin (180 mg/m^2)	
MAGNet	V.K.A. Polymers Pvt Ltd	Alpha-cypermethrin (261 mg/m^2)	
VEERALIN	V.K.A. Polymers Pvt Ltd	Alpha-cypermethrin (216 mg/m^2) Piperonyl Butoxide (79 mg/m^2)	
Yahe LN		75 denier	Deltamethrin (1.85 g/kg)

	Fujian Yamei Industry & Trade Co Ltd	100 denier	Deltamethrin (1.4 g/kg)
SafeNet	Mainpol GmbH	Alpha-cypermethrin (200 mg/m^2)	
Yorkool LN	Tianjin Yorkool International Trading Co., Ltd	75 denier	Deltamethrin (1.8 g/kg)
		100 denier	Deltamethrin (1.4 g/kg)
Panda Net 2.0	LIFE IDEAS Biological Technology Co., Ltd.	Deltamethrin (76 mg/m^2)	
Tsara Boost	NRS Moon netting FZE	Deltamethrin (440 mg/m^2) Piperonyl Butoxide (120 mg/m^2)	
Tsara Soft	NRS Moon netting FZE	Deltamethrin (80 mg/m^2)	
Tsara Plus	NRS Moon netting FZE	Roof	Deltamethrin (3 g/kg) Piperonyl Butoxide (11 g/kg)
		Sides	Deltamethrin (2.5 g/kg)

Table 1.2. Mode of action of active ingredients in WHO prequalified insecticidal nets.

Class	Active ingredient	Mode of action
Pyrethroid insecticide	Alpha-cypermethrin	Voltage-gated sodium channel
	Deltamethrin	
	Permethrin	
Pyrrole insecticide	Chlorfenapyr	Oxidative phosphorylation in mitochondria
Insecticide synergist	Piperonyl Butoxide	Metabolic enzymes (cytochrome P450s)
Insect growth regulator	Pyriproxyfen	Juvenile hormone analogue

1.2.1.1 Next-generation nets

Next-generation (or 2nd-generation) nets are defined as pyrethroid nets which contain secondary compounds that are non-pyrethroid insecticides, insecticide synergists or insect growth regulators, and as such, they aim to be effective against pyrethroid-resistant mosquitoes. Currently, 20 LLINs have WHO PQ listing (WHO, 2019a), of which 7 can be defined as next-generation nets. Five of these are pyrethroid-piperonyl butoxide (PBO)

nets, one is a dual active ingredient (AI) net, and one contains an insect growth regulator. Additional concepts for other next-generation LLINs include barrier-bednets, a novel bed-net designed with an additional roof barrier (Appendix 2, Murray *et al.*, 2020), or nets containing antimalarial compounds found to affect *Plasmodium* development (Paton *et al.*, 2019). Chapter 5 of this thesis reports on studies evaluating two of these nets.

Pyrethroid-PBO nets

Pyrethroid-PBO nets are a dual AI net (sometimes referred to as 'bi-treated nets') containing an insecticide (pyrethroid) plus a synergist (PBO). Synergists themselves are generally non-insecticidal, and function by improving the efficacy of the insecticide they are paired with. PBO targets specific metabolic enzymes (cytochrome P450s) within the mosquitoes. These enzymes usually function to detoxify or sequester pyrethroids, so by inhibiting them the PBO can restore the lethality of the pyrethroid in mosquitoes with metabolic resistance mechanisms (insecticide resistance mechanisms are discussed below).

Pyrethroid-PBO nets vary in design (e.g. some only contain PBO on the roof panel, while others have PBO throughout the net), AI concentrations, and pyrethroid insecticide used. There have been very few studies directly comparing the efficacy of different types of PBO nets (Gleave *et al.*, 2018). Consequently, there has been uncertainty amongst national malaria control programmes and LLIN procurement agencies under what conditions, and when, to distribute pyrethroid-PBO nets, and which brand of pyrethroid-PBO net to use. In 2017, the WHO released guidelines for their deployment which stated that pyrethroid-PBO net should be considered in areas where the major malaria vectors have pyrethroid-resistance (WHO, 2017a). This resistance should be intermediate (mosquito mortality in standard tests between 10-80% following pyrethroid exposure), at least partly conferred by monooxygenase-based resistance mechanisms, and deployment of pyrethroid-PBO nets should not compromise coverage of other ongoing effective vector control interventions. The guidelines were based mainly on epidemiological data from an ongoing cluster randomised control trial (RCT) in Tanzania, supported by a meta-analysis of entomological data from existing experimental hut trials, which was used to parametrise a malaria transmission dynamics model (Churcher *et al.*, 2016; Protopopoff *et al.*, 2018). The RCT reported that pyrethroid-PBO nets reduced malaria prevalence compared to pyrethroid-only LLINs 21 months after their deployment, with the meta-analysis and transmission

dynamics model also indicating that pyrethroid-PBO nets could avert clinical cases of malaria in some resistance settings. A subsequent Cochrane review of this data found that in areas of high pyrethroid resistance (mosquito mortality in standard tests < 30% following pyrethroid exposure) pyrethroid-PBO nets reduced mosquito mortality and blood-feeding rates, with limited evidence of any impact in low resistance settings (Gleave *et al.*, 2018). In the Cochrane review, the definition of high resistance overlaps with the definition of intermediate resistance used by WHO, and so, the results of the review agree with the current guidelines.

Knowledge gaps however still exist concerning the durability of pyrethroid-PBO nets (Katureebe *et al.*, 2019). Preliminary data from the third year of the Tanzania trial, and a second cluster randomised control trial embedded in a mass distribution campaign in Uganda (Staedke *et al.*, 2019), suggest that although pyrethroid-PBO nets perform better than pyrethroid-only nets in these settings, the improvements in efficacy may be diminished markedly within three years (Kleinschmidt, 2019).

Interceptor G2

Interceptor G2 (IG2) is a dual AI net coated with alpha-cypermethrin (a pyrethroid) and chlorfenapyr (a pyrrole). Chlorfenapyr targets the insect's mitochondria, uncoupling oxidative phosphorylation and disrupting the production of energy (Black *et al.*, 1994; Treacy *et al.*, 1994). Subsequently, it provides a novel mode of action for insecticidal nets, which previously only targeted the mosquito's nervous system. Positive results from initial laboratory assays (N'Guessan *et al.*, 2007; Kamaraju Raghavendra *et al.*, 2011) and hut trials (Mosha *et al.*, 2008; N'Guessan *et al.*, 2009) using chlorfenapyr to target insecticide-resistant mosquitoes led to the testing of mixture nets treated with pyrethroid + chlorfenapyr (Oxborough *et al.*, 2013; N'Guessan *et al.*, 2014). This led to the development of the dual AL product, Interceptor G2, which was launched by BASF in 2017.

Chlorfenapyr is a slow acting insecticide which has little repellent properties (N'Guessan *et al.*, 2007, 2009). It was combined with alpha-cypermethrin (fast acting and repellent) to increase the personal protection provided by the net. Interceptor G2 has been reported to have improved efficacy compared to Interceptor LN (alphacypermethrin-only LLIN) against pyrethroid-resistant mosquitoes in several experimental hut trials (N'Guessan *et al.*, 2016b;

Bayili *et al.*, 2017; WHO, 2017d; Camara *et al.*, 2018). However, replicating this level of efficacy under laboratory conditions in standard tests has proven to be a challenge (Oxborough *et al.*, 2015).

Attention has focused on establishing a laboratory assay which is predictive of the IG2-induced mortality levels observed in hut trials. However, in previous laboratory studies, Interceptor G2 has failed to reach 100% mortality in a pyrethroid-susceptible *An. gambiae* strain (Kisumu), which should be fully susceptible to the alpha-cypermethrin component of the net (N'Guessan *et al.*, 2016a; Camara *et al.*, 2018). Factors which may affect the efficacy of chlorfenapyr, such as temperature and time of exposure (Oxborough *et al.*, 2015), should not affect the efficacy of alpha-cypermethrin. Pyrethroids are considered relatively thermostable and their mode of action is not sensitive to mosquito metabolism, which could additionally be affected by temperature. These previous studies reported susceptibility to Interceptor LN, an alpha-cypermethrin only net, which contains twice the alpha-cypermethrin concentration of Interceptor G2. In Camara *et al.* (2018) study, a 30-minute cone exposure to Interceptor G2 increased mortality in susceptible Kisumu (from 42 – 81%) and resistant M'Bé (from 26 – 65%) strains, however, 100% mortality in the Kisumu strain was only achieved when nets were washed (both prior to and after the hut trial). These results suggest that the efficacy of the pyrethroid is potentially compromised when coated as a mixture on the net initially. A clearer understanding of how washing and storage affect the bioavailability of the insecticides on the net is essential and refinement of the net's formulation should be considered. In Camara *et al.* (2018) study no chemical analysis was conducted, so it is not possible to determine what concentrations of each insecticide were on the net when they were at their most effective.

Although novel for public health, chlorfenapyr has been widely used over the last 25 years in agriculture and the private consumer market. Its mode of action makes cross-resistance with existing insecticide classes in mosquitoes less likely, when resistance is driven by target site mutations. Although new to the market, so far, no evidence of resistance in mosquitoes has been documented (Agumba *et al.*, 2019; Dagg *et al.*, 2019; Stica *et al.*, 2019). Chlorfenapyr is a pro-insecticide (Black *et al.*, 1994), *i.e.* it is metabolised into its active lethal form only after entering the host. In insects, this is activated by mixed-function oxidases (metabolic enzymes), which are upregulated in some insecticide-resistant mosquitoes, raising the prospect of negative cross resistance in population that contain

these resistance mechanisms, as has seen observed in other insecticides (Corbel *et al.*, 2004).

Importantly, in lab studies, PBO has been reported to have an antagonistic effect on chlorfenapyr toxicity, where it inhibits the metabolic enzymes needed to activate chlorfenapyr into its lethal form (K. Raghavendra *et al.*, 2011; Kamaraju Raghavendra *et al.*, 2011; Yuan *et al.*, 2015). This could have undesirable effects in the field, should next-generation products such as Interceptor G2 and pyrethroid-PBO nets be distributed mosaically, as a method of insecticide resistance management (WHO, 2012), or in situations where the AIs are combined within sleeping spaces on bednets and in indoor residual spraying (IRS). Collecting further information on this interaction in the field will be vital.

1.2.2 Indoor residual spraying

Indoor residual spraying (IRS) is the application of residual insecticide to the interior walls of houses and sleeping spaces (WHO, 2015b). It can reduce malaria incidence in areas with stable and unstable malaria transmission (Pluess *et al.*, 2010), by targeting endophagic and endophilic mosquito behaviour, repelling mosquitoes from entering houses or killing susceptible individuals on contact. As such, it offers both personal and community protection depending on whether mosquitoes are exposed to, or influenced by, the insecticide before or after blood-feeding. By applying the insecticide to walls, rather than a bednet, the amount of human-insecticide contact is reduced, which allows a wider range of chemistries to be utilised, though far greater quantities are required to protect a household.

Historically, IRS was instrumental in the control and elimination of malaria in many countries, with IRS of DDT being the primary strategy employed during the global malaria eradication campaign (1955 – 1969) which eliminated malaria from Europe, North America, and the Caribbean (Kouznetsov, 1977; Carter and Mendis, 2002; Mabaso, Sharp and Lengeler, 2004). IRS is logistically challenging and requires large teams of personnel, specialist training and equipment. It is most effective at providing community protection through mosquito killing, so high household coverage is required for it to be successful, and due to the short residual efficacy of most IRS-insecticides it must be re-applied multiple

times a year. It relies on substantial collaboration from individuals receiving the intervention as it involves significant intrusion into their households. Consequently, IRS is an expensive form of vector control, and in the case of the global malaria eradication campaign, these costs fell heavily on malaria-endemic countries in Africa, contributing to the demise of the unsustainable campaign.

In recent years, documentation of successful IRS programmes led to strengthened support for this intervention, with the US Presidents Malaria Initiative (PMI) investing substantial funding to scale-up and monitor IRS programmes since 2005 (WHO, 2006; Kleinschmidt *et al.*, 2009; Pluess *et al.*, 2010). Until 2017, only two modes of action in mosquitoes (VGSC and acetylcholinesterase (AChE) inhibition) were targeted by IRS. Alongside this, the documentation of resistance to all approved insecticides classes at the time (Ranson and Lissenden, 2016), and the short residual efficacy of most products, made insecticide resistance management (WHO, 2012) of IRS products extremely challenging.
Currently four insecticide classes (carbamates, neonicotinoids, organophosphates and pyrethroids) are prequalified by WHO for use in IRS. Although most prequalified products are pyrethroids (19 or 24 products), in response to increasing pyrethroid resistance, a shift in use to non-pyrethroid IRS has occurred (Oxborough, 2016). In some locations, the organochloride, dichloro-diphenyl-trichlorethane (DDT), is also used, although no DDT product are prequalified by WHO (WHO, 2011b). The development of products with new or repurposed AIs (SumiShield - Sumitomo Chemical, containing clothianidin a neonicotinoid, prequalified by WHO 2017), dual AIs (Fludora Fusion, Bayer S. A. S, containing clothianidin and deltamethrin, prequalified by WHO 2018) and long-lasting efficacy (Actellic 300CS - Syngenta Crop Protection, a new formulation of the organophosphate pirimiphos methyl, converted to PQ listing) could overcome many of the challenges and associated costs related with the intervention (Rowland *et al.*, 2013; Agossa, Padonou, Fassinou, *et al.*, 2018; Agossa, Padonou, Koukpo, *et al.*, 2018; Ngwej *et al.*, 2019). Studies suggest that long-lasting IRS (LLIRS) formulations could outperform standard IRS formations depending on local conditions (Protopopoff *et al.*, 2018; Sherrard-Smith *et al.*, 2018). As new IRS products are begin developed and deployed, ensuring sustained susceptibility to these new classes will be vital. Currently discriminating doses of new AIs (clothianidin) are being established by WHO and monitoring the susceptibility to this compound is essential. Although novel to public health, neonicotinoids have been widely used in agriculture over the last 30 years for broad spectrum pest control.

Currently, the benefit of combining IRS with LLINs is unclear; contradictory results observed from studies are likely to be as a result of variation in local epidemiology and vector populations (WHO, 2014a; Protopopoff *et al.*, 2015; Choi, Pryce and Garner, 2019). Although the evidence is limited, adding pyrethroid-IRS to pyrethroid-LLINs has no apparent benefit (Choi, Pryce and Garner, 2019), and would conflict with the WHO recommendation to not deploy pyrethroid-IRS and LLINs together (WHO, 2012, 2014b). Currently, guidance on the deployment of next-generation nets and IRS, singularly and in combination is needed.

1.2.3 Other vector control tools

Most vector control interventions target the adult life-stage of the mosquito, however in some transmission settings control of the juvenile, larval and pupal, stage can be effective, depending on the type of larval control and local conditions (Tusting *et al.*, 2013; Choi, Majambere and Wilson, 2019). Larviciding aims to reduce mosquito density by killing juvenile stages before they develop into the adult form. Larval control can be larval source management, whereby mosquito breeding sites are actively removed by draining source water or habitat manipulation, or the use of larvicides. Larvicides can be chemical (e.g. temephos), microbial (e.g. *Bacillus thuringiensis israeliensis* (Bti)) or biological (e.g. larvivorous fish) and vary in their mode of action (e.g. suffocation, central nervous system targets, insect growth regulators). Larviciding is recommended by the WHO as a supplementary control measure (WHO, 2013b), however it is not widely adopted in the malaria control community. It is one of few interventions which can impact on exophagic mosquitoes, and as such could affect residual malaria transmission (transmission which can occur despite full coverage with effective LLINs and/or IRS, Killeen, 2014).

Volatile chemicals (repellents) interfere with mosquito host-seeking behaviour by interacting with mosquito olfactory receptors (Bohbot and Dickens, 2010; Bohbot *et al.*, 2011) and may have one or a number of different properties ranging from repellence to lethality. Repellents can be classified broadly into topical repellents (which are applied to skin), spatial repellents (e.g. passive emanators or coils) which disperse their active ingredient into the surrounding area though evaporation or heat, and insecticide treated clothing (ITC). Their primary objective is reducing host-vector contact through deterrence,

repellence, or contact-irritancy, though they can also cause temporary blood-feeding inhibition or death (Ogoma, Moore and Maia, 2012). Due to heterogeneity in study designs and lack of standardisation in evaluating repellent products there is currently insufficient evidence that repellents can prevent malaria (Maia *et al.*, 2018).

Space spraying is the outdoor application of insecticides as a fog, which aims to kill adult mosquitoes when they are at rest or during host-seeking, depending on the time of application. It is often used during disease outbreaks for other mosquito-borne disease such as dengue, or for controlling tsetse flies (Adam *et al.*, 2013), and is recommended for use in areas where IRS may not be possible (e.g. camps for refugees, WHO, 2013b). However, the evidence of its effectiveness in controlling mosquito-borne diseases is limited (Esu *et al.*, 2010; Bowman, Donegan and McCall, 2016; Pryce *et al.*, 2018).

1.2.3.1 Vector control tools in development

Attractive targeted sugar baits (ATSBs) are solutions sprayed on vegetations or in bait-stations, positioned inside or outside a house, which target the sugar-feeding behaviour of both female and male mosquitoes. They contain a lure (e.g. fruit juice) to attract mosquitoes and a toxin that kills them following contact or ingestion. Potentially, ATSBs could complement existing interventions and target residual malaria transmission. In field trials, ATSBs have been shown to reduce mosquito density and survival when deployed both as bait stations (Stewart *et al.*, 2013; Qualls *et al.*, 2015; Tenywa *et al.*, 2017) and when the solution is sprayed onto plants (Müller *et al.*, 2010; Beier *et al.*, 2012). However, questions remain on how best to deploy these products (Zhu *et al.*, 2015), and the impact they may have on non-target organisms. A recent hut trial has shown they can complement LLIN use, increasing mosquito mortality in an area with insecticide resistant *An. gambiae* (Furnival-Adams *et al.*, 2020).

Limited non-insecticidal approaches to mosquito control exist. Improved housing has long been associated with impact on malaria (Lindsay, Emerson and Charlwood, 2002), however this is challenging to evaluate due to the infinite possibilities of modification, and operationally it is difficult to "roll-out" as an intervention due to associated high-costs and logistics. In recent years there has been renewed vigour in evaluating housing improvements for controlling malaria. Housing condition is associated with mosquito

mortality, mosquito density, and malaria risk (Tusting *et al.*, 2017; Jatta *et al.*, 2018; Rek *et al.*, 2018; Lindsay *et al.*, 2019). Housing improvements such as screening windows and doors, improved roofing, and closing eaves can reduce indoor biting by inhibiting entry of host-seeking mosquitoes (Killeen *et al.*, 2019), while generally better housing will improve health and well-being (Von Seidlein *et al.*, 2019). This has led to the development of treated eaves tubes and ribbons, which aim to kill mosquitoes attempting to enter houses and reduce entry rates. These have been shown to be successful in controlling mosquito in semi-field trials (Sternberg *et al.*, 2016; Oumbouke *et al.*, 2018; Barreaux *et al.*, 2019; Mwanga *et al.*, 2019), and subsequently phase III studies are underway to evaluate the effects of screening and eaves tubes on malaria incidence in the field (Sternberg *et al.*, 2018).

Endectocides used to treat cattle and in mass drug administration campaigns (Foy *et al.*, 2019) have been posited as a vector control strategy to target residual malaria transmission (Chaccour *et al.*, 2013; Chaccour and Killeen, 2016). Studies have shown the drugs to reduce *Anopheles* survival (Chaccour, Lines and Whitty, 2010; Sylla *et al.*, 2010; Poché *et al.*, 2015), re-feeding (Kobylinski *et al.*, 2010; Kobylinski, Escobedo-Vargas, *et al.*, 2017), blood-meal digestion, fertility and fecundity (Fritz *et al.*, 2009; Sampaio *et al.*, 2016; Lyimo *et al.*, 2017; Dreyer *et al.*, 2019). They also have been documented to effect *Plasmodium* development (Kobylinski, Foy and Richardson, 2012; Kobylinski, Ubalee, *et al.*, 2017) when the mosquito takes a blood meal from a treated human or animal host.

Other methods for controlling vector's include targeted spraying of male mating swarms (Sawadogo *et al.*, 2017), lethal oviposition traps (Johnson, Ritchie and Fonseca, 2017), and altering mosquito populations using gene drive (Hammond *et al.*, 2016). The release of sterile males to reduce mosquito breeding and population density (Alphey *et al.*, 2010) have been tested in the field (Zheng *et al.*, 2019). Males are sterilised using irradiation (SIT) or *Wolbachia*-induced cytoplasmic incompatibility (IIT). Studies have also suggested *Wolbachia* can be used to reduce *Anopheles* refractoriness to *Plasmodium* infection (Bian *et al.*, 2013).

1.2.4 Evaluating vector control tools

The WHO evaluates vector control products for efficacy and suitability for malaria vector control. International agencies and country malaria control programmes generally rely on WHO recommendations when procuring products. In January 2017, the existing WHO Pesticide Evaluation Team (WHOPES) system transitioned to the WHO Prequalification Team Vector Control Group (PQT-VC) (WHO, 2017e). Manufacturers submit dossiers on the safety, quality, and efficacy of their product, which is reviewed by the PQT-VC. If the product falls within a new product class, and therefore requires epidemiological evidence of its efficacy, the Vector Control Advisory Group (VCAG), make recommendations on the public health value of the product class to the Malaria Policy Advisory Committee (MPAC) and the Strategic and Technical Advisory Group (STAG). Products that had previously received WHOPES recommendations were converted to Prequalification when specific criteria for each product were met.

A range of test procedures and bioassays are used in evaluating control tools and different techniques are used when evaluating chemistries for IRS (e.g. topical and tarsal testing), volatile insecticides (e.g. Peet-Grady chamber), or insecticidal nets. As this thesis focuses specifically on the effectiveness of bednets, the evaluation of insecticidal nets is discussed in detail.

The evaluation of insecticidal nets is a three-tiered system, from lab to large-scale field trials (Table 1.2, WHO, 2013a), with further guidance available for monitoring the durability of LLINs following field deployment (WHO, 2011a, 2013d). Products must pass pre-determined efficacy criteria in order to transition to the next phase of evaluation. In phase I testing these conditions are specific (i.e. mortality following exposure to test net washed x 20 in a cone bioassay ≥ 80%), whereas in phase II testing efficacy is dependent on equivalence or superiority to existing products (i.e. the net washed x 20 must perform as well as or better than the reference net in terms of mortality and blood-feeding). In phase III testing, a product is considered efficacious if after 3 years at least 80% of sampled nets are effective in WHO cone tests (same criteria as phase I testing). In phase I and II trials, both unwashed and washed nets are evaluated for efficacy, with x 20 washes used as a proxy for 3 year's field use. Phase I tests focus on lethality within 24-hours and do not consider long-term fitness effects, such as longevity and reproductive output, which may

impact on a mosquitoes vectorial capacity. This may constrain the scope of phase I tests when evaluating next-generation control tools, where novel modes of actions may not be captured by such tests.

Entomological data from experimental hut trials is often used in pre-qualification of vector control products (WHO, 2017a), and strong correlation between mortality from phase I bioassays and phase II experimental hut trials has been observed (Churcher *et al.*, 2016). In several studies, the data collected from these trials has been used to parametrise malaria transmission models to provide estimates of malaria impact (Churcher *et al.*, 2016; Sherrard-Smith *et al.*, 2018; Murray *et al.*, 2020). Experimental hut trials measure entomological efficacy using standardised structures built to represent local housing. In Africa, three hut designs (East-African, West-African, and Ifakara) are used to evaluate products (Okumu *et al.*, 2012; WHO, 2013a).

Table 1.3. Main parameters assessed and assays used in phase I, II and III studies evaluating insecticidal mosquito nets (WHO, 2013a).

Phase	Type of study	Parameters measured	Assay	Entomological outcomes measured
I	Laboratory	Regeneration of insecticidal activity Efficacy and wash-resistance	WHO cone bioassay	Knock-down Mortality
			Tunnel test	Knock-down Mortality Blood-feeding
II	Small-scale field trial	Wash-resistance Efficacy as measured by vector mortality and blood-feeding inhibition	Experimental hut trial	Deterrence Exophily Mortality Blood-feeding
III	Large-scale field trial	Long-lasting insecticidal efficacy Rate of loss or attrition of nets Physical durability of netting material Community acceptance Safety	Village trials	Knock-down Mortality

The efficacy of pyrethroid-only LLINs against malaria is well characterised, however bednets with novel AIs or modes of action (termed “first in class” products) additionally require evidence of public health impact from a minimum of two randomised-controlled trials conducted over two field seasons to receive full WHO support. “Second in class” products do not need to provide epidemiological evidence of impact, and are only required to show entomological evidence of non-inferiority compared to the first in class product (World Health Organization, 2018). This may create stagnation in the vector control market, due to the high costs and challenging logistics of epidemiological trials (Devine, Overgaard and Paul, 2019), with company’s waiting for others to establish the intervention class to reduce development costs. Innovation in the vector control market is hard to achieve due to low returns on product investment. Schemes such as the Innovative Vector

Control Consortium (IVCC) Vector Expedited Review Voucher (VERV) aim to increase innovation in the market by offering incentives to manufactures to develop novel public health insecticides (Ridley, Moe and Hamon, 2017). The VERV would reward the manufacturer with an expedited review of a second product, allowing them to bring more profitable investments to market at an earlier date.

1.3 Insecticide resistance

Insecticide resistance (IR) is defined as the ability of mosquitoes to survive exposure to a standard dose of insecticide, typically due to physiological and/or behavioural adaptations (WHO, 2016). With the exception of neonicotinoids, resistance to insecticide classes approved for use in public health, has been documented in all major African malaria vectors (Ranson and Lissenden, 2016; WHO, 2018a), with countries increasingly reporting resistance to multiple insecticide classes, and few describing full susceptibility. Between 2010 – 2016 only 13% malaria endemic countries reporting to WHO showed susceptibility to four classes (pyrethroids, DDT, carbamates and organophosphates) of insecticide (WHO, 2018a). Pyrethroid resistance was the most widespread, with 77% of reporting countries recording resistance in at least one major vector to at least one pyrethroid (WHO, 2018a). Pyrethroid resistance was initially documented in *An. gambiae* s.l. (Elissa *et al.*, 1993) and *An. funestus* (Hargreaves *et al.*, 2000) nearly 30 years ago, and in the last decade, a significant rise in pyrethroid resistance has been documented (Ranson and Lissenden, 2016; WHO, 2018a).

The mechanisms which underly insecticide resistance in *Anopheles* mosquitoes, historically have been classified into four main groups (target site, metabolic, penetration and behavioural). As major public health insecticides target just three neuronal proteins, cross-resistance, where resistance to one insecticide class also confers resistance to another insecticide class due to shared modes of action, is frequently documented amongst insecticides used in malaria control (Chandre *et al.*, 1999). Additional less well characterised mechanisms certainly contribute to the resistance phenotype, and novel gene families (hexamerins and α-crystallins) implicated in sequestration and regulation of down-stream effector genes have been shown to be associated with pyrethroid resistance (Ingham, Wagstaff and Ranson, 2018). Recently, a previously undescribed mechanism of insecticide resistance (expression of a sensory appendage protein, SAP2, which binds

pyrethroid insecticide) in *Anopheles* was identified, and is thought to act by sequestering the insecticide on contact (Ingham *et al.*, 2019). Determining other as yet unknown mechanisms and monitoring the prevalence and spread of IR mechanisms will be vital in maintaining effective malaria control and susceptibility to novel insecticides. Quick and accurate methods to track IR mechanisms are essential, and the development of a genotypic panel to screen for multiple IR mechanisms offers one solution to this (Lucas *et al.*, 2019). Genotypic measurements may allow us to document resistance in a population before it reaches the point where phenotypic expression occurs, as well as allow investigation into the origins of insecticide resistance.

1.3.1 Target-site resistance

Target-site resistance occurs when a genetic mutation alters the site at which the insecticide binds. In *Anopheles*, target-site mutations have been reported in most insecticide target sites, including acetylcholinesterase (conferring organophosphates and carbamates resistance), gamma amino butyric acid receptor (conferring cyclodiene resistance), and the VGSC (resulting in resistance to pyrethroids and DDT resistance). In *An. gambiae* and *An. coluzzii* three mutations (L995F, L995S, N1570Y) in the VGSC are frequently documented to cause pyrethroid resistance (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000; C. M. Jones *et al.*, 2012; Silva, Santos and Martins, 2014), with a whole genome sequence study identifying 20 additional non-synonymous nucleotide substitutions in the VGSC gene (Clarkson *et al.*, 2018) whose role in resistance remains to be defined.

1.3.2 Metabolic Resistance

Metabolic resistance is complex and acts by increasing the rate at which an insecticide is catabolized or sequestered by an insect before reaching its target. Commonly this occurs due to qualitative or quantitative modifications to detoxification enzymes involved in its pathway. Three enzyme groups have been highly characterised in metabolic resistance; carboxylesterases (COEs), glutathione S-transferases (GSTs), and cytochrome P450s (CYPs). P450s are the most extensively studied enzyme class in *An. gambiae* s.l., with CYP6M2 and CYP6P3 being the best characterised (Müller *et al.*, 2008; Stevenson *et al.*, 2011), and recently functionally validated (Adolfi *et al.*, 2019). However, other members of these enzyme families (*e.g.* CYP6Z2, CYP6Z3 and GSTD1) are also known to metabolise

pyrethroids (Ranson *et al.*, 2001; Yunta *et al.*, 2016) and many more (*e.g.* GSTD7, GSTD3, GSTE5, GSTMS3, COEAE8O, CYP4C28 and CYP12F2) are upregulated in resistant mosquito populations but their ability to metabolise insecticides have not yet been determined (Ingham, Wagstaff and Ranson, 2018). In *An. funestus*, CYP6P9a, CYP6P9b and CYP6P4 have been associated with pyrethroid resistance (Wondji *et al.*, 2009, 2012; Riveron *et al.*, 2013; Mugenzi *et al.*, 2019).

1.3.3 Penetration resistance

Pyrethroids are typically deployed as contact insecticides for malaria control (in LLINs or IRS), and hence must pass through the mosquito's cuticle to reach their target site. Penetration resistance occurs when insecticide diffusion into the mosquito is reduced due to changes in its cuticle, either by altering cuticular composition, or by increasing its thickness (Wood *et al.*, 2010; Balabanidou *et al.*, 2016). It is often measured using insecticide penetration assays or by comparing the cuticle thickness of susceptible vs resistant mosquitoes (Yahouédo *et al.*, 2017). Few studies have investigated the role of penetration resistance in *Anopheles* (Balabanidou, Grigoraki and Vontas, 2018), although cuticle thickening has been reported in *Culex* mosquitoes (Stone and Brown, 1969; Apperson and Georghiou, 1975) and bed-bugs (Koganemaru, Miller and Adelman, 2013; Lilly *et al.*, 2016). Elevation of cuticular pre-cursor genes have been documented in resistant *Anopheles* populations (Vontas *et al.*, 2007; Djouaka *et al.*, 2008), and one study shows resistant *Anopheles* to have thicker leg cuticles compared to susceptible mosquitoes due to cuticular hydrocarbon enrichment (Balabanidou *et al.*, 2019).

1.3.4 Behavioural resistance

Behavioural resistance can be characterised as any alteration to baseline mosquito behaviour that reduces the risk of physical contact with the insecticide targeting them. The most widely deployed vector controls tools, LLINs and IRS, target mosquito vectors which bite and rest inside houses, predominately at night. Changes to mosquito biting times (e.g. a switch to biting people before they enter a bednet, biting location (e.g. a switch to outdoor biting), or host-preference (e.g. a switch to biting non-human animals) are commonly hypothesised behavioural resistance mechanisms, but convincing evidence of these, or studies linking them to control interventions are limited (Takken, 2002; Gatton *et*

al., 2013). Behavioural changes as a result of selective pressure from an intervention acting on a genetic level or causing altered behavioural preferences is contested (Govella, Chaki and Killeen, 2013). Sibling-species can be impacted differently by an intervention, and the behavioural differences could be the result of behavioural plasticity in the species or the existence of multiple cryptic species in the population (Kitau *et al.*, 2012). Behavioural resistance is challenging to define, and difficult to measure as it requires longitudinal studies which characterise behaviour prior to and after the distribution of the intervention. Additionally, studies are often confounded when they fail to report nuances, such as sibling-species composition changes (Russell *et al.*, 2011) which would impact observed behaviour and interpretation of the results.

Several studies have reported a shift in peak biting times to early-evening or early-morning following mass-distribution of LLINs (Bugoro *et al.*, 2011; Moiroux *et al.*, 2012; Sougoufara *et al.*, 2014; Thomsen *et al.*, 2017). Though the direction of the time-shift varied between these studies, it always resulted in peak biting occurring at times when human hosts were less likely to be protected by LLINs. Increases in outdoor biting have also been reported (Moiroux *et al.*, 2012), as well as changes to host preference (Charlwood and Graves, 1987) as a response to LLINs, both of which would also decrease net effectiveness, and increase the vector population contributing to residual malaria transmission (Killeen, 2014). Often studies lack the historical pre-intervention data required to attribute changes in behaviours to an intervention (Ojuka *et al.*, 2015), and in cases where no evidence for a behavioural effect is observed it is difficult to determine if this is because the phenomenon does not exist, or that sufficient data is lacking in these populations. Studies looking at how physiological insecticide resistance affects mosquito behaviour on an individual genetic level are limited (Porciani *et al.*, 2017).

1.3.5 Insecticide resistance monitoring

In 2012, WHO released the “Global Plan for Insecticide Resistance Management”, a strategy document which provides guidance for countries on their Insecticide Resistance Management (IRM) policies, such as rotation of insecticides or mosaic distribution of interventions (WHO, 2012). It highlights the necessity for timely entomological and resistance monitoring to ensure the suitability of deployed insecticides against local mosquito populations. Current methods for monitoring insecticide resistance are based on

classifying phenotypic resistance, which is typically measured using standardised tests, such as WHO susceptibility bioassays (WHO, 2016) and CDC bottle assays (Centers for Disease Control, 2012). These tests expose local mosquito populations (wild-collected or larval-reared females) to pre-defined 'discriminating doses' of insecticide (defined by WHO (2016) as " a concentration of an insecticide that, in a standard period of exposure, is used to discriminate the proportions of susceptible and resistant phenotypes in a sample of a mosquito population"), and record mosquito mortality at 1 and 24-hours post-exposure. In 2016, following increasing evidence that these tests may not detect changes in insecticide resistance (Toe *et al.*, 2014; Bagi *et al.*, 2015), WHO updated their guidance on insecticide resistance monitoring to include additional testing of resistant populations at x5 and x10 discriminating doses to provide further information on resistance intensity and "strength" of phenotypic resistance (WHO, 2016).

Monitoring of IR has increased, and the establishment of online platforms such as IR-Mapper and the WHO Malaria Threats Map make this data more easily accessible (Knox *et al.*, 2014; WHO, 2017c). Between 2010 – 2016, 86% of malaria-endemic countries reported phenotypic IR monitoring data to WHO (WHO, 2018a). However, the reporting of IR mechanisms (35%) or resistance intensity data (10%) was extremely low. Collection of this data is essential if current malaria control gains are to be maintained. Resistance intensity has been observed to increases dramatically over a short period of time in field (Toe *et al.*, 2014), and understanding the mechanisms which drive resistance in a population are vital when targeting resistant vector with different intervention.

1.3.6 The impact of insecticide resistance on malaria control

The implications of insecticide resistance for malaria control remain controversial (Ranson *et al.*, 2011). In the last 20-years, alongside the scaling up of insecticide-based control tools (particularly LLINs), a significant rise in pyrethroid resistance has been documented (Hemingway *et al.*, 2016; Ranson and Lissenden, 2016; Implications of Insecticide Resistance Consortium, 2018; WHO, 2018a). A direct link between operational control tool failure and vector insecticide resistance has yet to be rigorously confirmed, partly due to the complexity of malaria epidemiology, numerous confounding factors and the absence of historical data (Ranson *et al.*, 2011; Alout *et al.*, 2017). The most frequently cited example of pyrethroid resistance impacting on malaria control comes from reports in South Africa,

where malaria cases increased following a switch from DDT to pyrethroids for IRS. It was found that the major malaria vectors in the area were resistant to pyrethroid and susceptible to DDT, and a switch back to DDT-IRS resulted in a reduction in malaria cases (Hargreaves *et al.*, 2000; Brooke *et al.*, 2001; Maharaj, Mthembu and Sharp, 2005). Studies with pyrethroid-PBO nets have shown reductions in malaria compared to pyrethroid-only LLINs (Protopopoff *et al.*, 2018; Kleinschmidt, 2019). As the action of PBO is only to reverse resistance to pyrethroids, this is compelling evidence that insecticide resistance is eroding the efficacy of standard nets.

Recently, studies have shown that in areas of pyrethroid resistance first-generation LLINs (*i.e.* those containing pyrethroids-only) still provide malaria protection to net users (Lindblade *et al.*, 2015; Bradley *et al.*, 2017; Ochomo *et al.*, 2017; Kleinschmidt *et al.*, 2018). Kleinschmidt *et al.* (2018) found that the level of protection was not related to phenotypic resistance, but it was not possible to determine if the same nets offered more protection prior to the emergence of resistance. Malaria rates were higher in non-net users compared to users even when LLIN coverage was high, suggesting the community effect of LLINs may have fallen (Kleinschmidt *et al.*, 2018). Given the personal protection offered by nets as a physical barrier, it is likely that resistance will erode gains in community protection (through reduced net lethality) before failure in personal protection is documented. This is supported by a malaria transmission model, parametrised using a meta-analysis of lab bioassays and experimental hut trials, which estimated that pyrethroid resistance impacted community protection at lower resistance levels than those required to impact on personal protection (Churcher *et al.*, 2016). Despite the difficulty in confirming a link between resistance and operational failure, it is beyond doubt that the entomological efficacy of standard LLINs is decreasing (Hemingway *et al.*, 2016; Protopopoff *et al.*, 2018), and progress in reducing malaria transmission is stalling (WHO, 2019b). Continued innovation in the field is needed to diversify control tools in order to reverse this trend (Zaim and Guillet, 2002; Hemingway *et al.*, 2006).

1.4 Sub-lethal effects of insecticide exposure

In the pest control sector, the dual importance of lethal and sub-lethal effects has been recognised, and it is recommended that these effects on target and non-target organisms be measured routinely in insecticide impact assessments (Stark and Banks, 2003; Desneux,

Decourtye and Delpuech, 2007). Unfortunately, the practise has not yet been adopted in the public health sector. The efficacy of an insecticide on mosquitoes is generally measured using two indices: 1-hour knockdown and 24-hour mortality. Such outcomes form the basis of most phenotypic bioassays to measure insecticide resistance and also assays to evaluate the efficacy of vector control tools (WHO, 2013a, 2016). These assays were designed for pyrethroids, which act rapidly to cause knockdown and death. These assays to not detect sub-lethal (e.g. reduced fecundity, altered host-seeking preference and/or blood-feeding behaviours) or delayed (e.g. reductions in longevity) effects that could also result in significant impacts on mosquito vectorial capacity and subsequent disease transmission. This could have dramatic implications for both our understanding of insecticide resistance, and the evaluation of vector control products with differing, slower, or novel modes of action.

In mathematical modelling, a mosquito's ability to transmit disease (vectorial capacity) is defined in the classic Ross-Macdonald model by four parameters: The ratio of mosquitoes to humans, the human biting rate, the mosquito's daily survival, and the parasite's extrinsic incubation period, (Smith *et al.*, 2012; Brady *et al.*, 2016). A mosquito's vectorial capacity is inextricably linked to its disease transmission potential, therefore anything that affects these parameters could significantly affect malaria transmission. Additionally, effects on the malaria parasite which alter its extrinsic incubation period will also impact on disease transmission. Some studies have shown insecticidal net exposure to affect *Plasmodium* development in mosquitoes, although much further work is needed in this area (Alout *et al.*, 2014, 2016; Kristan *et al.*, 2016).

1.4.1 Delayed mortality and longevity

To transmit malaria, a mosquito must acquire *Plasmodium* by biting an infected host, survive long enough to become infectious, and then take another blood meal to pass on the parasite. It takes between 9-16 days for *Plasmodium* to complete their extrinsic incubation period and for sporozoites to be present in the mosquitoes' salivary glands (Beier, 1998; Vaughan, 2007; Paaijmans *et al.*, 2010), meaning that only older mosquitoes can transmit malaria. Therefore, anything that reduces the mosquito's lifespan, also decreases its lifetime disease transmission potential.

It is well documented that mosquitoes are more susceptible to insecticides as they age (Rowland and Hemingway, 1987; Lines and Nassor, 1991; Glunt, Thomas and Read, 2011; Rajatileka, Burhani and Ranson, 2011; Jones *et al.*, 2012; Collins *et al.*, 2019; Machani *et al.*, 2019). Village trials have shown a shift in age-structure of *Anopheles* spp. populations, from predominantly older to younger *Anopheles*, following use of insecticidal nets (Magesa *et al.*, 1991; Vulule *et al.*, 1996), suggesting the intervention was exerting a stronger selective pressure on older mosquitoes, which is likely to affect disease transmission, even if the tool is not killing younger mosquitoes.

Prior to this thesis, only two studies have documented how insecticide exposure affects the longevity of insecticide resistant survivors, despite its importance for disease transmission (Viana *et al.*, 2016; Tchakounte *et al.*, 2019). Viana *et al.* (2016) observed reductions of up to 50% in mosquito lifespan in moderate and highly resistant strains of *An. gambiae,* and they estimated that this delayed mortality could reduce the malaria transmission potential of these populations by two thirds. As insecticide resistance intensity increased in the populations tested, the magnitude of this reduction in malaria transmission potential decreased, suggesting that the observed effects could be eroded by intensification of resistance. In the second study, in semi-field populations, PermaNet 2.0 exposure was observed to reduce longevity of *An. gambiae (F7)* and *An. funestus (F1)* compared to unexposed mosquitoes. Notably, it is unclear if this study distinguished between immediate (within 24-hours) and delayed (>24-hours) mortality, and so it is not possible to determine the impact of the insecticide is delayed or not (Tchakounte *et al.*, 2019).

As most insecticidal tools are designed to rapidly kill (due to their pyrethroid component), the majority of efficacy studies record mosquito's mortality at 24-hours post-exposure, though for products with a known slower mode of action this has been extended for up to 120-hours in some instances (WHO, 2013a; Agossa, Padonou, Koukpo, *et al.*, 2018). Subsequently, possible sub-lethal effects of LLINs may continue to be undocumented, and their true impact on mosquitoes underrepresented.

1.4.2 Blood-feeding

The malaria parasite is transmitted by female *Anopheles* during blood-feeding. Subsequently, anything which impacts on their willingness or ability to locate a human host

or blood-feed will affect malaria transmission. Insecticidal bednets inhibit blood-feeding initially because they are a physical barrier to mosquito biting. In experimental hut trials, lower blood-feeding risk has been observed in insecticide treated arms compared to untreated, suggesting that insecticidal bednets cause additional effects on mosquito's ability or willingness to blood-feed beyond the physical barrier (Strode *et al.*, 2014). These effects were observed to persist regardless of mosquito insecticide resistance level. Experimental hut trials separate the number of unfed and blood-fed mosquitoes by location (inside bednet, main hut, or veranda), and calculate blood-feeding inhibition (BFI) as the reduction in blood-fed mosquitoes in insecticide treated huts compared to untreated huts. Due to the design of experimental hut trials, they are unable to distinguish blood-feeding inhibition due to spatial repellence (the mosquito is inhibited from contacting the bednet in the first instance) or contact-irritation (the mosquito contacts the bednet but is inhibited from feeding by sub-lethal insecticide toxicity). Therefore, it is difficult to assess using hut trials whether the blood-feeding inhibition occurs prior to or after insecticidal net contact.

In lab tests, inhibition of blood-feeding following insecticide exposure has been reported in several strains using varied exposure methods and insecticides. Strode *et al.* (2014) reviewed blood-feeding inhibition in *Anopheles* following exposure to insecticidal netting in tunnel tests. Mosquito blood-feeding is recorded as an outcome for all WHO tunnel tests (WHO, 2013a). Reduction in blood-feeding has been observed in *Aedes aegypti* following topical exposure to pyrethroids (d-phenothrin, d-allethrin and tetramethrin) (Liu, Todd and Gerberg, 1986), and dieldrin (Duncan, 1963). And, blood-feeding inhibition has been observed in resistant *An. gambiae* and *An. funestus* strains following expose for 1 – 10 minutes (depending on their resistance level) to PermaNet 2.0 compared to untreated netting in lined WHO tubes (Glunt *et al.*, 2018). None of these studies, however, investigate how long blood-feeding inhibition persisted for in surviving mosquitoes (although Liu, Todd and Gerberg (1986) did established the effect did not persist in the F2-generation). One field study, observed no blood-feeding inhibition following PermaNet 2.0 exposure compared to untreated, however blood-feeding ability was first tested 3-days post exposure (Tchakounte *et al.*, 2019).

1.4.3 Reproductive output

If insecticide exposure alters mosquito reproductive output through negative effects on fertility and fecundity this could have a significant impact on mosquito density and subsequently the biting rate on humans. *Anopheles* mosquitoes are anautogenous, meaning they require a blood-meal to develop their eggs (Clements, 1993). *Anopheles* reproduction is therefore intimately linked to blood-feeding, and so it can be difficult to determine which stage in the gonotrophic cycle is affected by insecticide exposure. Duncan (1963) for example, observed topical exposure to dieldrin to reduce oviposition in *Ae. aegypti*, however, the study also recorded a reduction in blood-feeding. Tests were conducted on cages of females, so results were at a group level, and so it was not possible to control for the effect of blood-feeding. Topical treatment of d-phenothrin or d-allethrin has also been observed to reduce oviposition in *Ae. aegypti*, however, tetramethrin application had no effect, suggesting impact could be insecticide specific (Liu, Todd and Gerberg, 1986).

Oral toxicants and spatial repellents have been observed to affect *Anopheles* blood-feeding and reproductive output. Ingestion of ivermectin has been observed to delay time to re-blood-feeding (Kobylinski *et al.*, 2010; Kobylinski, Ubalee, *et al.*, 2017), inhibit egg development, laying and hatching (Fritz *et al.*, 2009; Sampaio *et al.*, 2016; Lyimo *et al.*, 2017; Dreyer *et al.*, 2019; Mekuriaw *et al.*, 2019). Similarly, volatile pyrethroids, such as transfluthrin and metofluthrin, have been shown to reduce egg laying, dispersal and hatching in *Aedes* mosquitoes (Bibbs *et al.*, 2018, 2019). Knowledge, however, on how insecticides delivered mainly through cuticular penetration (such as in LLINs and IRS) impact on mosquito blood-feeding and reproductive output are limited. Reproductive output is not measured in standard efficacy assays and is often only assessed when evaluating vector control products whose primary mode of action effects mosquito reproduction (e.g. Pyrethroid-PPF nets containing mosquito juvenile hormone analogues) (Ngufor *et al.*, 2014, 2016; Koffi *et al.*, 2015; Toé *et al.*, 2019).

Recently, two studies have considered the impact of insecticidal net exposure on aspects of *Anopheles* reproductive output (Hauser, Thiévent and Koella, 2019; Mulatier *et al.*, 2019). Mulatier *et al.* (2019) investigated the impact of permethrin exposure on several life history traits. Resistant *An. gambiae* were given the opportunity to blood-feed through

permethrin-treated material for 1-hour and the size of their blood-meal and post-exposure fertility and fecundity were recorded. The authors observed no effect on the blood-meal size, number of eggs laid, number of descendants produced, or emergence rate between those exposed to permethrin-treated nets or controls after either a single or double exposure. Using a susceptible *An. gambiae* strain (Kisumu) Hauser, Thiévent and Koella (2019) observed no difference in oviposition when mosquitoes were exposed to Olyset Plus (a pyrethroid-PBO net) compared to untreated, and no difference in egg number when controlling for both mosquito size and blood-meal volume. Egg number increased with blood-meal volume (measured as haematin as a proxy), however, the amount of blood ingested was not affected by net type, regardless of feeding duration. The authors observed a decrease in both biting attempts and time blood-feeding following Olyset Plus exposed mosquitoes compared to controls.

1.5 Mosquito behaviour

In mosquitoes, host-seeking behaviour has been defined as the orientation from a distance in search of a potential blood-meal (Bowen, 1991; Takken, 1991). Mosquitoes use a number of sensory modalities (e.g. olfactory, thermal and visual cues) to locate and select preferred host species and individuals (Bowen, 1991; Zwiebel and Takken, 2004; McMeniman *et al.*, 2014). These senses work in combination, gating, augmenting and modifying responses to other cues, both antagonistically and synergistically, and differentiating the behavioural effect of single cues is challenging. The ability of these cues to mediate mosquito behaviour and their degree of influence, varies spatially. Over long-distances visual and olfactory cues play a major role in activating a behavioural response, whereas at short-ranges thermal cues and moisture levels are more important (Sutcliffe, 1994; Cardé, 2015).

Mosquitoes are initially stimulated to orient towards a host by carbon dioxide (CO_2), which activates take-off and sustains flight (Rudolfs, 1922). Studies have shown that mosquitoes respond to CO_2 when it is delivered in intermittent pulses or when concentration is varied, rather than in response to steady flow or at a defined threshold (Omer and Gillies, 1971; Gillies, 1980). It is likely this is how CO_2 is perceived by mosquitoes under natural circumstances as air currents affect odour plumes causing concentration changes. At short distances olfactory cues (e.g. CO_2, lactic acid) and heat work in combination to stimulate

mosquito landing and probing behaviour (Howlett, 1910; Gillies, 1980; McMeniman *et al.*, 2014). CO_2 alters attractiveness to odour (e.g. pulsed CO_2 has been observed to significantly enhance *Ae. aegypti* attractiveness to lactic acid (Acree *et al.*, 1968)), and heat (e.g. *Ae. aegypti* landed on a heated target when pulsed CO_2 was present, but this effect was absent in homozygous Gr3 mutants which could not detect CO_2 (McMeniman *et al.*, 2014)), and heat enhances the effect of odour (Healy *et al.*, 2002; Olanga *et al.*, 2010; McMeniman *et al.*, 2014). In nature, mosquitoes search for hosts in sensory-rich environments, where they are unlikely to encounter cues singularly.

Body odour cues, from volatile organic compounds contained in sweat, have been observed to stimulate host-seeking at short and long-range, prompting flying, landing, and probing behaviour (De Jong and Knols, 1995; Gibson and Torr, 1999; Healy and Copland, 2000). Body odour cues play an important role in host preference, enabling mosquitoes to discriminate between humans and other animals. Composition and production rate of volatile organic compounds vary between and within species, this results in differences in host attractiveness, which in turn affects disease transmission (Acree *et al.*, 1968; Zwiebel and Takken, 2004; Smallegange, Verhulst and Takken, 2011).

Mosquito eyes have a poor resolution but high sensitivity (Muir, Thorne and Kay, 1992), and visual cues are used when following odour plumes using optomotor anemotaxis (upwind flight using optical feedback from the ground to assess progress) even in extremely low light settings (Gillies, 1980; Gibson and Torr, 1999). Responses to visual cues vary depending on if the mosquito is active during the day (diurnal) or night (nocturnal). Diurnally-active species have been shown to respond better to colour and brightness (Allan, 1987), whereas nocturnal species are believed to be limited to distinguishing only conspicuous objects, using visual contrast to mediate flight (Bidlingmayer, 1994).

1.5.1 The impact of insecticidal net exposure on mosquito behaviour

The potential impacts of insecticidal net exposure on mosquito behaviour at a population level (e.g. changes to peak biting times or shifts in host-preference) are discussed above (Section 1.3.4 Behavioural resistance). At an individual level, host-seeking, host-choice and blood-feeding are key behaviours which influence a mosquito's vectorial capacity and disease transmission potential. The most effective control methods exploit these

characteristic behaviours, *e.g.* in the case of *Anopheles*, human-occupied insecticidal nets act as a baited-trap, capitalising on the anthropophilic and endophilic behaviours of the vector. The nets effectiveness is based on its ability to kill the mosquito (conferring both personal and community protection) or alter its behaviour in a way that reduces infectious bites (personal protection). Such behavioural impacts could be through effects on host-seeking or blood-feeding. However, nets might also be simply repellent. Repellence encompasses both non-contact or true repellence, where the mosquito is repelled by the LLIN after detecting volatile compounds in the air which emanate from the net, or contact irritancy, also termed excito-repellency, where the mosquito moves away from the net following physical contact (Grieco *et al.*, 2007).

Net repellence provides personal protection if it inhibits mosquitoes from biting. However, a modelling study found non-repellent lethal nets were the most effective at providing malaria protection, even when they offered no personal protection (the mosquito was killed only after feeding) (Killeen *et al.*, 2011). Lethal but slightly repellent nets were superior to wholly repellent net, highlighting the importance of community protection to both net-users and non-users alike. Under semi-field conditions, LLINs are frequently documented to stimulate mosquito exit from experimental huts (Strode *et al.*, 2014). In individual experimental hut studies, differences in deterrence (reduction in hut entry of mosquitoes) and exophily (proportion of mosquitoes captured in veranda/exit traps) between huts containing insecticidal or untreated nets points to impact of insecticide exposure. In some cases, this can be explained by variations in natural mosquito behaviour, (Okumu *et al.*, 2013); for example, high exiting rates were observed for *An. arabiensis*, which is a more exophagic and exophilic vector (Sinka, Bangs, *et al.*, 2010), for all study arms, with little difference between treated and control huts.

Net repellence varies depending on insecticide (Siegert, Walker and Miller, 2009). For example, compared to untreated netting, non-lethal exposure to permethrin (Olyset Net) and deltamethrin (PermaNet 2.0) netting caused reduced landing and increased flight in *An. gambiae*. However, the response was stronger to permethrin netting compared to deltamethrin (Siegert, Walker and Miller, 2009). Net repellence has also been seen to vary between mosquito species (Kawada *et al.*, 2014), and effects could possibly be alerted by insecticide exposure history (Mulatier *et al.*, 2019). Following a secondary exposure to permethrin-treated nets, Mulatier *et al.* (2019) observed significantly higher blood-feeding

rates in resistant *An. gambiae* pre-exposed to permethrin-treated nets compared to mosquitoes pre-exposed to control nets, suggesting pre-exposure to the insecticide reduced its blood-feeding inhibition effect. In the secondary exposure, flight-activity was increased in permethrin-exposed mosquitoes compared to untreated, suggesting permethrin irritancy is still present, although this may not affect blood-feeding. Flight activity was not affected by permethrin pre-exposure and was not measured in the first exposure.

Several lab studies have shown that insecticide exposure can reduce subsequent host-seeking and biting behaviours, with this impact persisting for up to 48-hours following insecticide exposure (Glunt *et al.*, 2018; Thiévent *et al.*, 2019). Using the same exposure method discussed previously (LLIN lined WHO tubes), Glunt *et al.* (2018) observed reductions in "host-seeking" (defined as mosquitoes probing at cage sides where host cues were present) following LLIN exposure in resistant *Anopheles* strains and field-caught *An. funestus*. Reductions in host-seeking at 1-hour post-exposure were observed in both *An. arabiensis* (90% reduction) and *An. funestus* (80% reduction) lab-strains, and *An. funestu*s (95% reduction) collected from the field. The field-caught *An. funestu*s were of unknown age, insecticide exposure history, and resistance level, however, were collected from an area of known pyrethroid resistance. In the *An. arabiensis* strain inhibition was seen to persist until 24-hours post-exposure (30% reduction), when the experiment finished. In susceptible *An. gambiae* LLIN exposure has been shown to affect mosquito biting behaviour (Hauser, Thiévent and Koella, 2019). In this study, time to start of biting was increased while time to blood-feeding and proportion attempting to bite decreased following pyrethroid-PBO net (Olyset Plus) exposure compared to untreated.

Under more naturalistic setting, room-scale video tracking systems have been used to visualise mosquito behaviour in response to a whole human-baited net, both using laboratory (Parker *et al.*, 2015; Angarita-Jaimes *et al.*, 2016) and wild-reared field (Parker *et al.*, 2017; Murray *et al.*, 2020) mosquito populations. In their original study, Parker *et al.* (2015) observed reductions in flight activity and net contact when susceptible *An. gambiae* were exposed to deltamethrin net (PermaNet 2.0) compared to controls, however, no repellent effects were seen prior to contact confirming the effect to be as a result of contact irritancy. The authors observed ~75% of mosquito flight activity to be concentrated on the roof of the net. This led to the development of a novel bed-net design (McCall,

2015), which was later shown to target and kill wild-pyrethroid-resistant *An. gambiae* s.l. in Burkina Faso with greater efficacy than standard net designs (Murray *et al.*, 2020). These studies provide a key example of how understanding nuances in individual mosquito behaviour can lead to the development of improved vector control tools to target resistant population.

1.6 Aims and objectives

This project investigated the effects of sub-lethal pyrethroid exposure on, the life-history traits and behaviour of wild pyrethroid-resistant *Anopheles gambiae* s.l., and the efficacy of insecticidal bednets. The specific aims were:

1. To determine the impact of insecticide exposure on the longevity of *Anopheles* by measuring daily survival in laboratory and semi-field hut trials (Chapter 3).
2. To design and conduct assays to investigate the impact of insecticidal nets on the long-term fitness of *Anopheles sp.* by measuring reproductive output and ability to blood-feed after exposure (Chapter 4).
3. To evaluate the feasibly of using novel video benchtop assays for describing and quantifying behaviours of *Anopheles* at the insecticidal net interface (Chapter 5).
4. To use the assays developed in objective 3 to characterise the effects of exposure to standard and next-generation nets on the behaviour of *Anopheles* (Chapter 5).

Chapter 2. General methods and characterisation of mosquito population

2.1 Study sites

Burkina Faso is a landlocked country in west-Africa. It is one of the 11 high burden malaria countries highlighted by WHO with an estimated 12,725 malaria deaths and 7.8 million malaria cases in 2018 (WHO, 2019b). Administratively, it is divided into thirteen regions and 45 provinces. Comoé Province (within Cascades region), in the south-west of the country, is where the work reported in this thesis was conducted. Banfora, the province's capital, is located ~450 km south-west of Ouagadougou, the country's capital. Ecologically, the country is divided into three zones; the humid savannah in the south-west, the arid savannah across the centre, and the Sahel in the north. The western-savannah is characterised by a rainy reason from May to September. In the study area, cotton and cereals cultivation are the major agricultural practices, with pesticide use in the cotton industry accounting for >90% of the pesticides used in the country (Ouedraogo *et al.*, 2011).

An. gambiae, *An. coluzzii* and *An. arabiensis* are the major mosquito vectors in the study area. Insecticide resistance in these populations is driven by multiple insecticide resistance mechanism (Ingham, Wagstaff and Ranson, 2018; Ingham *et al.*, 2019; Namountougou *et al.*, 2019; Williams *et al.*, 2019), and insecticide resistance intensity has been observed to dramatically increase over a short time period in the study area (Toe *et al.*, 2014). Standard pyrethroid-only LLINs do not kill the local mosquito population, however next-generation pyrethroid-PBO nets have shown efficacy in experimental hut trials (Toe *et al.*, 2014; Toe *et al.*, 2018)

Field experiments were conducted in Burkina Faso over a three-year period (2016 – 2018). Laboratory bioassays were conducted at CNRFP's insectary in Banfora (10°37' N, 04°46' W), and experimental hut studies at their field station in Tengrela (10°40' N, 04°50' W). The huts are located on the outskirts of Tengrela village adjacent to rice growing fields. Mosquito populations were collected from Tengrela, Yendere (10°12' N, 04°58' W),

Mangodara (9°54' N, 04°20' W), Sitiena (10°36'N, 4°48'W), Toumousenni (10°37' N, 04°55' W), and Toundoura (10°11' N, 04°40' W).

2.2 Mosquito collection, rearing and identification

Mosquitoes used for tests were defined as either 'wild-entering' or 'larval-reared' adults. Wild-entering adults were naturally host-seeking mosquitoes entering the experimental huts in Tengrela. Larval-reared mosquitoes were collected as larvae or pupae from field locations and transported to CNRFP's insectaries in Banfora for rearing. Larvae were collected from June – October each year. Mosquitoes were reared at 25 ± 3°C and 75 ± 25% relative humidity. The daily light: dark cycle was dependant on natural light entering the room. Larvae were fed daily on a dried cat or fish food, and adults were provided with 10% glucose solution soaked onto cotton wool. Pupating mosquitoes were separated daily to ensure the date of emergence was known and only F0 adult females were used for experiments. Adults were morphologically identified as *An. gambiae* complex (Gillies, 1968; Gillies and Coetzee, 1987). A random sub-sample of all test mosquitoes from Tengrela (2016 – 2018) and Yendere (2018) were identified to species level using SINE PCR (Santolamazza *et al.*, 2008). Molecular ID confirmed *An. coluzzii* to be the dominant species of mosquitoes collected from Tengrela, while *An. gambiae* s.s. were more abundant in mosquitoes collected from Yendere (Table 2.1). Species composition of *An. gambiae* s.l. from Mangodara, Toumousenni, and Toundoura has been reported elsewhere (Sanou, 2020).

Table 2.1. SINE PCR (Santolamazza et al., 2008) species identification of a sub-sample of mosquitoes used in all tests from the study side in Burkina Faso from 2016 – 2018.

Study site	Year	Total ID	*% An. coluzzii*	*% An. gambiae*	*% An. coluzzii/ gambiae* hybrid	*% An. arabiensis*	% Unknown
Tengrela	2016	146	81.51	2.74	0.68	0.00	15.07
	2017	437	87.41	2.97	1.14	0.23	8.24
	2018	125	84.00	10.40	0.00	0.00	5.60
Yendere	2018	203	2.46	90.15	0.49	0.49	6.40

2.3 Mosquito status

For wild-entry mosquitoes age, mating status, feeding status, and previous insecticide exposure is unknown. For larval-reared mosquitoes age range is known, mosquitoes are held with males prior to testing but spermatheca were only dissected for some females during fertility assays (Chapter 4), so mating status is unknown. Mosquitoes were non-blood-fed and not exposed to insecticide prior to testing.

In experimental hut trials and WHO tube assays mosquitoes were not starved prior to testing. In video cone tests, video baited box tests and re-feeding assays, adult mosquitoes were sugar and water starved prior to testing. The duration of starving, and the times of tests are described in detail in each study's methodology.

2.4 Mosquito resistance testing

WHO susceptibility and intensity bioassays (WHO, 2016) were used throughout the testing period, during the wet season (June – November), to determine the insecticide resistance level of the field populations to pyrethroids and organophosphates. 3 to 5-day-old non-blood-fed female mosquitoes were exposed to deltamethrin (0.05, 0.25, 0.50, 0.75, 1.00%) or control papers for 1-hour, or fenitrothion (1.00%) for 2-hours. Mosquitoes were held for 24-hours with access to 10% glucose solution. Mosquito knockdown was counted after 1-hour, and mortality after 24-hours. Mortality was not corrected using Abbott's formula (Abbott, 1987) as control mortality was always <5%.

The Tengrela population was susceptible to the organophosphate fenitrothion (2016; 94.44% 24hr mortality, 90 mosquitoes tested; 2017, 100% 24hr mortality, 98 mosquitoes tested), and extremely resistant to deltamethrin, with less than 80% mortality in all three years when exposed to 5 x the discriminating dose (Figure 2.1). Previous testing in the same study area documented the *An. coluzzii* population to be extremely resistant to permethrin (2014; 14% 24hr mortality, n = 101 mosquitoes, Toe *et al.*, 2018).

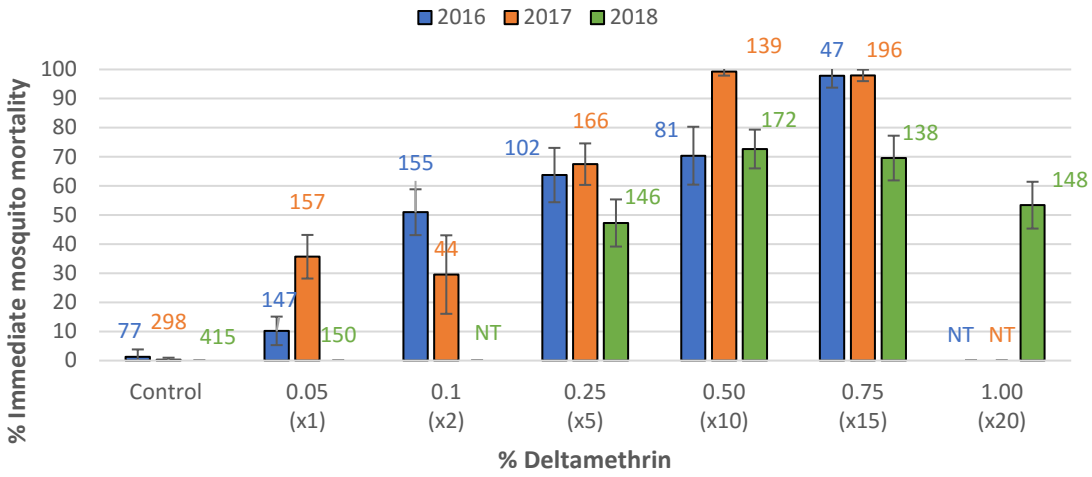


Figure 2.1. 24-hour mortality of An. gambiae s.l. from Tengrela following exposure to deltamethrin in WHO tube bioassays from 2016-2018.

Error bars show 95% confidence intervals for the population proportion. Numbers above bars show the number of mosquitoes tested.

WHO tube bioassays conducted in 2018 showed that the mosquito populations in all other study sites were also highly resistant to deltamethrin (Figure 2.2) with <20% 24-hour mortality after exposure to the standard discriminating dose.

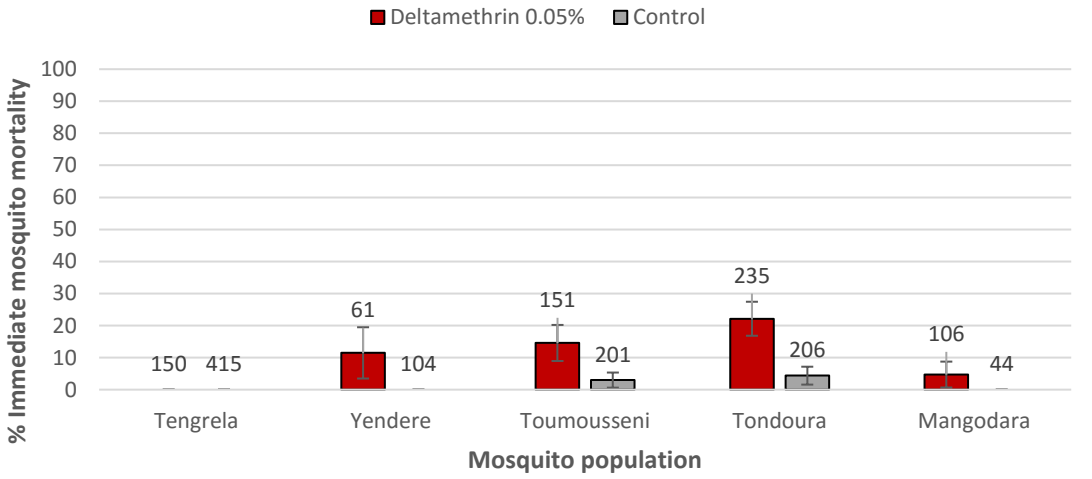


Figure 2.2. 24-hour mortality of An. gambiae s.l. from different study locations following exposure to deltamethrin 0.05% or untreated control papers in WHO tube assays in 2018.

Error bars show 95% confidence intervals for the population proportion. Numbers above bars show the number of mosquitoes tested.

2.5 Net Types

Mosquitoes were exposed to untreated nets, Olyset Net (Manufactured by Sumitomo Chemical Ltd and sourced by CNRFP), PermaNet® 2.0, PermaNet® 3.0 (manufactured and provided by Vestergaard Frandsen), and Interceptor® G2 (manufactured and provided by BASF). Untreated nets were tailored in Burkina Faso from locally sourced netting. Untreated nets were confirmed as non-insecticidal (2016, 0.00% mortality, 99 mosquitoes tested; 2017, 1.89% mortality, 106 mosquitoes tested; 2018 3.52% mortality, 142 mosquitoes tested) with a WHO cone bioassay using Kisumu, a susceptible *An. gambiae* laboratory stain (Williams *et al.*, 2019). The Kisumu strain was originally collected from Kisumu, Kenya, in 1975 and has been held in colony in both LSTMs and CNRFPs insectaries for several years.

Olyset Net is a 150-denier polyethylene first-generation net impregnated with permethrin (20 g/kg ± 3g/kg). PermaNet 2.0 is a 100-denier polyester first-generation net impregnated with deltamethrin (1.4 g/kg ± 25%). PermaNet 3.0 is a next-generation pyrethroid + PBO combination net. The sides are made of 100 denier polyester impregnated with 2.1 g/kg ± 25%). The roof is 100-denier polyethylene treated with deltamethrin (4 g/kg ± 25%) and piperonyl butoxide (25 g/kg ± 25%). Interceptor G2 is a next-generation combination net made of 100-denier polyester treated with alpha-cypermethrin (2.4 g/kg) and chlorfenapyr (4.8 g/kg). Nets were not aired before testing in the 2016 experimental hut trial due to time constraints. All other nets or net fragments/pieces were aired (indoors in a large ventilated room away from direct sunlight) for a minimum of 1 week before testing, and all testing took place within six months of nets being aired. Nets were acclimatised to the temperature of the testing room or experimental hut for at least 1-hour before experiments began.

2.6 Hosts

Humans acted as hosts to attract mosquitoes during the following experimental assays: experimental hut trials, video cone tests, baited box test, and re-feeding assays. Volunteers were requested to refrain from using scented substances before the experiments began. In experimental hut trials, volunteers were requested to remain inside the hut throughout the

night where possible, and if they should exit the huts, to ensure the doors were quickly closed behind them. A trial supervisor was present overnight to assist volunteers.

2.7 Ethics

Ethical approval for the for experimental hut trials was received from the Research Ethics Committees at the Liverpool School of Tropical Medicine (LSTM Research Protocol 16-38, Liverpool) and Centre National de Recherche et de Formation sur le Paludisme (CNRFP Deliberation no. 2016-9-097, Ouagadougou). Informed written consent was obtained from all volunteers, and no mosquito-borne infections, or adverse effects, were reported during the study.

Chapter 3. Longevity of adult female *Anopheles gambiae* following sub-lethal pyrethroid exposure[1]

3.1 Introduction

Insecticide resistance status in mosquito vectors is assessed based on standardised tests using pre-defined discriminating doses (WHO, 2016). Typically, these assays measure mosquito knockdown and immediate mortality (i.e. within 24-hours of exposure), and although this provides valuable data for resistance monitoring, its predictive use for malaria epidemiology is unclear, particularly as these discriminating doses are not representative of insecticide concentrations mosquitoes encounter in the field following contact with vector control tools. Additionally, these tests fail to measure any sub-lethal and delayed effects (e.g. impaired feeding, reduced longevity) which could impact on the mosquitoes' vectorial capacity and subsequently affect their malaria transmission potential. Furthermore, by focusing solely on mortality at 24-hours post-exposure, these tests may not be fit-for-purpose for evaluating 2nd-generation LLINs containing slow-acting insecticides or insect growth regulators, or other novel vector control tools whose primary mode of action may not include rapid knockdown and mortality like the pyrethroids. It is vital that we improve our knowledge of the long-term impacts that all currently available classes of bednet have on mosquitoes, including importantly their impact on pyrethroid-resistant field populations, in order to have a better understanding of how resistance is impacting on vector control currently, and in the future.

Delayed mortality, defined as death occurring >24-hours post-insecticide exposure, has been documented in moderate and highly insecticide-resistant mosquito strains following LLIN exposure (Viana *et al.*, 2016). Mathematical models predicted that, when the delayed

[1]At the time of writing, some of the data reported in this chapter has been included in two independent publications:

Murray, G.P.D., Lissenden, N., Jones, J. *et al*. (2020). Barrier bednets target malaria vectors and expand the range of usable insecticides. Nature Microbiology, 5, 40–4. doi:10.1038/s41564-019-0607-2 (Appendix 2);

Hughes, A., Lissenden, N., Viana, M., Toé, K. H., and Ranson, H. (2020). *Anopheles gambiae* populations from Burkina Faso show minimal delayed mortality after exposure to insecticide-treated nets. Malaria Journal, 13(11):17 (Appendix 3).

mortality is considered, exposure to LLINs reduced the mosquito's malaria transmission potential by two thirds, with half of this being as a result of delayed effects, despite the presence of pyrethroid resistance. In wild populations, it is frequently documented that pyrethroid exposure fails to kill resistant mosquitoes within 24-hours (WHO, 2018a). However, data on mosquito lifespan following LLIN exposure in wild populations is restricted to limited evidence from one study (Tchakounte *et al.*, 2019), in which the authors did not separate out the effects of immediate and delayed mortality.

In this chapter, the effect of insecticide exposure on mosquito longevity was investigated in the pyrethroid-resistant mosquito populations from the study site in Burkina Faso.

The objectives of these experiments were to:

- Determine if mosquito longevity was reduced following single or multiple exposures to LLINs in WHO cone bioassays.
- Determine if mosquito longevity was reduced following exposure to LLINs in a semi-field environment in experimental hut studies.
- Determine if the longevity of mosquitoes was affected by the concentration of pyrethroids in WHO tube tests.
- Determine if mosquito age at the time of exposure affected mosquito longevity.

3.2 Methods

3.2.1 Study sites, mosquitoes and net treatments

Hut trials were performed at the experimental hut station in Tengrela, Burkina Faso, and all laboratory bioassays at the CNRFP insectaries in Banfora, Burkina Faso. Mosquitoes used for tests were defined as either 'wild-entering' or 'reared-release' adults, and were collected, reared and identified using the methods described in Chapter 2. Mosquitoes were exposed to untreated, PermaNet 2.0, or Olyset Net netting during tests (full nets specifications can be found in Chapter 2, Section 2.5).

3.2.2 Longevity testing: WHO tube bioassays

WHO tube bioassays (WHO, 2016) were used to compare mosquito longevity following exposure to insecticide papers. Mosquitoes from multiple populations were exposed to the discriminating doses of deltamethrin (0.05%), bendiocarb (0.1%), malathion (5%), propoxur (0.1%) or an untreated control for 1 hour. To establish if increasing the concentration of insecticide affected mosquito longevity, populations were also exposed to deltamethrin papers treated at 0.05, 0.25, 0.50, 0.75, or 1.00%. Mosquitoes were 3 to 5-days-old non-blood-fed larval-reared females. After exposure mosquitoes were provided with 10% glucose solution soaked onto cotton wool. Mortality was recorded daily until no mosquitoes remained alive, and dead mosquitoes were stored in silica. All longevity tube exposures were conducted in 2018.

3.2.3 Longevity testing: WHO cone bioassays

The effect of varied LLIN exposure on *An. gambiae* longevity

This experiment aimed to establish if mosquito longevity was affected by different durations of exposure to insecticide-treated netting (PermaNet 2.0). Mosquitoes were exposed to untreated or PermaNet 2.0 netting singularly or multiple times depending on the experimental regime (Table 3.1) using a WHO cone bioassay (WHO, 2013a). Mosquitoes were reared from larvae, 3-to-8-days-old on first exposure (Table 3.2), female, and non-blood-fed. Each exposure lasted for 3-minutes and after exposure mosquitoes were provided with 10% glucose solution soaked onto cotton wool. Mortality was recorded daily until no mosquitoes remained alive, and dead mosquitoes were stored in silica. Mosquitoes were held in an experimental hut in Tengrela or the insectary in Banfora. A preliminary study showed no difference in survival based on mosquito holding locations (P = 0.204, 25 mosquitoes stored in an experimental hut, 25 mosquitoes stored in the Banfora insectary).

The different exposure regimes approximate various types of exposure to LLINs that mosquitoes may experience in the wild (Viana *et al.*, 2016). Regime A (single exposure) provided a baseline level of net contact to compare untreated and treated netting. Regime B (exposure every 4 days for 4 exposures) simulates the level of net contact a mosquito might encounter once every gonotrophic cycle whist host seeking. Regime C (daily

exposure for 5 days) simulates the net contact a mosquito might encounter if it is repeatedly prevented from obtaining a blood meal.

Table 3.1. Exposure regime summarising number and day of exposures for each longevity experiment carried out in Burkina Faso using a WHO cone bioassay.

Experiment ID	Net exposure (times exposed)	Day of exposure												
		0	1	2	3	4	5	6	7	8	9	10	11	12
A	Single (x 1)	✓												
B	Multiple (x 4)	✓				✓				✓				✓
C	Multiple (x 5)	✓	✓	✓	✓	✓								

Table 3.2. Summary of experimental factors for each longevity experiment carried out in Burkina Faso using a WHO cone bioassay.

Different populations of mosquitoes were exposed to untreated or PermaNet 2.0 netting, single or multiple times and their post exposure longevity was recorded.

Experiment ID	Net exposure (times exposed)	Mosquito population	Age (days) at first exposure	Date conducted
A	Single (x 1)	Yendere	3 – 5	July 2018
A	Single (x 1)	Tengrela	5 – 8	September 2017
B	Multiple (x 4)	Tengrela	4	August 2018
C	Multiple (x 5)	Tengrela	4	September 2018

The effect of *An. gambiae* age at *LLIN* exposure on longevity

A preliminary assay also looked at the effect of age at first exposure on mosquito immediate mortality, with a subset of mosquitoes additionally scored for longevity. In this

assay larval-reared mosquitoes were 1 or 7 days-old at first exposure. Mosquitoes were only exposed to PermaNet 2.0 in a standard 3-minute cone assay.

3.2.4 Experimental hut trials

Experimental hut trials were used to expose mosquitoes to untreated or treated netting under semi-field conditions. Mosquitoes could contact the nets throughout the night, and as a result, the overall duration of net contact is unknown. Mosquito longevity data were collected from three different hut trials; a reared-release trial, a wild-entry trial, and a trial assessing the efficacy of a novel design of bed net (referred to as the barrier bed net trial, Murray *et al.*, 2019). The methodological differences (Table 3.3.) and the aims of these trials are discussed below. All trials were replicated in 2016 and 2017.

The six experimental huts in Tengrela are situated between the rice fields and the village (Figure 3.1). The huts were built in 2013 to the standard West-African design (WHO, 2013a), and have been used previously for phase II net efficacy trials (Toe *et al.*, 2018). The huts (3.5 x 2 x 2m high) are made of concrete with a corrugated iron roof, polythene ceiling and a veranda trap. They are housed on top of a concrete base with a water-filled moat to prevent the entry of ants and other scavengers. In their standard configuration, the hut windows are shuttered and made from angled metal containing a 1 cm entry slit. This design permits mosquito entry into the huts but impedes their exit.

In all hut trials nets were unwashed with holes, based on standard WHO guidelines (WHO, 2013). Six 4 cm^2 holes were cut into the nets (two on each of the long sides and one on the short sides of the net). Nets were aired for a minimum of one week prior to experiments (except for the 2016 hut trials where nets were used on the same day, without airing). Nets were hung in the main sleeping area of the hut over a sleeping mat. Volunteers rotated between huts daily, and nets between huts weekly, to control for biases in the hut or volunteer attractiveness. Not all six huts were used in all trials. Trials ran on non-consecutive days, and in some cases did not complete full rotations through huts due to logistical constraints. Because of this, some net treatments or volunteers spent unequal time in each hut (Appendix 1, Table A1.2 – A1.8).

Figure 3.1. Photograph of the six experimental huts in Tengrela, Burkina Faso.
The huts are built to the standard West-African design (WHO, 2013) and are situated between Tengrela's rice growing fields and the village, ~ 100 m from the rice fields and ~200 m from human habitation.

Volunteers entered the huts at ~20:00 and positioned themselves under the nets. Nets were tucked under the sleeping mat. Volunteers slept under the nets and exited the huts the following morning after mosquito collection at ~06:30. Mosquitoes were individually captured using glass universal tubes and placed into labelled bags to separate samples by collection location (i.e. in net, in the veranda, in the main hut). Huts were checked for uncaught mosquitoes using an electronic Prokopack aspirator. As it was not possible to identify their collection location, mosquitoes collected with a Prokopack aspirator were stored separately. Following collection, mosquitoes were morphologically identified (Gillies, 1968; Gillies and Coetzee, 1987), sexed, recorded as dead or alive, and scored for abdominal status (unfed, partially-fed, blood-fed, semi-gravid/gravid). Dead female *Anopheles* mosquitoes were stored in silica, and male *Anopheles* and non-*Anopheles* were recorded and discarded.

Surviving female *Anopheles* were transferred from universal tubes and pooled into paper cups (maximum 10 mosquitoes per cup). In 2016 trials, mosquitoes were pooled into unfed, and blood-fed (partially blood-fed, fully blood-fed, semi-gravid, and gravid) groups.

In 2017 trials, mosquitoes were pooled into unfed, blood-fed (partially blood-fed, fully blood-fed), and gravid (semi-gravid, gravid) groups. Cups were stored in racks in an experimental room in Tengrela and mosquitoes were provided with 10% glucose solution. Cups were identifiable by experimental day, hut number, collection location, and feeding status. Mortality was recorded daily until no mosquitoes remained alive, with dead mosquitoes stored in silica.

The primary outcome of interest from these hut trials was the effect of LLIN exposure on mosquito longevity. However, the modes of action of LLINs stem beyond this effect, and so the standard outcomes of an experimental hut trial were also collected and defined as follows:

- Deterrence: the reduction in hut entry of mosquitoes in treatment huts relative to untreated huts (note this data was not available for reared-release trials).
- Exophily: mosquitoes found in veranda as a proportion of the total number collected in the hut.
- Induced exophily: the increase in mosquitoes in the veranda in treatment huts compared to untreated huts.
- Blood-feeding inhibition: the reduction in blood-feeding mosquitoes in treatment huts compared to untreated huts.
- Dead when collected and 24-hour mortality: mosquitoes found dead on collection or dying within the first 24-hours as a proportion of those collected. The standard WHO definition defines mosquitoes dead when collected as immediate mortality and those dying by 24-hours as delayed mortality (WHO, 2013a). In the following trials, 24-hour mortality was instead described as immediate mortality to align with definitions used for tube and cone assays, and to differentiate it from delayed mortality which referred to post-exposure longevity.
- Longevity: the day of mosquito death, where day 0 is the day of its collection from the experimental huts.
- Personal protection (%) calculated as:

$$100 \times \frac{(Blood\ fed\ mosquitoes\ in\ untreated\ hut - Blood\ fed\ mosquitoes\ in\ treated\ hut)}{Blood\ fed\ mosquitoes\ in\ untreated\ hut}$$

- Killing effect (%) calculated as:

$$100 \times \frac{(Mosquitoes\ killed\ in\ treated\ nets - Mosquitoes\ killed\ in\ untreated\ nets)}{Total\ mosquitoes\ collected\ in\ untreated\ hut}$$

Experimental hut trials of reared and release *An. gambiae* ('reared-release')

To standardise the age range of the mosquitoes tested, reared-release trials used adult female mosquitoes reared from larvae collected locally. Mosquitoes were reared from larvae to adults, which at 5-to-8-days-post-emergance, were released into the huts on the evening of each experimental day and recaptured the following morning. Huts were sealed to inhibit the exit of released mosquitoes and the entry of wild mosquitoes. Window shutters were closed, the entries filled with untreated netting, and the interior side screened (Figure 3.2). The door frames were covered with overlapping untreated netting. In the evening, volunteers positioned themselves under the test nets. Mosquitoes were acclimatised to the test hut for > 10-minutes before ~25 *Anopheles* females were manually released into the hut and allowed to host-seek.

Figure 3.2. Photographs of the adaptations to the experimental huts for the reared-release experimental hut trials.
Doors were covered with overlapping untreated netting (left). Windows entries were filled with untreated netting and the interior screened with untreated mesh (right).

Experimental hut trials of wild-entering *An. gambiae* ('wild-entry')

The primary objective of the wild-entry trial was to examine how LLIN exposure affected mosquito reproductive output (reported in Chapter 4). Subsequently, in this trial longevity

was only recorded for non-blood-fed mosquitoes, as blood-fed mosquitoes were used to assess reproductive output. Huts were unaltered from the standard WHO protocol (WHO, 2013a) and mosquitoes were those freely entering huts while host-seeking. The age and previous insecticide exposure of this population is unknown.

Experimental hut trial evaluating the efficacy of barrier bednets ('barrier bednet')

The barrier bed net trials aimed to assess the efficacy of a novel design of bed net. Data on mosquito longevity was collected as a secondary outcome for all net treatment arms. The primary outcomes of these studies were to assess the effect of different net treatments, with and without different treated barriers, on immediate mosquito mortality, blood-feeding inhibition, deterrence, and repellency. Huts were unaltered from the standard WHO protocol (WHO, 2013a) and mosquitoes were those naturally host-seeking and entering huts. The main results of the 2017 trial are published elsewhere (Murray *et al.*, 2019; Appendix 2), but here the effects of the different barrier treatments on mosquito longevity are reported.

Table 3.3. Summary of experimental factors in longevity hut trials.

Mosquitoes were exposed to untreated or treated nets under semi-field conditions and their post-exposure longevity was recorded.

Trial ID	Date conducted	Number of nights	Net treatments	Number of huts used
Reared-release	26 September – 3 October 2016	6	Untreated PermaNet 2.0	2
	10 – 22 September 2017	10	Untreated PermaNet 2.0	2
Wild-entry	10 – 21 October 2016	10	Untreated PermaNet 2.0	2
	2 – 14 July 2017	12	Untreated PermaNet 2.0 Olyset Net	6
Barrier bednet	26 September – 20 October 2016	16	Untreated PermaNet 2.0 PermaNet 2.0 + PN2 Barrier PermaNet 2.0 + OP Barrier	4
	16th July – 25th August 2017	36	Untreated PermaNet 2.0 PermaNet 2.0 + PN2 Barrier PermaNet 2.0 + NPI Barrier PermaNet 2.0 + OP Barrier Untreated + OP Barrier	6

Abbreviations: PN2 (PermaNet 2.0); NPI (non-pyrethroid insecticide); OP (organophosphate fenitrothion).

3.2.5 Data analysis

In all experiments, to compare immediate mortality (within 24-hours following exposure) Pearson Chi-Square was used. When the assumptions of this test were violated due to low expected frequencies Fisher's Exact Test was used. If a mosquito was censored (e.g. mosquito escaped) during the 24-hours following exposure, it was removed from the immediate mortality analysis. Cox proportional hazard regression (Cox, 1972) was used to examine the effect of all predictor variables (i.e. date, blood-feeding status, hut, net treatment, and collection locations (e.g. in net, in veranda)) on mosquito survival post-exposure (longevity). When variables were non-significant, they were removed from the regression and it was re-run.. When experiments were stratified by blood-feeding status mosquitoes were pooled into blood-fed (partially-blood-fed, blood-fed, semi-gravid, and gravid) and unfed groups.

Kaplan-Meier curves were produced to visualise daily survival probabilities, and box and whisker plots were used to visualise the spread of the data. When investigating the effects on longevity, immediate mortality (mortality 24-hours following any exposure) was excluded from all data sets. In experimental hut trials, immediate mortality additionally included mosquitoes that were dead on collection in the huts, as well as those that died within the first 24-hours. Results from each year were analysed separately. The analysis was conducted in IBM SPSS Statistics 24.

In hut trials, the proportion of mosquitoes collected dead (mortality), blood-fed (blood-feeding), and in the veranda (exophily) were compared between treated and untreated nets using generalised linear mixed effects models (GLMMs) with a binomial distribution and logit link function. The number of mosquitoes entering huts was compared using GLMMs with Poisson distribution and a log link function, or a negative binomial distribution to account for overdispersion. Sleeper, hut, and day were all included as random effects in the models, except in cases where they failed to converge and were re-ran as fixed effects. The analysis was conducted within R statistical software version 3.4.1 (2017-06-30) (R Core Team, 2017) using the lme4 (Bates *et al.*, 2015), and glmmADMB (Fournier *et al.*, 2012) packages. Model parameters are listed in Appendix 1, Table A1.9

3.3 Results

3.3.1 Mosquito longevity: WHO tube assays

This experiment investigated if the immediate mortality and longevity of mosquitoes was affected by exposure to the discriminating doses of several insecticides in WHO tube tests. Additionally, it aimed to establish if increasing the dose of deltamethrin affected mortality or longevity.

Following exposure to the discriminating dose of deltamethrin (0.05%), no difference in immediate mortality (Figure 3.3) or longevity (Table 3.4) were observed compared to untreated paper controls in all study populations.

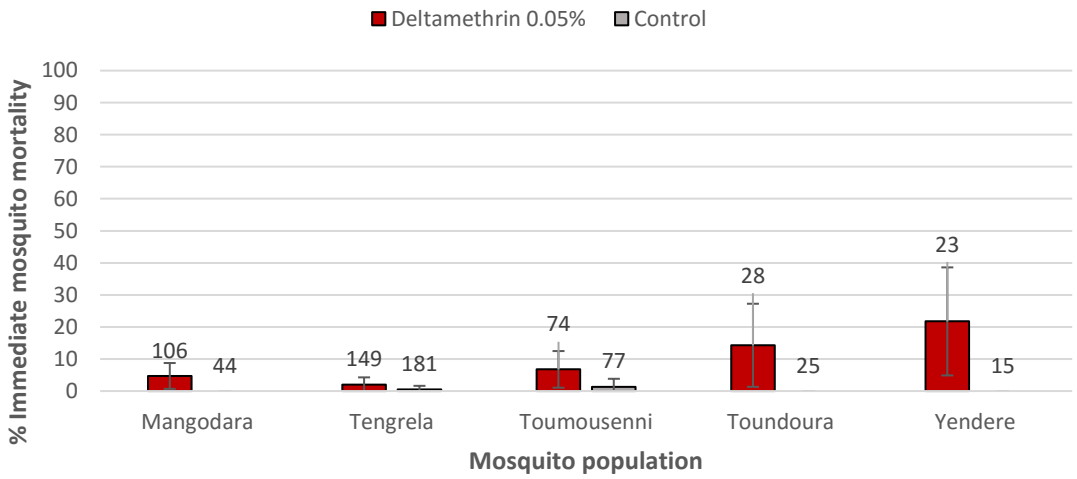


Figure 3.3. 24-hour mortality of An. gambiae s.l from WHO longevity tube assay. *Mosquitoes from the different locations were exposed to deltamethrin 0.05% (red) or untreated control (grey) papers. Error bars show 95% confidence intervals for the population proportion. Numbers above bars show the number of mosquitoes tested. Mangodara P = 0.322, Tengrela P = 0.331, Toumousenni P = 0.210, Toundoura P = 0.113, Yendere P = 0.136.*

Table 3.4. Summary of longevity analysis of An. gambiae s.l. from multiple study sites following exposure to the discriminating dose of deltamethrin, or control papers, in a WHO tube assay.

Survival was compared between treatment and control using Cox regression. Immediate mortality (within 24-hours) was excluded from the analysis.

Mosquito population	Insecticide	Total mosquitoes	Median survival (days)	P-value
Mangodara	Control	44	11.5	0.308
	Deltamethrin 0.05%	101	11	
Tengrela	Control	180	9	0.172
	Deltamethrin 0.05%	146	10	
Toumousenni	Control	70	10	0.568
	Deltamethrin 0.05%	69	10	
Toundoura	Control	25	10	0.334
	Deltamethrin 0.05%	24	13	
Yendere	Control	15	11	0.345
	Deltamethrin 0.05%	18	10	

Mosquitoes from Tengrela were also exposed to increasing concentrations of deltamethrin. The discriminating dose had no effect on immediate mortality compared to untreated, but as the concentration was increased, a significant difference was observed following exposure to concentrations >0.05% (Figure 3.4). When immediate mortality was excluded, a significant difference in longevity was observed at doses >0.50% (Figure 3.5, Table 3.5). These results were additionally modelled using a Bayesian state-space survival model developed by Viana *et al.* (2016). This quantified the daily survival rate and the magnitude of any observed delayed mortality effect in each exposure and found evidence of delayed mortality at deltamethrin concentrations > 0.05% (Hughes *et al.*, 2020, Appendix 3).

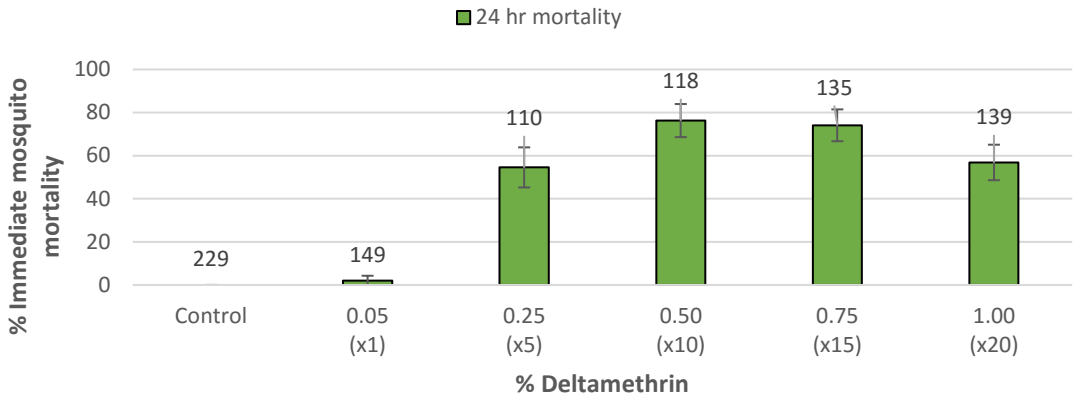


Figure 3.4. The 24-hour mortality of An. gambiae s.l. from Tengrela following exposure to deltamethrin (0.05, 0.25, 0.50, 0.75, 1.00%) or an untreated control WHO tubes.

Error bars show 95% confidence intervals for the population proportion. Numbers above bars show the number of mosquitoes tested. Deltamethrin 0.05% P = 0.136, 0.25% P = 0.000, 0.50% P = 0.000, 0.75% P = 0.000, 0.75% P = 0.000.

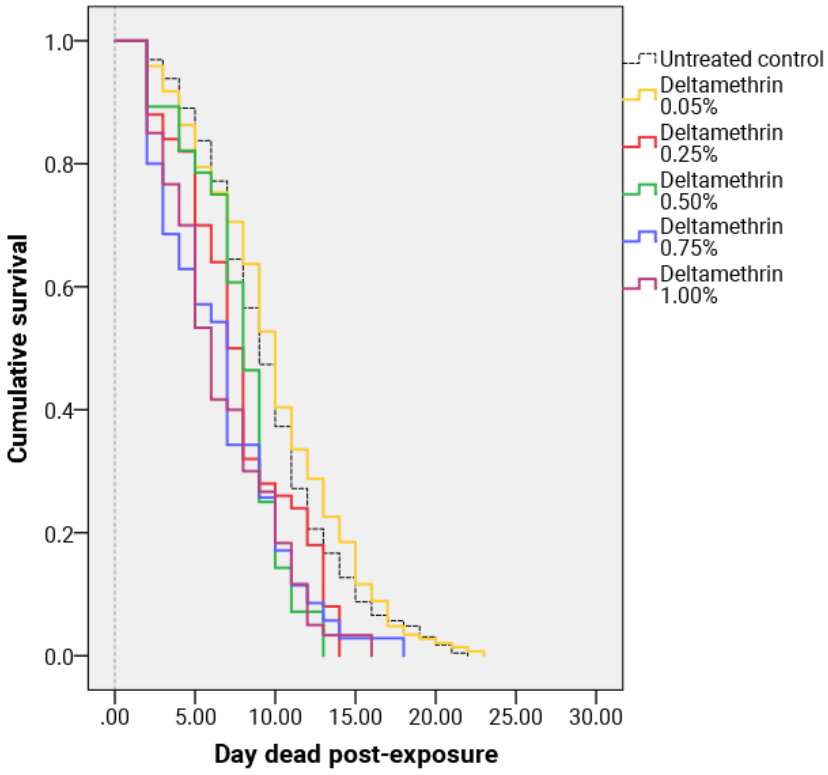


Figure 3.5. The longevity of An. gambiae s.l. from Tengrela following exposure to increasing concentration of deltamethrin, or control papers, in a WHO tube assay.

Following exposure mosquito mortality was counted daily until no mosquitoes remained alive. Kaplan Meier survival curves show day dead post-exposure. The dashed grey line shows the day of exposure.

Table 3.5. Summary of longevity analysis of An. gambiae s.l. from Tengrela following exposure to increasing concentration of deltamethrin, or control papers, in a WHO tube assay.

*Survival was compared between treatment and control using Cox regression. Immediate mortality (within 24-hours) was excluded from the analysis, * indicate statistical significance (P < 0.05).*

Insecticide	Total mosquitoes	Median survival (days)	P-value
Control	180	9	0.172
Deltamethrin 0.05%	146	10	
Control	159	8	0.130
Deltamethrin 0.25%	50	7.5	
Control	159	8	0.098
Deltamethrin 0.50%	28	8	
Control	159	8	0.013*
Deltamethrin 0.75%	35	7	
Control	109	8	0.007*
Deltamethrin 1.00%	60	6	

Mosquitoes were also exposed to organophosphates (i.e. malathion), and carbamates (i.e. propoxur, bendiocarb) and their post-exposure longevity recorded. Greater susceptibility to these insecticide classes resulted in increased immediate mortality compared to the pyrethroids, and therefore low numbers surviving for longevity analysis (Appendix 1, Table A1.1)

3.3.2 Mosquito longevity: WHO cone bioassays

The effect of varied *LLIN* exposure on An. gambiae longevity

Immediate Mortality: The Kisumu susceptible strain showed high immediate mortality with PermaNet 2.0 (98% mortality, n = 48 mosquitoes). Following a single exposure to PermaNet 2.0, 1-hour knockdown and immediate mortality were extremely low in mosquitoes from Yendere (Figure 3.6; 1.94% KD, 1.94% mortality) and Tengrela (Figure 3.6; 10.64% KD, 5.32% mortality). In both locations, there was no difference in mortality following exposure to PermaNet 2.0 compared to untreated net (Yendere P = 1.000; Tengrela P = 1.000).

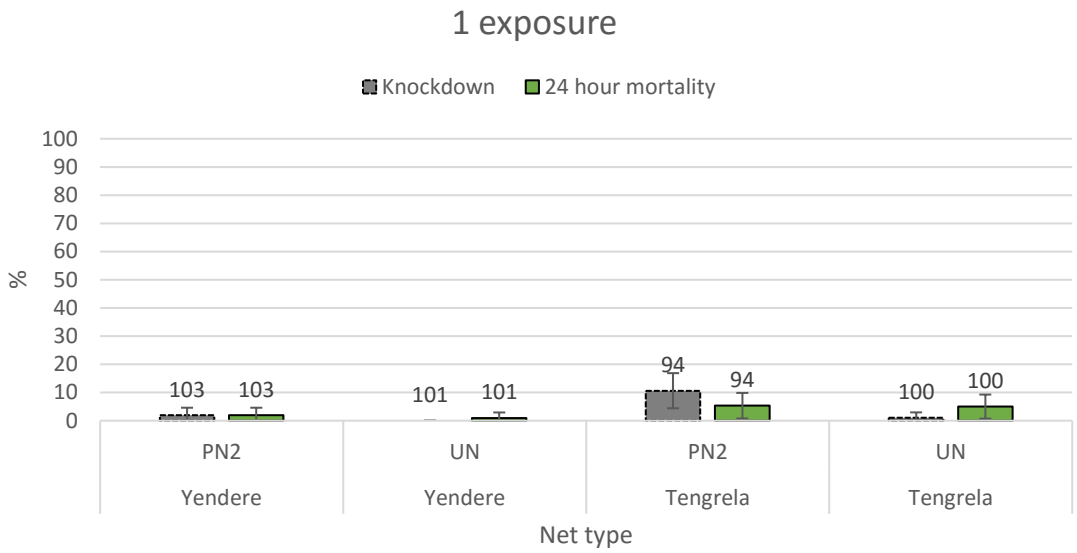


Figure 3.6. 1-hour knockdown and 24-hour mortality of Yendere and Tengrela An. gambiae s.l. after WHO cone bioassay exposure.

Mosquitoes were exposed to PermaNet 2.0 or untreated on Day 1 and their mortality recorded. Error bars show 95% confidence intervals for the population proportion. Numbers above bars show the number of mosquitoes tested. Abbreviations: PN2 = PermaNet 2.0, UN = Untreated net.

When Tengrela mosquitoes were exposed to netting 4 times (every three days), immediate mortality rates increased with each subsequent exposure (Figure 3.7; PermaNet 2.0, 0.85 - 62.50 %; Untreated, 0.00 – 65.52 %). However, a significant difference between net types was only observed after the third exposure (P = 0.011) when mortality was 31.46% for PermaNet 2.0 and 15.66% for the untreated net. As this experiment was observing cumulative mortality the sample size decreased with each round of exposure and by the 4th exposure, fewer than 30 mosquitoes were exposed to each net (PermaNet 2.0 = 24 mosquitoes, untreated = 29 mosquitoes).

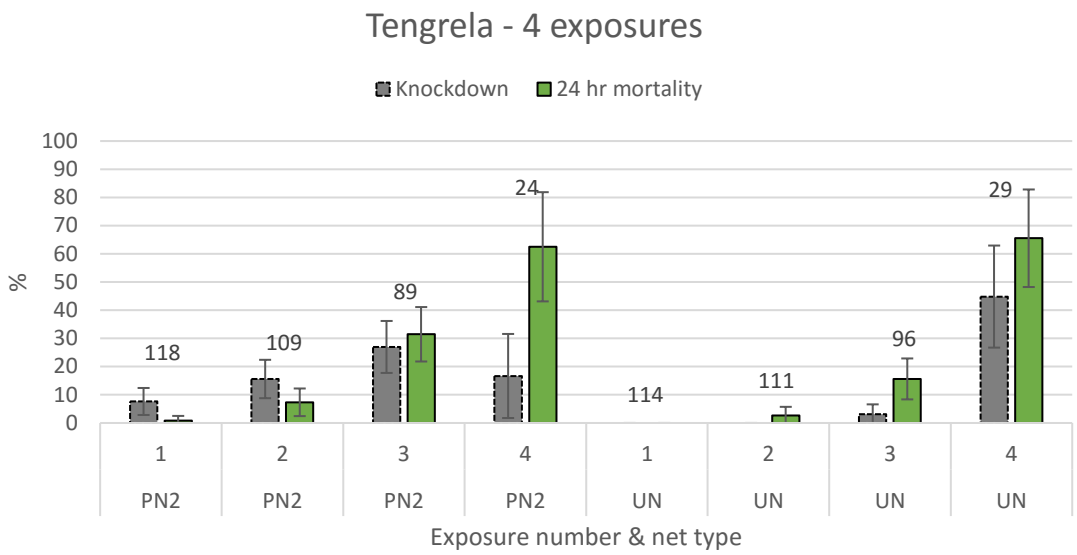


Figure 3.7. 1-hour knockdown and 24-hour mortality of Tengrela An. gambiae s.l. after WHO cone bioassay exposure.

Mosquitoes were exposed to PermaNet 2.0 or untreated net on Days 1, 4, 8 and 12 and their mortality recorded. Error bars show 95% confidence intervals for the population proportion. Numbers above bars show the number of mosquitoes tested. Numbers below the graph show the number of exposures. Abbreviations: PN2 = PermaNet 2.0, UN = Untreated net.

When mosquitoes were exposed daily for 5 exposures, a significant difference in immediate mortality between net treatments was seen for exposure 4 (Figure 3.8; PermaNet 2.0, 11.55% mortality; Untreated, 2.7% mortality; P = 0.032), and exposure 5 (Figure 3.8; PermaNet 2.0, 15.36% mortality, Untreated, 4.44% mortality; P = 0.017). However, the mortality in the treatment arm was still low.

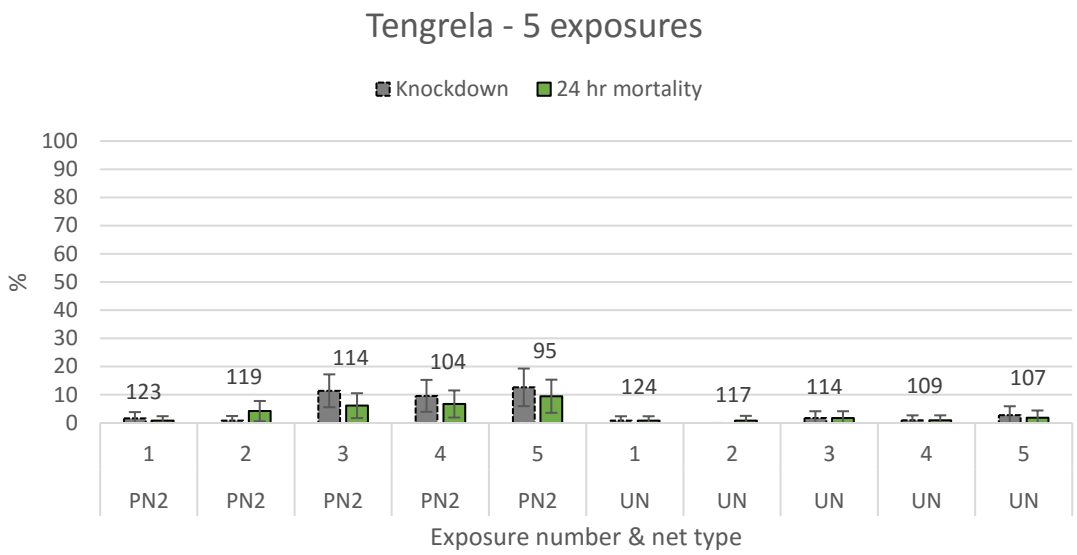


Figure 3.8. 1-hour knockdown and 24-hour mortality of Tengrela An. gambiae s.l. after WHO cone bioassay exposure.

Mosquitoes were exposed to PermaNet 2.0 or untreated net on Days 1, 2, 3, 4 and 5 and their mortality recorded. Error bars show 95% confidence intervals for the population proportion. Numbers above bars show the number of mosquitoes tested. Numbers below the graph show the number of exposures. Abbreviations: PN2 = PermaNet 2.0, UN = Untreated net.

Longevity: A single exposure to PermaNet 2.0 did not affect daily survival 24-hours post-exposure in either population tested (Figure 3.9; Yendere P = 0.518; Tengrela P = 0.266).

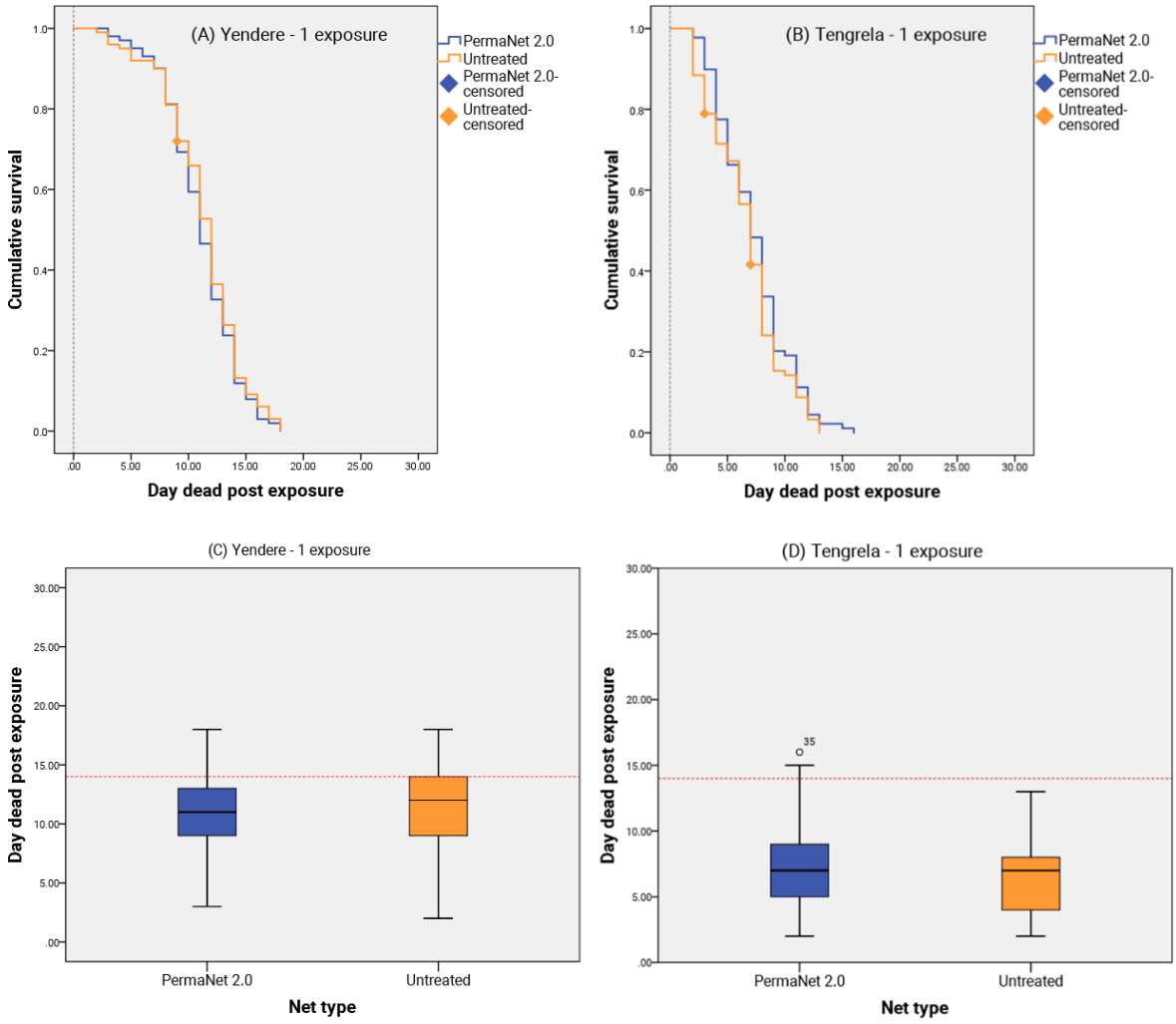


Figure 3.9. The longevity of Yendere (A, C) and Tengrela (B, D) An. gambiae s.l. following a single 3-minute exposure to PermaNet 2.0 (blue) or untreated net (orange) in a WHO cone bioassay.

(A – B) Kaplan Meier survival curves show day dead post-exposure. Dashed grey lines show day of exposure. Diamond points show censored data. (C-D) Box and whisker plots of days dead post exposure. Dashed red lines show day 14 to correspond with the average extrinsic incubation period of the malaria parasite, Plasmodium falciparum. In A-D immediate (within 24-hours of exposure) mortality is excluded.

In all multiple exposure assays, mosquitoes were 4 days-old at first exposure. When Tengrela mosquitoes were exposed to netting every 3 days for four exposures (Day 0, 4, 8, 12), immediate mortality on days 1, 5, 9, and 13 were removed from subsequent longevity analysis. In this experiment net treatment had no effect on longevity (P = 0.718). A similar absence of effect was also seen when mosquitoes were exposed to netting daily for five days (P = 0.097). Mortality on days 1-5 was excluded (Figure 3.10).

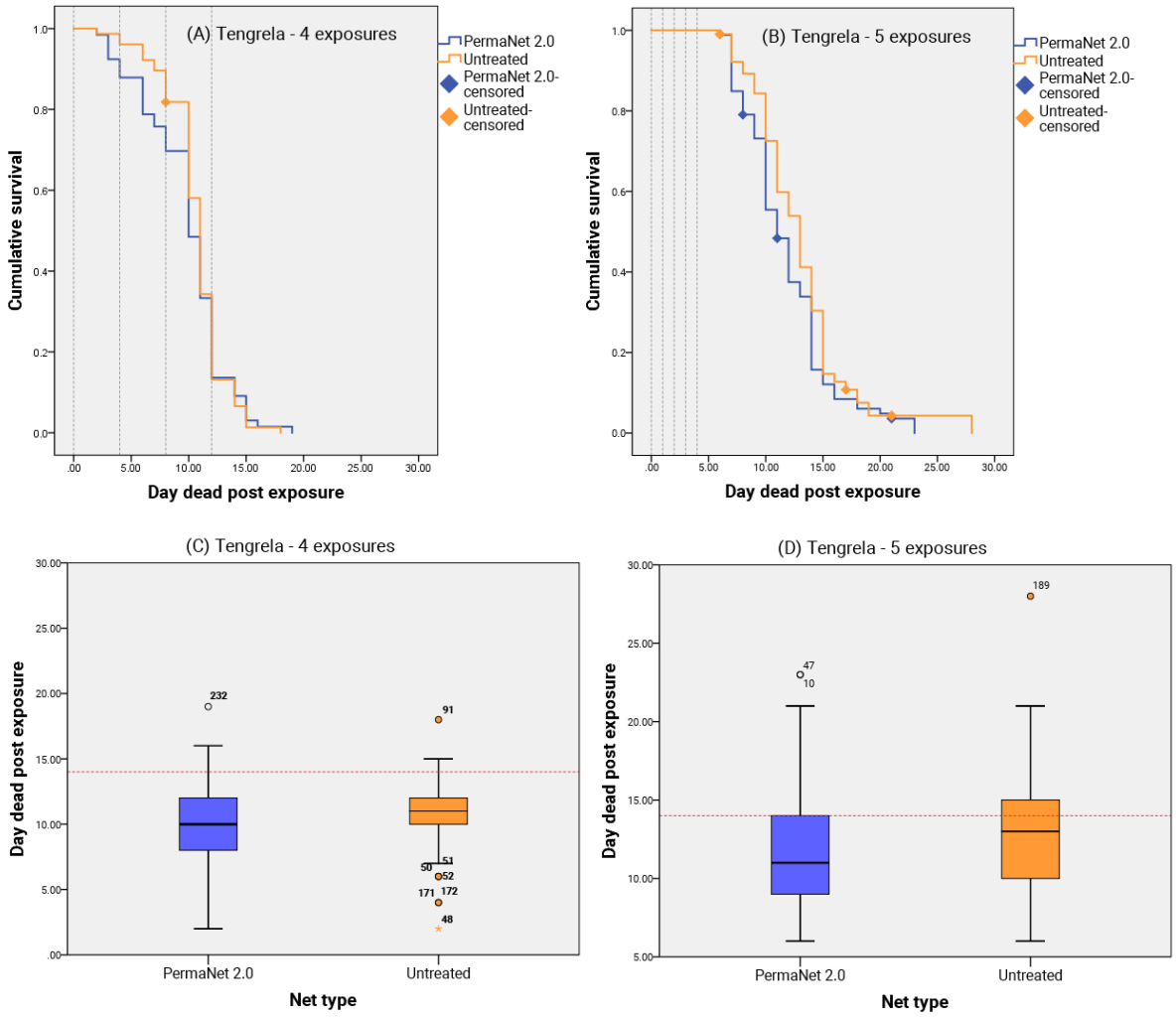


Figure 3.10. The longevity of Tengrela mosquitoes following multiple exposures in a 3-minute WHO cone bioassay. Mosquitoes were exposed every 3 days for 4 exposures (A, C) or daily for 5 exposures (B, D), to PermaNet 2.0 (blue) or untreated net (orange).

(A – B) Kaplan Meier survival curves show day dead post-exposure. Dashed grey lines show the day of exposure. Diamond points show censored data. (C-D) Box and whisker plots of days dead post-exposure. Dashed red lines show day 14 to correspond with the average extrinsic incubation period of the malaria parasite, Plasmodium falciparum (Vaughan, 2007)*. In A-D immediate (within 24-hours of exposure) mortality is excluded.*

The effect of *An. gambiae* age at LLIN exposure on longevity

When mosquitoes of differing age were exposed to PermaNet 2.0 in cone tests, immediate mortality significantly increased from 2.00% in 1-day-olds (n = 100 mosquitoes) to 11.88% in 7-day-olds (n = 101 mosquitoes) (P = 0.006). Excluding immediate mortality, mosquitoes

exposed at 1-day-old (n = 27 mosquitoes) survived significantly longer than mosquitoes exposed at 7-days-old (n = 23 mosquitoes), with those exposed at 1-day-old having a 0.311-fold (0.169 – 0.571) reduction in risk of death (
Figure **3.11**, P = 0.000). However, when comparing age at death, mosquitoes that were exposed at 1-day-old were on average younger (11-days-old), than those exposed at 7-days-old (15-day-olds), despite their post exposure longevity being greater.

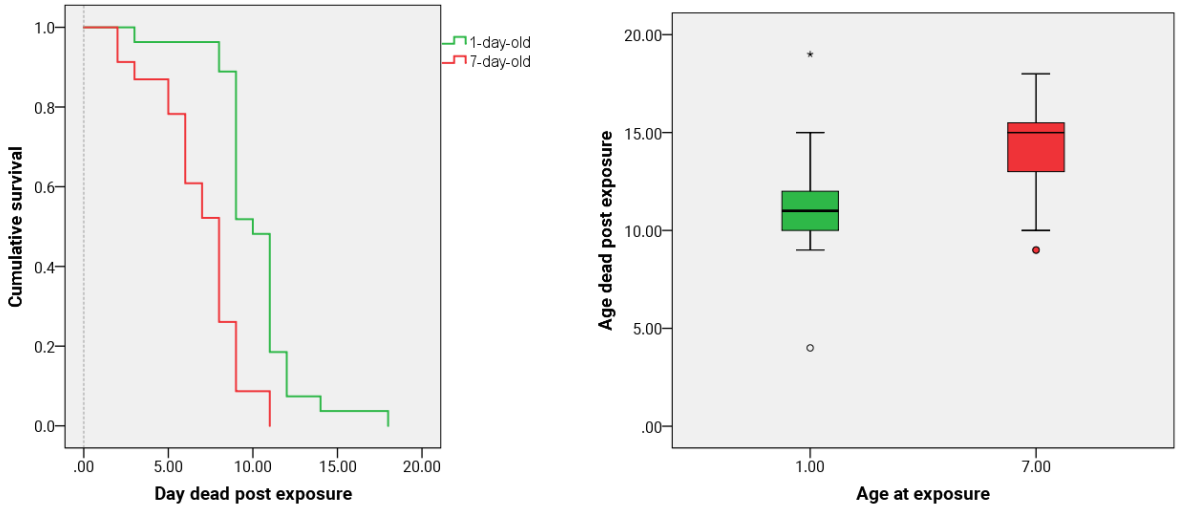


Figure 3.11. The longevity (A) and age at death (B) of Tengrela mosquitoes following a single 3-minute WHO cone bioassay.

Mosquitoes were exposed to PermaNet 2.0 netting at 1 (green) or 7-days-old (red). Immediate (within 24-hours of exposure) mortality is excluded from both A & B. In A the dashed grey line shows the day of exposure.

3.3.3 Mosquito longevity: Experimental hut trials

Experimental hut trials of reared and release *An. gambiae* ('reared-release')

Over the two-year study, 782 female *Anopheles* were released into huts. In the 2016 trial, equal numbers of mosquitoes were released into huts daily except for day four (19 released into untreated and 18 released into PermaNet 2.0). In the 2017 trial, mosquito numbers were limited, so unequal numbers of mosquitoes were released into huts daily to allow the maximum number of mosquitoes of the correct age to be used each day. A total of 493 female *Anopheles* were recaptured (63%) across all huts (Table 3.6). Recapture rates

were greater in untreated compared to PermaNet 2.0 huts over the two years. Non-target mosquitoes (including *Culex* spp. and *Mansonia* spp.) were collected in all huts in both years, indicating that the various methods used to seal the hut were not fully successful.

Table 3.6. Summary of mosquitoes collected in 2016 and 2017 reared-release trials.

	2016		**2017**	
	Untreated net	**PermaNet 2.0**	**Untreated net**	**PermaNet 2.0**
Female *Anopheles* (recapture rate)	113 (78.47%)	75 (52.45%)	185 (74.90%)	120 (48.39%)
Male *Anopheles*	0	0	1	0
Culex	12	46	1	0
Mansonia	0	0	15	16

Table 3.7. Summary of outcomes for Anopheles females collected in reared-release experimental hut trials conducted in 2016 and 2017.

Outcomes were calculated based on recaptured mosquitoes only. Asterisks show when treatment was statistically significant compared to untreated control of the same year (P > 0.05, GLMMs).

	2016		**2017**	
	Untreated net	**PermaNet 2.0**	**Untreated net**	**PermaNet 2.0**
Total collected	113	75	185	120
% exophily (95% CI)	27.43 (19.21 – 35.66)	46.67* (35.38 – 57.96)	28.65 (22.13 – 35.16)	29.17 (21.03 – 37.30)
Induced exophily	-	41.21	-	1.78
% blood-fed (95% CI)	80.53 (73.23 – 87.83)	22.67* (13.19 – 32.14)	54.05 (46.87 – 61.24)	18.33* (11.41 – 25.26)
Blood-feeding inhibition	-	71.85	-	66.08
% mortality (95% CI)	11.01 (5.13 – 16.89)	50.00* (38.61 – 61.39)	16.22 (10.90 – 21.53)	45.50* (33.66 – 51.34)
% personal protection	-	81.32	-	78
% killing effect	-	22.94	-	11.35

In the 2016 trial, experimental day, hut, net treatment, and collection location (e.g. in net, in veranda) had no effect on mosquito longevity. Only blood-feeding status significantly affected mosquito longevity when included with all variables (P = 0.001). When non-significant variables were excluded from the regression, blood-fed mosquitoes had a 0.561-fold (0.384 – 0.819) lower risk of death (Figure 3.12a, P = 0.003). The median survival time post-collection was 8 days for blood-fed and 7 days for unfed mosquitoes (Figure 3.12c). In 2017, date (P = 0.005) and blood-feeding status (P = 0.000) both significantly affected mosquito longevity. When non-significant variables were removed from the model, and results were stratified by day to account for this variation, blood-fed mosquitoes had a 0.450-fold (0.327 – 0.618) reduction in the risk of death compared to unfed mosquitoes (Figure 3.12b, P = 0.000). The median survival time was 10 days for blood-fed and 7 days for unfed mosquitoes (Figure 3.12d).

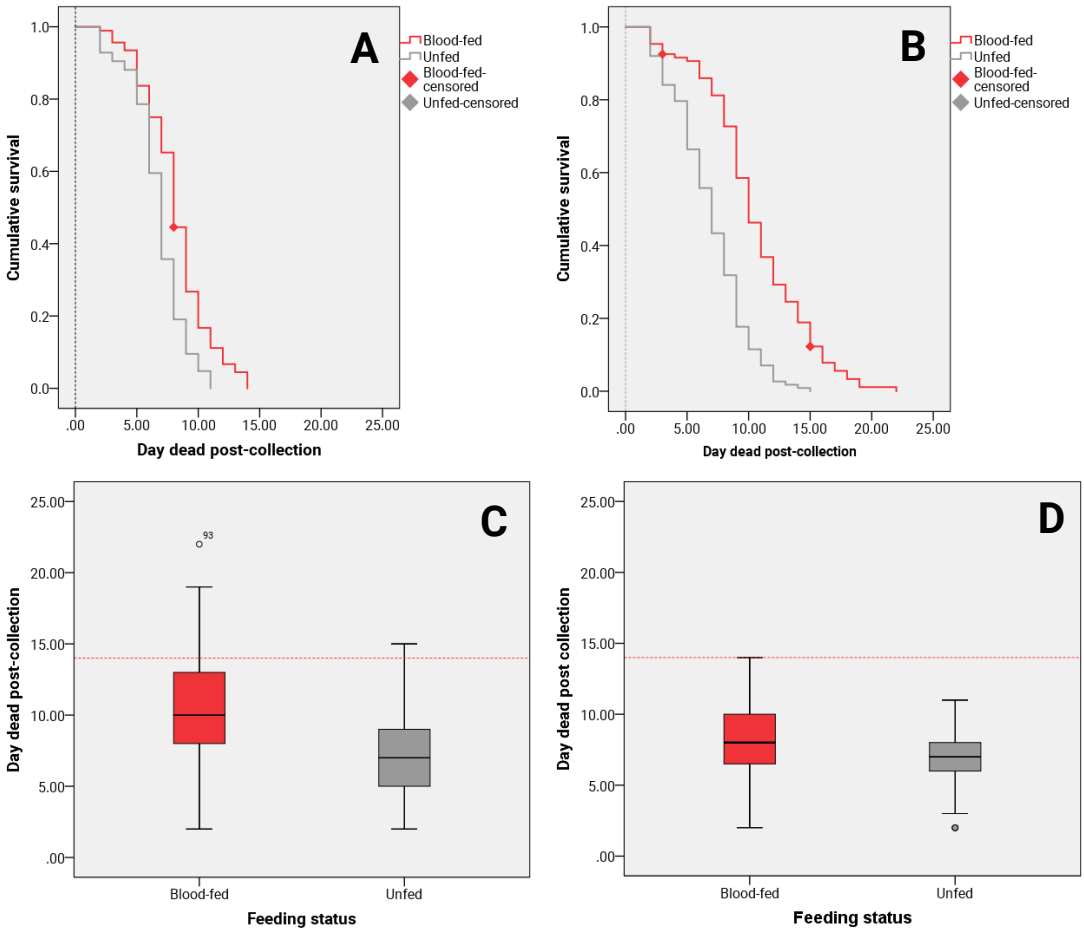


Figure 3.12. The longevity of blood-fed and non-blood-fed female Anopheles in reared-release hut trials.

Data for untreated and PermaNet 2.0 huts is combined. Kaplan Meier survival curves show the proportion alive each day in the 2016 (A) or 2017 (B) trials. Dashed grey lines represent day of insecticide exposure in the hut trial. Diamond points show censored data. Box and whisker plots of day dead post-exposure in the 2016 (C) or 2017 (D) trials. Dashed red lines show day 14 to correspond with the average extrinsic incubation period of the malaria parasite, Plasmodium falciparum (Vaughan, 2007). *Immediate mortality (dead on collection or within 24-hours of exposure) is excluded. 2016 trial: blood-fed mosquitoes n = 92, unfed mosquitoes n = 42; 2017 trial: blood-fed mosquitoes n = 107, unfed mosquitoes n = 113.*

To evaluate the impact of net type on longevity, data were stratified into unfed and blood-fed groups to account for the effects of obtaining a blood meal. In the reared-release trials, where mosquito age range was standardised, net treatment had no effect on mosquito longevity in either the 2016 (P = 0.137) or 2017 (P = 0.603) trial (Figure 3.13).

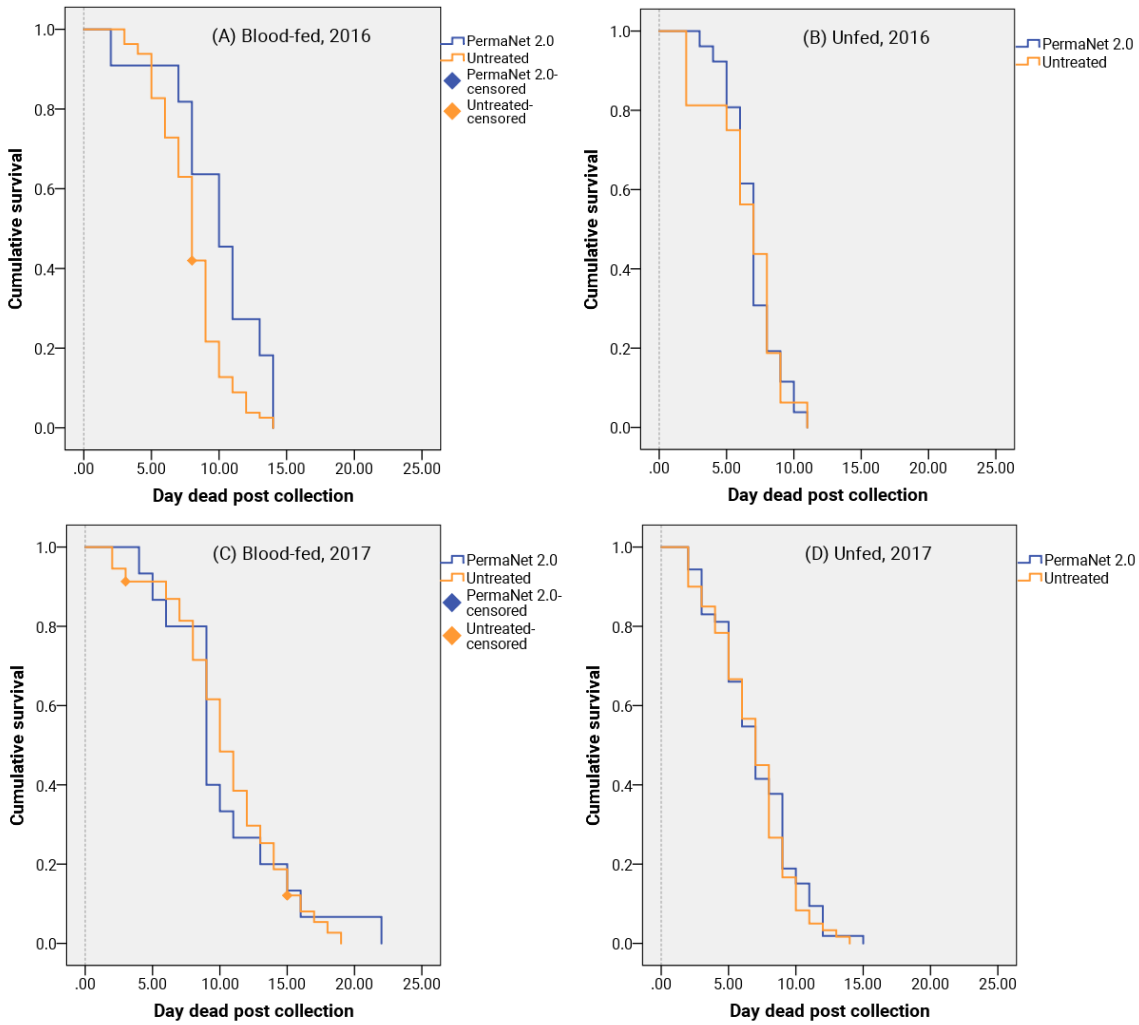


Figure 3.13. The longevity of female Anopheles after exposure in reared-release hut trials*. Kaplan Meier survival curves show the proportion alive each day in the 2016 (A, B) or 2017 (C, D) trials, separated by blood-fed (A, C) and unfed (B, D) groups. Dashed grey lines represent day of insecticide exposure in the hut trial. Diamond points show censored data. Immediate mortality (dead on collection or within 24-hours of exposure) is excluded. 2016 trial, blood-fed: PermaNet 2.0 mosquitoes n = 11, untreated mosquitoes n = 88; unfed: PermaNet 2.0 mosquitoes n = 26, untreated mosquitoes n = 16. 2017 trial, blood-fed: PermaNet 2.0 mosquitoes n = 15, untreated mosquitoes n = 9; unfed: PermaNet 2.0 mosquitoes n = 53, untreated mosquitoes n = 60.*

Experimental hut trials of wild-entering An. gambiae ('wild-entry')

Across the two years *Anopheles*, Aedes, *Culex*, and *Mansonia* mosquitoes were collected in the wild-entry hut trials. In total, 908 female *Anopheles* were collected during 22 experimental nights from huts (Table 3.8). Lower mosquito numbers in 2017 might have

been due to the trial being conducted early in the rainy season (July), whereas mosquito numbers in 2016 (October) were comparable to other hut trials conducted at this site (Toe *et al.*, 2018). In 2016, 59.76% (n = 932) of mosquitoes collected were non-target (non-*Anopheles* and male *Anopheles*), with male *Anopheles*, being the most abundant (40.99%, n = 382 mosquitoes). In 2017, 56.53% (n = 693) of mosquitoes collected were non-target mosquitoes, with *Mansonia* being the most abundant (44.37%, n = 544 mosquitoes).

Table 3.8. Summary of mosquitoes collected in 2016 and 2017 wild-entry trials.

	2016		**2017**		
	Untreated net	**PermaNet 2.0**	**Untreated net**	**PermaNet 2.0**	**Olyset Net**
Female *Anopheles* (per night/ per hut)	206 (20.6)	169 (16.9)	194 (8.08)	144 (6.00)	195 (8.13)
Male *Anopheles*	213	169	33	59	19
Culex	7	1	7	10	9
Mansonia	89	78	237	161	146
Aedes	0	0	7	5	0

Table 3.9. Summary of experimental hut trial results for Anopheles females collected in wild-entry trials conducted in Tengrela, Burkina Faso in 2016 and 2017.

Asterisks show when treatment was statistically significant compared to untreated control of the same year (P > 0.05, GLMMs).

	2016		**2017**		
	Untreated net	**PermaNet 2.0**	**Untreated net**	**PermaNet 2.0**	**Olyset Net**
Total collected	206	169	194	144*	195
Deterrence	-	17.96	-	25.77	-0.52
% exophily	27.18 (21.11 - 33.36)	28.40 (21.60 - 35.20)	15.46 (10.38 - 20.55)	16.67 (10.58 - 22.75)	26.67 (20.46 - 32.87)
Induced exophily	-	4.29	-	7.22	42.01
% blood-fed	54.85 (48.06 - 61.65)	46.15 (38.64 - 53.67)	69.07 (62.57 - 75.58)	52.78 (44.62 - 60.93)*	50.77 (43.75 – 57.79)
% feeding inhibition	-	15.86	-	23.59	26.5
% mortality	4.93 (1.95 - 7.90)	8.38 (4.18 - 12.59)	5.29 (2.10 - 8.48)	13.57 (7.90 - 19.24)*	18.85 (13.30 – 24.39)*
% personal protection	-	30.97	-	43.28	26.12
% killing effect	-	1.97	-	4.76	13.76

In the wild-entry trials, only non-blood-fed mosquitoes without visible signs of a blood meal, were retained for post-collection longevity analysis. Net treatment had no significant effect on mosquito longevity in either 2016 (P = 0.405) or 2017 (P = 0.867). In 2016, the median survival time was 8 days for unfed mosquitoes collected from both untreated and PermaNet 2.0 huts. In 2017, median survival time was 12 days for untreated and Olyset Net and 13 days for PermaNet 2.0 (Figure 3.14).

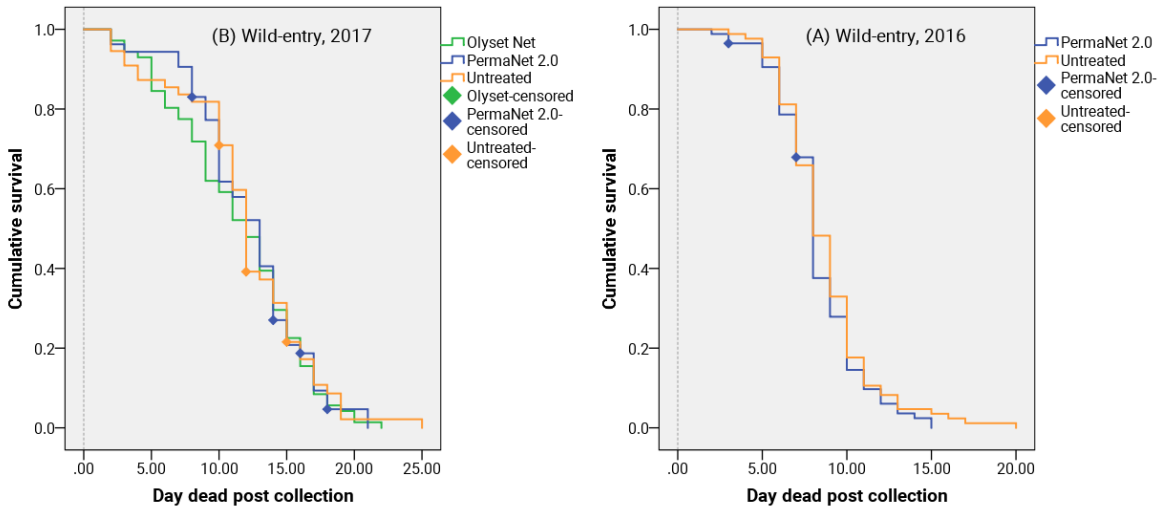


Figure 3.14. The longevity of non-blood-fed female Anopheles after exposure in wild-entry hut trials.

Kaplan Meier survival curves show the proportion alive each day in the 2016 (A) or 2017 (B) trials. Dashed grey lines represent day of insecticide exposure in the hut trial. Diamond points show censored data. Immediate mortality (dead on collection or within 24-hours of exposure) is excluded. 2016 trial: PermaNet 2.0 mosquitoes n = 85, untreated mosquitoes n = 85; 2017 trial: Olyset Net mosquitoes n = 71, PermaNet 2.0 mosquitoes n = 53, untreated mosquitoes n = 55.

Reared-release vs wild-entry

Comparing the longevity of unfed mosquitoes from the reared-release and wild entry trial, wild mosquitoes (unknown age) from the wild-entry trial lived significantly longer post-collection than reared (age range known) mosquitoes from the reared-release trial. This effect was seen regardless of treatment, in both years tested (Figure 3.15, 2016 P = 0.000, 2017 P = 0.000).

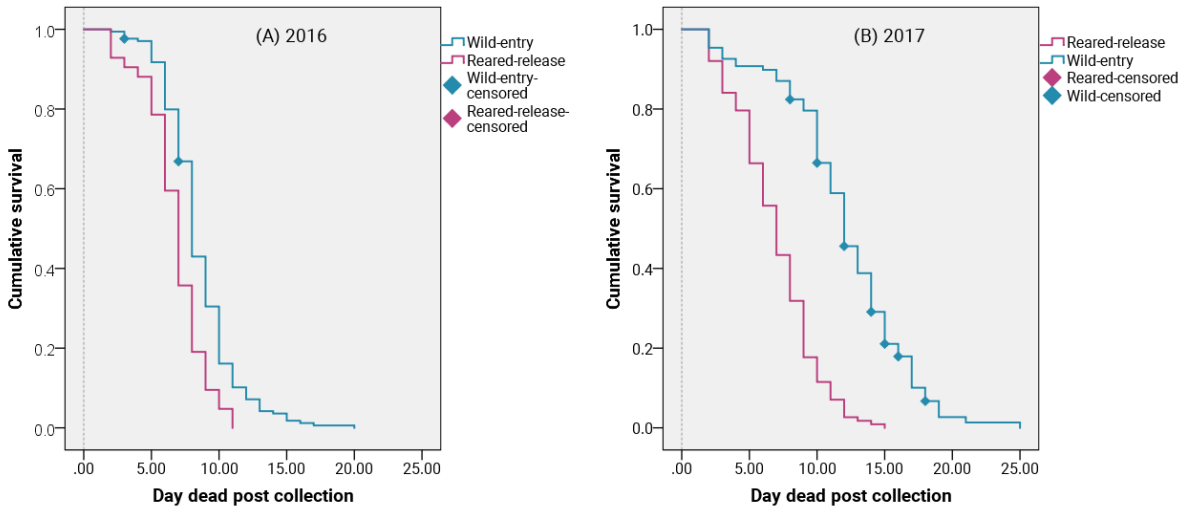


Figure 3.15. Comparison of longevity of female Anopheles after exposure in the wild-entry and reared-release trials.

Kaplan Meier survival curves show the proportion alive each. The dashed grey line represents day of insecticide exposure in the hut trial. Diamond points show censored data. Immediate mortality (dead on collection or within 24-hours of exposure) is excluded.

Experimental hut trial evaluating the efficacy of barrier bednets ('barrier bednet')

In the 2016 trial, 848 female *Anopheles* females were collected across all treatment arms (Table 3.10). Net treatment, blood-feeding status, and collection locations had no impact on mosquito longevity. Only experimental day significantly affect mosquito longevity when included with all variables (P = 0.000). Stratifying the model by date, net treatment had no effect (P = 0.946) on mosquito longevity (Figure 3.16).

Table 3.10. Summary of experimental hut trial results for Anopheles females collected in barrier bed net trial conducted in Tengrela, Burkina Faso in 2016.

Asterisks show when treatment was statistically significant compared to untreated control (P > 0.05, GLMMs). Abbreviations: PN2B = PermaNet 2.0 barrier, OPB = Organophosphate fenitrothion barrier.

	Untreated	**PermaNet 2.0**	**PermaNet 2.0 + PN2B**	**PermaNet 2.0 + OPB**
Total collected	249	219	202	178
Deterrence	-	12.05	18.88	28.51
% exophily	16.87 (12.22 – 21.52)	31.51* (25.35 – 37.66)	30.69* (24.33 – 37.05)	24.72* (18.38 – 31.06)
Induced exophily	-	86.79	81.97	46.55
% blood-fed	58.63 (52.52 – 64.75)	45.66 (39.06 – 52.26)	43.56* (36.73 – 50.40)	42.70* (35.43 – 49.96)
% feeding inhibition	-	22.12	25.70	27.18
% mortality	6.50 (3.42 – 9.59)	7.44 (3.93 – 10.95)	10.95 (6.63 – 15.26)	16.29* (10.87 – 21.72)
% personal protection	-	31.51	39.73	47.95
% killing effect	-	0	2.44	5.28

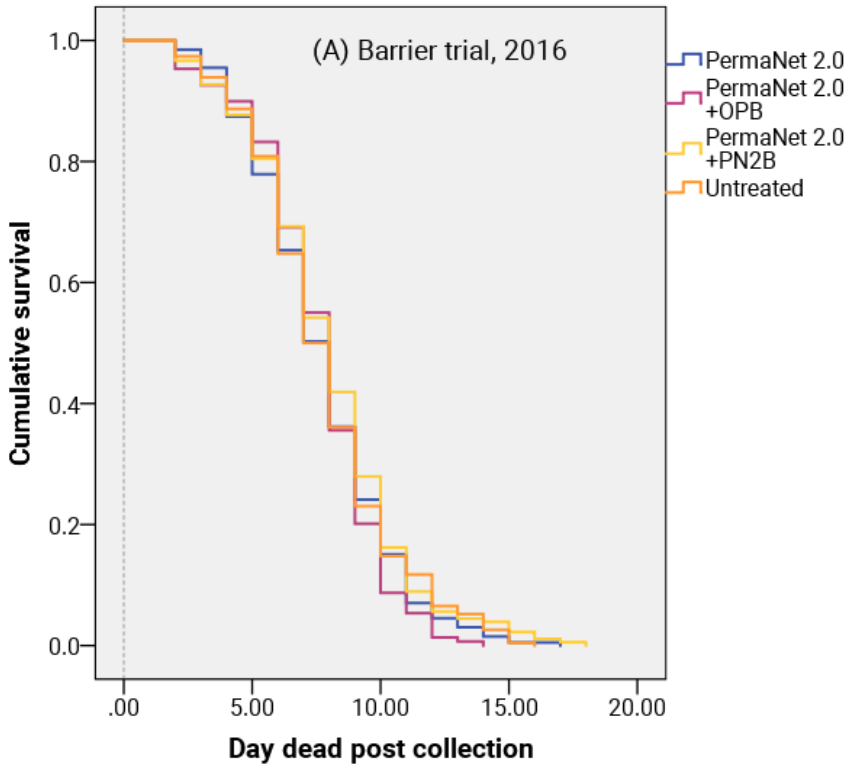


Figure 3.16. The longevity of female Anopheles after exposure in the 2016 barrier bed net trial.

Kaplan Meier survival curves show the proportion alive each day. The dashed grey line represents day of insecticide exposure in the hut trial. Immediate mortality (dead on collection or within 24-hours of exposure) is excluded. Abbreviations: OPB = Organophosphate fenitrothion barrier, PN2B = PermaNet 2.0 barrier. PermaNet 2.0 mosquitoes n = 199, PermaNet 2.0 + OPB mosquitoes n = 149, PermaNet 2.0 + PN2B mosquitoes n = 179, untreated net mosquitoes n = 230.

In the 2017 trial, 2402 *Anopheles* females were collected across all treatment arms (Table 3.11). Net treatment, and collection locations had no impact on mosquito longevity, whereas experimental day (P = 0.000) and blood-feeding status (P = 0.000) significantly affected longevity. Stratifying the model by date, blood-fed mosquitoes had a 0.811-fold (0.733 – 0.897) reduced risk of death compared to unfed mosquitoes (P = 0.000). Net treatment had no effect (Figure 3.17) on mosquito longevity when results were stratified by feeding status (P = 0.061), or date (P = 0.268).

Table 3.11. Summary of experimental hut trial results for Anopheles females collected in barrier bed net trial conducted in Tengrela, Burkina Faso in 2017.

Asterisks show when treatment was statistically significant compared to untreated control (P > 0.05, GLMMs). Abbreviations: PN2B = PermaNet 2.0 barrier, OPB = Organophosphate fenitrothion barrier, NPIB = Non-pyrethroid insecticide barrier.

	Untreated	**PermaNet 2.0**	**PermaNet 2.0 + PN2B**	**PermaNet 2.0 + OPB**	**PermaNet 2.0 + NPIB**	**Untreated + OPB**
Total collected	532	378*	388	350*	412	342*
Deterrence	-	28.95	27.02	34.21	22.56	35.71
% exophily	22.93 (19.36 – 26.50)	35.19* (30.37 – 40.00)	35.31* (30.55 – 40.06)	26.29 (21.67 – 30.90)	34.47* (29.88 – 39.06)	21.05 (16.73 – 25.37)
Induced exophily	-	9.02	12.30	-24.59	16.39	-40.98
% blood-fed	63.53 (59.44 – 67.62)	39.68* (34.75 – 44.61)	45.10* (40.15 – 50.05)	32.57* (27.66 – 37.48)	38.35* (33.65 – 43.04)	48.83* (43.53 – 54.13)
% feeding inhibition	-	37.54	29.01	48.73	39.64	23.14
% mortality	8.40 (6.02 – 10.77)	13.40* (9.95 – 16.86)	13.64* (10.16 – 17.11)	46.06* (40.79 – 51.34)	16.09 (12.51 – 19.67)	50.74* (45.42 – 56.06)
% personal protection	-	55.62	48.22	66.27	53.25	50.59
% killing effect	-	1.15	1.34	21.76	4.01	24.43

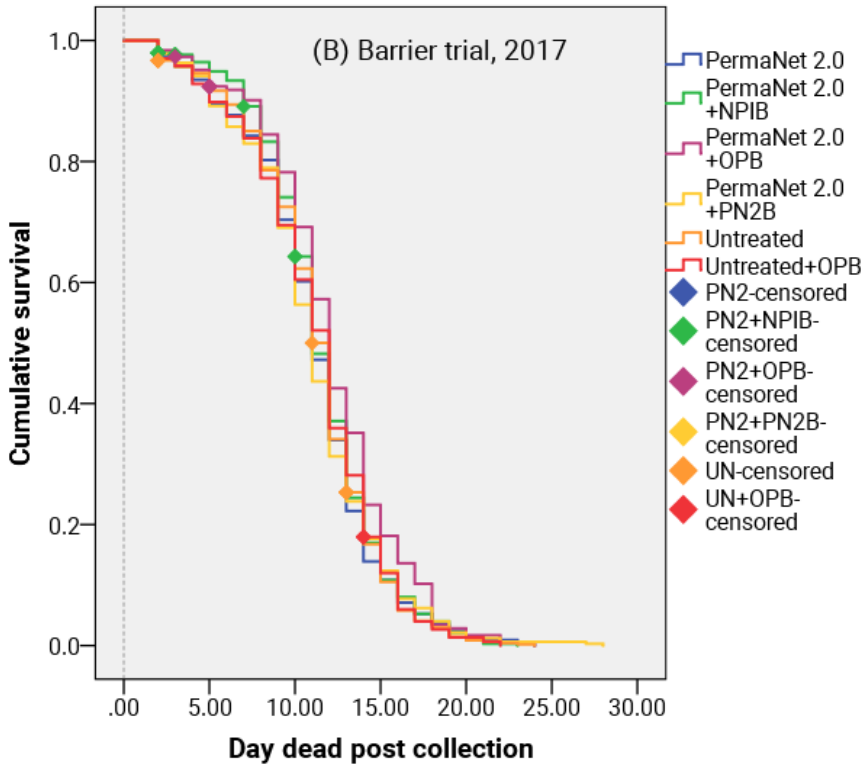


Figure 3.17. The longevity of female Anopheles after exposure in the 2017 barrier bed net trial.

Kaplan Meier survival curves show the proportion alive each. The dashed grey line represents day of insecticide exposure in the hut trial. Diamond points show censored data. Immediate mortality (dead on collection or within 24-hours of exposure) is excluded. Abbreviations: NPIB = Non-pyrethroid insecticide barrier, OPB = Organophosphate fenitrothion barrier, PN2B = PermaNet 2.0 barrier. PermaNet 2.0 mosquitoes n = 323, PermaNet 2.0 + NPIB mosquitoes n = 339, PermaNet 2.0 + OPB mosquitoes n = 185, PermaNet 2.0 + PN2B mosquitoes n = 323, untreated net mosquitoes n = 480, untreated net + OPB mosquitoes n = 167.

3.4 Discussion

The aim of this series of experiments was to investigate if insecticide exposure impacted the post-exposure life-span (referred to as longevity) of wild insecticide resistant *An. gambiae s.l.*, that were not killed immediately following insecticide exposure. To explore this, mosquitoes were exposed to LLINs or insecticides using three different exposure methods (i.e. WHO tube bioassays, WHO cone bioassays and experimental hut trials). In experimental hut trials, mosquitoes of known age range and physiological status ('reared-

release'), or with unknown life histories ('wild-entering') were used. This allowed the effect of insecticide exposure to be assessed against both standardised, and more field representative, mosquito populations.

No differences were observed in mosquito longevity post-exposure following contact with insecticide-treated netting compared to untreated netting in any of the cone bioassays. Single exposure tests represented the minimum level of net contact a mosquito might encounter in the field. Tests were carried out with two distinct mosquito populations collected from different study sites (Tengrela and Yendere). Molecular ID confirmed *An. coluzzii* to be the dominant vector species in Tengrela and *An. gambiae* s.s. to be the dominant vector species in Yendere. A single exposure to an LLIN had no effect on immediate or delayed mortality in either of the two major malaria vectors in this region. The two populations were studied in different field seasons (2017 and 2018), so inherent natural variation precluded investigating if longevity differences existed between the *An. coluzzii* and *An. gambiae* s.s. populations

With the high rates of LLIN usage in Burkina Faso today, it is unlikely that a highly pyrethroid-resistant mosquito will contact a net just once in its lifetime, and therefore the impact of multiple exposures was investigated. The different exposure regimes used were chosen to replicate previous studies conducted against moderate and highly resistant colonised mosquitoes (Viana *et al.*, 2016). Using laboratory colonies, Viana *et al.* (2016) demonstrated that LLIN contact resulted in substantial delayed mortality effects in mosquitoes that were not killed immediately following exposure. Using the same exposure methods (WHO cone bioassay) and regimes, the results of this current study showed no evidence of delayed or sub-lethal effects in wild highly pyrethroid-resistant populations. Applying the Bayesian state-space model (Viana *et al.*, 2016) supports the absence of any delayed mortality from PermaNet 2.0 in these experiments (Hughes *et al.*, 2020, Appendix 3).

Viana *et al.* (2016) reported high levels of immediate mortality following cone exposure. This ranged from 60-100% in moderately resistant mosquitoes, and 3-61% in highly resistant mosquitoes. The magnitude of delayed mortality was different between their test populations, both of which had different resistance mechanisms. This led them to hypothesise that ongoing selection for resistance could erode the mitigatory effects that

they observed. The populations tested in the current study are highly pyrethroid-resistant with multiple underpinning resistance mechanisms (Toe, C. M. Jones, *et al.*, 2014; Ingham, Wagstaff and Ranson, 2018; Toe *et al.*, 2018; Ingham *et al.*, 2019; Williams *et al.*, 2019). Immediate mortality was extremely low, ranging from 0 - 5.32% following a single exposure. Subsequently, the absence of evidence for any delayed effects in the current study, supports their hypothesis that delayed mortality effects could be lost as resistance intensifies.

Evidence of reduced longevity following LLIN exposure has been reported from Cameroon, where unexposed mosquitoes lived significantly longer than mosquitoes exposed to PermaNet 2.0 netting (Tchakounte *et al.*, 2019). A greater reduction in life span was observed in *An. funestus* and results of susceptibility assays conducted on the same population showed it to be less resistant to pyrethroids than *An. gambiae* from the same study area. These results also support the hypothesis that delayed effects could decrease as resistance intensifies. It should be noted, however, that it is unclear if in this study mosquitoes were compared to unexposed mosquitoes or an untreated control, so it is possible that experimental handling could affect the results. Moreover, results may not have been separated into immediate and delayed effects of the exposure. Following exposure to PermaNet 2.0, the authors report some immediate mortality (17.5% *An. funestus* mortality, 16.6% *An. gambiae* mortality), which could have impacted on the results reported if they were not separated.

The results reported in this chapter show repeated insecticidal net exposure is having little impact on the immediate mortality, and no impact on the longevity, of highly pyrethroid-resistant mosquito populations. In the multiple exposure assays, mosquito mortality remained well below the 90% threshold for insecticide susceptibility. In the five-exposures assay immediate mortality was extremely low for all exposures (< 10% mortality) and only significantly different between PermaNet 2.0 and untreated following the 4^{th} and 5^{th} exposure (Figure 3.8). In experiments in which mosquitoes were exposed to PermaNet 2.0 every fourth day, immediate mortality increased with each subsequent exposure, but never exceeded 65% (Figure 3.7). Significantly higher mortality, to PermaNet compared to untreated, was only detected following the third exposure when mosquitoes were 12-days-old. It is well documented that mosquito susceptibly to insecticides increases with age, with several studies in both laboratory and field populations showing differences in resistance

profiles when different age mosquitoes are tested (Lines and Nassor, 1991; Glunt, Thomas and Read, 2011; Christopher M. Jones *et al.*, 2012; Collins *et al.*, 2019; Machani *et al.*, 2019). The significant difference in immediate mortality observed was lost by the fourth exposure (16-days-old). However, as mortality reduced mosquito numbers at every exposure throughout the assay, fewer than 30 mosquitoes remained per treatment arm by the fourth exposure. This small sample size leads to low statistical power. Additionally, similar mortality levels were observed in the untreated arm so mortality at this age could simply be mosquito senescence, and not the result of LLIN exposure.

Other studies have also documented repeated LLIN exposure to have little impact on mosquito mortality and longevity. Glunt *et al.* 2011 observed that previous exposure to low doses of permethrin had no effect on subsequent insecticide susceptibility in *An. stephensi* when mosquitoes were aged-matched, and Viana *et al.* (2016) did not document a relationship between the number of exposures and immediate mortality in their study. Mulatier *et al.* (2019) found no evidence that multiple exposures to permethrin-treated netting while blood-feeding affected survival rates of KdrKis mosquito, a resistant strain which carries pyrethroid-resistant alleles.

In the current study, excluding immediate mortality, mosquitoes that were 1-day-old when exposed survived significantly longer post-exposure than mosquitoes exposed at 7-day-olds. However, the median age at death was lower in mosquitoes exposed at 1-day-old compared to mosquitoes exposed at 7-days-old. If the younger mosquito group were surviving longer post-exposure because of their age it would be expected that there would be no difference in their average age at death. These results should be interpreted with caution, as sample sizes for longevity estimates were small (fewer than 30 mosquitoes per study arm) and no untreated net was tested.

Future studies should examine the effect of age on survival post-exposure, at ages when mosquitoes could be infectious (i.e. > 14-days-old). In order to transmit malaria, mosquitoes must survive long enough for the *Plasmodium* parasite to mature into its infective form. This can take a minimum of 9 days (Beier, 1998), although typically it occurs between 10-16 days and is *Plasmodium* species-dependent (Vaughan, 2007). Under natural conditions, few mosquitoes survive long enough to transmit malaria (Gillies and Wilkes, 1965; Charlwood *et al.*, 1997), but addressing knowledge gaps on how LLIN exposure

affects the longevity of this older group is critical, as they are the cohort capable of transmitting malaria.

Cone assays have long provided a simple reproducible means of exposing mosquitoes to LLINs in a standardised way. However, the 3-minute exposure time is greater than the duration of net contact accumulated by the majority of mosquitoes during a typical response to a human baited bednet (Parker *et al.*, 2017). Hence, experimental hut trials were used to expose mosquitoes to LLINs for a more realistic duration. Due to the design of huts, it is not possible to ascertain whether contact with the net occurred, or exact duration of exposure. Therefore, when the current experimental hut data was analysed mosquitoes were classified into the treatment arms based on the huts they were collected from.

In the experimental hut trials, immediate mortality was increased, and blood-feeding was reduced in huts with LLINs compared to untreated nets, however significant differences were only observed in some trials. Excluding these immediate effects, no difference between the longevity of mosquitoes exposed to LLINs or control nets was observed, and after applying the Bayesian state-space model (Viana *et al.*, 2016) to the results no evidence of delayed mortality was identified (Hughes *et al.*, 2020).

It is important to note the different effects observed in the cone and hut trials. In cone bioassay exposures, the performance of PermaNet 2.0 with these resistant populations was no different to an untreated net with no impact on the mosquitoes based on the outcomes measured (e.g. knockdown and mortality). In experimental hut trials however, some impacts on immediate mortality and blood-feeding inhibition were observed. The difference between the two methods shows the importance of applying a variety of evaluation methods when assessing vector controls tools. The results of the hut trial suggest PermaNet 2.0 still offers some protective value against resistant populations in this setting. In hut trials using wild-entry mosquitoes, mortality following exposure to pyrethroid-only netting ranged between 7 – 14% (Untreated 4 – 9%) and was comparable to other trials conducted in the area which tested pyrethroid-only netting (Toe *et al.*, 2018). Blood-feeding inhibition levels were between 15 – 38%, suggesting some mosquitoes were still prevented from blood-feeding, even if they were not killed by the net. Unlike phase I trials, efficacy criteria for phase II trials are not based on set percentage thresholds, and a

test LLIN can proceed to phase III trials only if it performs as well as, or better than, the reference net (both washed 20 times as a proxy for 3-years field use), in terms of mortality and blood-feeding inhibition. Phase I trials offer a standardised method for evaluating products, but their suitability for evaluating all vector control tools, particularly those with novel modes of actions, has previously been called into question (Hughes, 2018).

In the hut trials, blood-feeding status had a significant effect on mosquito longevity with blood-fed mosquitoes surviving significantly longer post-collection than non-blood-fed mosquitoes. This effect was seen, regardless of net treatment, or in cases where collection day affected results when it stratified by date. Using laboratory-reared resistant mosquitoes Spillings *et al.* (2008) observed that a single blood meal increased the resistance level of fed mosquitoes compared to unfed. During blood meal digestion mosquitoes upregulate enzymes to detoxify harmful products from the blood meal. Subsequently, the authors suggested that these enzymes were providing an additional benefit following exposure by assisting in insecticide detoxification. Other laboratory studies have observed that acquiring a blood meal improves survival (Glunt, Thomas and Read, 2011) and increases longevity (Oliver and Brooke, 2014) in mosquitoes. However, studies investigating if this effect occurs in field populations are limited. Recently, Machani *et al.* (2019) documented reduced mortality following exposure to deltamethrin in WHO tube bioassays when comparing blood-fed *An. gambiae* to unfed, regardless of the mosquito's age. The result of the current study in the Tengrela population support this, showing a similar effect of obtaining a blood meal is observed when exposed to an LLIN.

In the hut trials, wild entry mosquitoes survived longer post-exposure than reared-release mosquitoes. The huts are situated between the rice fields and the village, so, it is anticipated that a large proportion of wild entry mosquitoes may be newly eclosed females seeking their first blood meal (Service and Townson, 2002), whereas reared-release mosquitoes were 5-to-8-days-old. The presumed difference in age structure may explain the difference in survival. Additionally, by collecting and rearing mosquitoes in the insectary for release, reared mosquitoes may include those of lower fitness which in the wild may have died before reaching the huts.

Single and repeated exposure to LLINs in cone bioassays and huts trials showed no impact on mosquito longevity. Subsequently, WHO tube bioassays were used to investigate if

increasing the amount of insecticide mosquitoes were exposed to induced delayed mortality. The results showed evidence of a delayed mortality at concentrations of > 5× the discriminating dose of deltamethrin (Hughes *et al.*, 2020). These results suggest pyrethroids can induce sub-lethal effects, even in the highly pyrethroid-resistant populations, but under standard exposure conditions, these effects are rarely evident. In longevity tube bioassays a significant reduction in immediate mortality was documented between 0.75% (74.07% mortality) and 1.0% (56.83% mortality) deltamethrin (P = 0.003). This could be as a result of poor stability in papers impregnated with high concentrations of insecticide. Future tests could compare longevity effects between similar net types with increased insecticide concentrations (i.e. PermaNet 2.0 vs PermaNet 3.0 sides, to establish if similar effects are observed in response to treated netting.

These results show limited impact of LLIN exposure on a highly-pyrethroid resistant mosquito population, and no evidence of delayed mortality following exposure to insecticide concentrations currently in field use. Future experimental hut trials should consider including longevity as a standard outcome to establish if the effects seen here occur in other populations with differing insecticide resistance levels or mechanisms. If in other populations, resistant mosquitoes do not survive long enough to become infectious, or if the infectious population is significantly reduced, this would impact on malaria transmission, as highlighted by Viana *et al.* (2016). The absence of delayed mortality in the Tengrela population might partly explain why a reduction in malaria cases has not been observed in this area (WHO, 2018b), and the efficacy of LLINs in this setting is called into question.

3.5 Conclusion

Previous studies have shown a delayed mortality effect in insecticide-resistant mosquito populations that survive the initial exposure to pyrethroids, but subsequently show reduced longevity compared to non-exposed mosquitoes. The objective of the studies reported in this chapter were to determine whether pyrethroid exposure, mainly in LLINs, exerted a delayed mortality effect on pyrethroid-resistant mosquitoes from the study site in Burkina Faso, using both laboratory bioassays and more realistic exposure methods in experimental huts. In all assays, the level of immediate mortality induced by LLIN exposure was low. The results of both cone and field trials showed no significant impact of LLIN

exposure on post-exposure survival, and delayed mortality was only observed when mosquitoes were exposed to very high concentrations of pyrethroids in WHO tube bioassays.

Chapter 4. The effect of insecticidal net exposure on *Anopheles gambiae* blood-feeding and reproductive output

4.1 Introduction

Mosquito vectorial capacity is influenced by blood-feeding in several ways. Directly, malaria transmission is inextricably linked with blood-feeding as this is when *Plasmodium* transmission occurs. Indirectly, *Anopheles* mosquitoes are anautogenous, they require a blood meal in order to develop their eggs and reproduce. Therefore, impacts on blood-feeding impact reproductive output which impacts population growth and vector density. Blood-feeding may also impact parasite transmission, and a recent study has reported blood-feeding to reduce the extrinsic incubation period and increases competence of several viruses in *Aedes* mosquitoes (Armstrong *et al.*, 2020). Consequently, anything that affects a mosquito's willingness or ability to blood-feed will dramatically affect their disease transmission potential both on an individual and population level.

Blood-feeding is measured as a standard outcome in WHO tunnel tests and experimental hut trials (WHO, 2013a). These assays are run overnight, and the number of mosquitoes (dead and alive) which obtain a blood meal are recorded the following morning. Blood-feeding inhibition following LLIN exposure has been documented in several lab studies using tunnel tests, lined-WHO tubes, and net-covered cups to expose mosquitoes (Strode *et al.*, 2014; Glunt *et al.*, 2018; Hauser, Thiévent and Koella, 2019; Mulatier *et al.*, 2019). Time to initiate blood-feeding was increased and time spent blood-feeding decreased by insecticidal net exposure in one study (Hauser, Thiévent and Koella, 2019). Evidence from another trial suggests prior insecticide exposure may impede the blood-feeding inhibition effect at subsequent exposures (Mulatier *et al.*, 2019).

In experimental hut trials, blood-feeding inhibition is often observed in huts containing insecticide treated nets compared to untreated nets. As data on blood-feeding typically combines total numbers of blood-fed mosquitoes, regardless of whether they are dead or alive, a reduction in blood feeding in huts with insecticidal nets suggests that the insecticide itself affects a mosquito's ability or willingness to blood-feed (Strode *et al.*, 2014). However, due to the experimental design of hut trials, it is not possible to determine

if, or when, contact with the net occurred. Additionally, if the mosquito was blood-fed, we do not know whether the mosquito entered the hut blood-fed, or, if not whether blood-feeding was synchronous with exposure (the mosquito feeds through the net) or the mosquito passed through the net and fed directly on the host. If feeding occurs after exposure, we do not know how long after, and blood-feeding is only characterised at one time point (mosquitoes are scored as unfed or blood-fed the following morning). Hence standard experimental hut studies cannot determine whether blood-feeding inhibition is immediate or delayed, nor if it is a temporary or persistent affect. In the field, if feeding inhibition only lasts for a few hours, it may be possible for a mosquito to obtain a blood meal the same night. However, if the effects persist for longer a mosquito may delay feeding until the following night, or later, which would increase the length of their gonotrophic cycle and decrease their chances of survival long enough to take two blood meals, thus reducing their disease transmission potential.

Insecticide exposure could also affect mosquito population density via its effect on reproductive output, without affecting blood-feeding. If insecticide exposure alters a mosquito's fecundity (ability to lay eggs or amount of eggs laid) or fertility (surviving progeny) it will affect population growth and vector density. The results of this could be two-fold. A reduction in fecundity could simply result in a reduction of the ratio of mosquitoes to humans. Conversely, in areas with limited breeding sites (due to the local ecology or time in the rainy season) and high mosquito numbers, a reduction in fecundity could plausibly lead to an increase in the mosquito population or improved mosquito fitness, due to density dependant survival in constrained breeding pools (Gimnig *et al.*, 2002; Muriu *et al.*, 2013). A number of studies have considered how oral toxicants (e.g. endectocides) or spatial repellents affect mosquito reproductive output (Bibbs *et al.*, 2019; Mekuriaw *et al.*, 2019), but studies investigating the impact of pyrethroid-only nets on mosquito reproduction are limited (Mulatier *et al.*, 2019).

Reproductive output is not typically measured in standard insecticidal net efficacy tests, unless the control tool being evaluated specifically targets this outcome (e.g. Pyrethroid-PPF nets) (Ngufor *et al.*, 2014, 2016; Koffi *et al.*, 2015; Toé *et al.*, 2019). Some studies evaluating pyrethroid-PPF nets used a standard pyrethroid-only net as a comparator and found minor or no differences in reproductive output when comparing mosquitoes exposed to Olyset Net (permethrin) to untreated nets. In experimental hut trials in Benin

(Ngufor *et al.*, 2014, 2016), both reductions and increases in reproductive output were observed depending on the trial type, however, samples sizes were extremely small (< 10 mosquitoes) or not reported for egg/larvae calculations, making it difficult to draw conclusions from the results. No differences in reproductive output were observed between pyrethroid-only nets and controls in experimental hut trials in Côte d'Ivoire (Koffi *et al.*, 2015) or cone assay exposures using resistant-mosquito strains (Toé *et al.*, 2019). Recently, studies using susceptible and resistant *An. gambiae* strains have found no evidence that pyrethroid-only or pyrethroid-PBO net exposure impacts on reproductive output (Hauser, Thiévent and Koella, 2019; Mulatier *et al.*, 2019).

The aim of this chapter was to determine if insecticidal net exposure either effects wild pyrethroid-resistant mosquitoes' ability to blood-feed, or their reproductive output. The specific objectives of these experiments were to:

- Determine if mosquito ability to take a blood-meal was reduced following single exposure to LLINs in WHO cone bioassays, and if so, if this effect persisted over a 24-hour period.
- Determine if mosquito egg production, oviposition, and egg viability were reduced following exposure to LLINs in a semi-field environment using experimental hut studies.

4.2 Methods

4.2.1 Study sites, mosquitoes and net treatments

Hut trials were performed at the experimental hut station in Tengrela, Burkina Faso, and laboratory bioassays at the CNRFP insectaries in Banfora, Burkina Faso. Mosquitoes used for tests were either wild-entry or larval-reared adults, collected, reared and identified using the methods described in Chapter 2, section 2.2. Mosquitoes were exposed to untreated, PermaNet 2.0, Olyset Net, PermaNet 3.0 sides, PermaNet 3.0 roof, and Interceptor G2 depending on the trial. Full nets specifications are listed in Chapter 2, section 2.5.

4.2.2 Blood-feeding assay

Mosquitoes were exposed to test netting in a standard 3-minute WHO cone bioassay (WHO, 2013a), and their subsequent ability to take a blood meal from a human host following exposure was assessed. Tests were performed on untreated net, Olyset Net, PermaNet 3.0 sides, PermaNet 3.0 roof, and Interceptor G2.

Blood-feeding tests were conducted over 12 non-consecutive days between August – October 2018. Mosquitoes were 5 to 7-days-old, non-blood-fed larval-reared females, which had been starved of sugar and water for a minimum of 24 hours prior to exposure. On each experimental day at least 1 untreated netting replicate was conducted alongside test nets. Test nets and mosquitoes were acclimatised to the testing room for >1 hour before experiments began. Testing began after ~22:00 to coincide with peak *Anopheles* biting times in Burkina Faso (Dambach *et al.*, 2016; Epopa *et al.*, 2019). The recorded temperature in the testing room ranged from 27.8 – 28.6°C, and humidity between 59.3 – 99.9%.

Two experiments were run:

- Experiment A: No host present, mosquitoes were exposed in batches of 5 to test netting using a standard 3-minute WHO cone bioassay unaltered from the WHO protocol.
- Experiment B: Host present, mosquitoes were exposed in batches of 5 to test netting using the standard 3-minute WHO cone bioassay, which was additionally baited with a human host and recorded using a smartphone. The exposure was recorded to investigate mosquito behaviour at the LLIN interface (discussed in Chapter 5).

Following net exposure, mosquitoes were transferred into paper cups or paper buckets. At 1, 8 and 24-hours following net exposure (~23:00, 06:00, 22:00, respectively) mosquitoes were offered a human blood-meal. At each time point, the operator placed their forearm over the paper cup/bucket for 20 minutes. After the 20 minutes, mosquitoes were scored as knockdown (1-hour), dead (24-hour), able to feed, or unable to feed (Table 4.1). No knockdown or dead mosquitoes were removed from cups/buckets until 24-hours post-exposure, but blood-fed mosquitoes were counted and separated from unfed mosquitoes at each time point. Blood-fed mosquitoes were not offered the opportunity to blood-feed

again and were provided with 10% glucose solution soaked onto cotton wool. Unfed mosquitoes were starved of sugar and water until they blood-fed or until 24-hours post-exposure. All mosquitoes were stored in paper cups for follow up after 24-hours (max 15 mosquitoes per cup). Three day's following exposure blood-fed mosquitoes were provided the opportunity to oviposit. Cotton wool soaked with water was placed in the base on the cup and kept moist for 3 days. Any eggs laid were discarded. Mortality was recorded daily until no mosquitoes remained alive, and dead mosquitoes were stored in silica.

Table 4.1. Definitions used for classifying mosquito status in the blood-feeding assay.

Mosquito status	Definition
Knockdown	The mosquito is immobile or unable to stand or take off, at 1-hour following net exposure
Dead	The mosquito is immobile or unable to stand or take off, at 24-hours following net exposure
Unable to feed	The mosquito does not imbibe blood within the 20-minute offered. The mosquito may not attempt to take a bloodmeal or the mosquito may attempt to probe, but a blood meal is not visible by eye.
Able to feed	The mosquito does imbibe blood within the 20-minute offered. The mosquito probes and a blood-meal is visible by eye

Blood-feeding assay: Data analysis

Outcomes from the blood-feeding assay were defined as follows:

- Knockdown: the number of mosquitoes defined as knockdown at 1-hour as a proportion of the number of mosquitoes exposed, excluded those lost-to-follow up in the first 24-hours.
- Mortality: the number of mosquitoes defined as dead at 24-hour as a proportion of the number of mosquitoes exposed, excluding those lost-to-follow up in the first 24-hours.
- Blood-feeding: the number of mosquitoes which successfully obtained a blood meal within the specified time period as a proportion of the number of mosquitoes offered a blood meal during that time period, including mosquitoes knocked down or dead.

- Longevity: the day of mosquito death, where day 0 is the day of its net exposure.

Binary logistic regression was used to examine the effect of independent variables (date, host present, net type, temperature, and humidity) on blood-feeding at 1-hour post-exposure. No effect of host-presence was observed, therefore experiment A and B were combined, and analysis was conducted on the pooled data set. Immediate mosquito mortality (within 24-hours of exposure) and blood-feeding were compared between untreated and treated nets using Pearson Chi-Squared. When the assumptions of this test were violated due to low expected frequencies Fisher's Exact Test was used. For blood-feeding, results are cumulative (e.g. blood-feeding at 8-hours is the proportion that fed within 8 hours and so includes those which already fed at 1-hour). Cox proportional hazard regression (Cox, 1972) was used to examine the effect of the net type on post-exposure longevity. Unfed mosquitoes and immediate mortality were excluded from longevity analysis. Obtaining a blood-meal significantly impacts on mosquito longevity (Chapter 2), and due to the nature of the blood-feeding assay very few unfed mosquitoes remained per treatment precluding analysis of this sub-group. Additionally, due to the design of the assay unfed mosquitoes had been starved for 48 hours before being provided with access to sugar (24 hours prior to exposure and 24 hours after exposure when offered blood-meals), which could significantly impact their longevity. The analysis was conducted in IBM SPSS Statistics 24.

4.2.3 Reproductive output hut trial

The aim of this experiment was to assess the effect of insecticidal net exposure on mosquito reproductive output. To achieve this, wild mosquitoes were exposed to LLINs using an experimental hut trial. The methodology for the hut trial is described in full in Chapter 3, Section 3.2.4. Hut trials were performed at the experimental hut station in Tengrela, Burkina Faso in 2016 and 2017. Mosquitoes were exposed to untreated, PermaNet 2.0, (2016 and 2017) or Olyset Net (2017 only). Full nets specifications are provided in Chapter 2, section 2.5. Following net exposure, only blood-fed mosquitoes were retained for reproductive output assessments, which were conducted at the CNRFP insectaries in Banfora, Burkina Faso. Methodologies for assessing reproductive output were altered between the 2016 and 2017 trials and are clarified below.

Reproductive output hut trial: 2016

In the 2016 trial, blood-fed mosquitoes were transferred from the experimental huts in Tengrela to the insectaries in Banfora. Mosquitoes were kept in cups for 3 days to allow them to become gravid, and their mortality was recorded daily. On day 3, surviving mosquitoes were transferred into individual 50 ml universal tubes for forced oviposition, using a technique adapted from Morgan *et al.* (2010). Oviposition tubes contained cotton wool soaked with water and covered with damp filter paper. They were covered with untreated netting and placed in constant darkness for 3 days. During this time, mosquitoes were fully sugar-starved. Each day they were recorded as having died or laid eggs. Laid eggs were counted using a dissection microscope and egg papers were floated into individual plastic pots containing water. Hatched larvae were fed daily on dried fish food and emerging larvae were counted a week after eggs were floated. On day 6 post-collection any remaining mosquitoes which had not died, or laid eggs were dissected. Dissected mosquitoes were scored as having no visible eggs, normal, abnormal, or underdeveloped eggs (Table 4.2, Figure 4.1) based on definitions modified from Koama *et al.* (2015). When dissected ovaries were normal, retained eggs were counted. When no visible eggs were seen ovaries were dried and observed for parity (Detinova, 1945; Beklemishev, Detinova and Polovodova, 1959), and spermatheca dissections were carried out to check for the presence of sperm to indicate if the female had mated (Table 4.2). All mosquitoes were stored in silica.

Table 4.2. Definition of ovary status, parity and mating used in reproductive output tests. *Mosquito ovaries were dissected and classified using definitions adapted from Koama et al. (2015). When no visible eggs were seen mosquito's parity and mating status were confirmed.*

Mosquito status	Description
No visible eggs	No follicular development is seen
Undeveloped ovaries	Some follicular development is observed, but eggs are small, translucent, or have not formed into their distinctive oval shape
Abnormal ovaries	Eggs are rounded in shape and/or discoloured
Normal ovaries	Eggs are oval-shaped and a solid white colour
Nulliparous	Ovary skein tracheoles are tight suggesting mosquitoes have not laid an egg batch
Parous	Ovary skein tracheoles are unravelled suggesting mosquitoes have laid at least one egg batch
Mated	Mosquito was inseminated. Sperm was present in the spermatheca
Unmated	Mosquito was not inseminated. Sperm was absent from the spermatheca

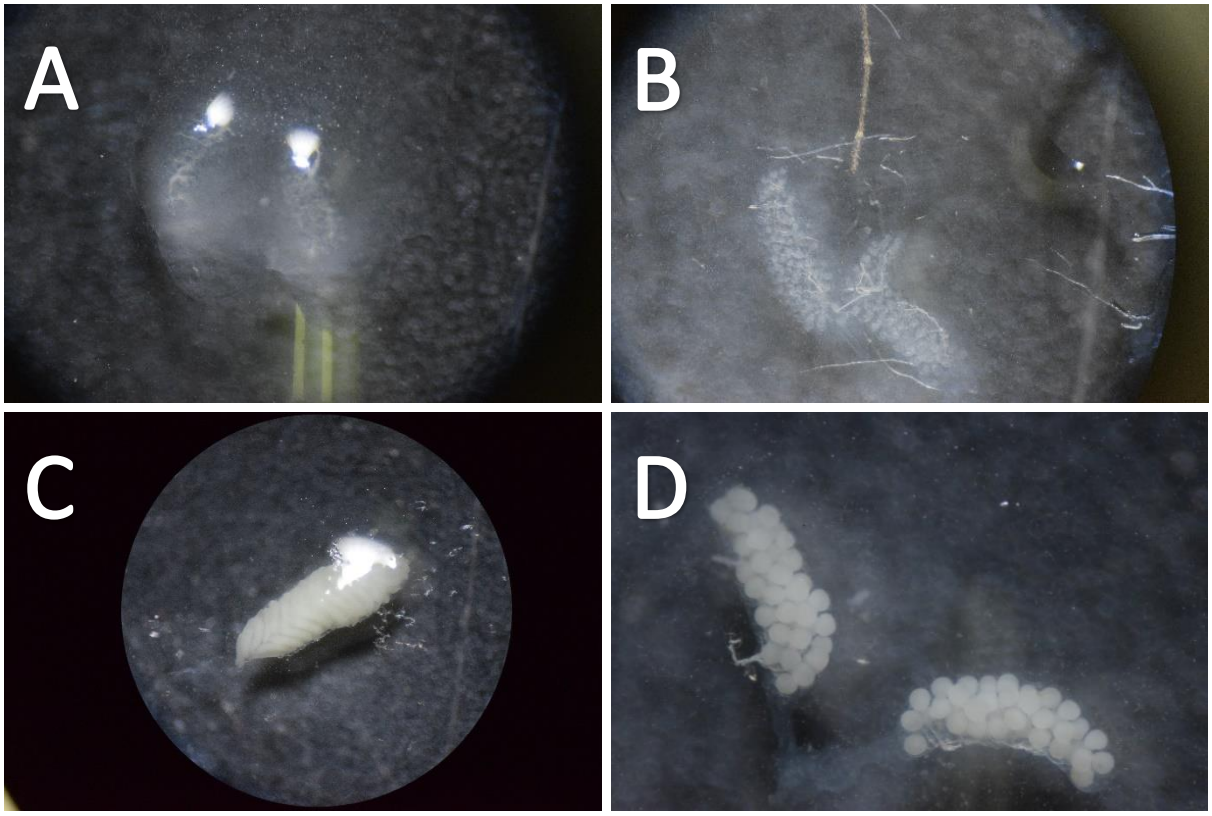


Figure 4.1. Photograph of mosquito ovaries observed through a dissection microscope. *(Scale: x 200 magnification). Example of ovaries with no visible eggs (A), underdeveloped (B), normal (C), and abnormal (D) ovaries, as defined by Table 4.2*

Reproductive output hut trial: 2017

The trial was repeated in 2017. The methodology for the hut trial exposure remained the same, however, the reproductive output methodology was altered to improve mosquito oviposition rates. Blood-fed mosquitoes were still transferred from the experimental huts in Tengrela to the insectaries in Banfora. Mosquitoes were kept in cups for 3 days to allow them to become gravid, and their mortality was recorded daily. However, on day 3 post-collection mosquitoes either underwent forced oviposition procedures (Experiment A: Oviposition) or were dissected immediately (Experiment B: Dissections). In experiment A, mosquitoes remained in 50 ml universal tubes without a time limit, until they laid eggs or died. Forced oviposition, egg and larval counting, and dissection procedures were the same as described in the 2016 trial. The same definitions were used for classifying mosquito ovaries (Table 4.2, Figure 4.1). All mosquitoes were stored in silica.

Reproductive output hut trial: Data analysis

Outcomes from the reproductive output trial were defined as follows:

- Mortality: Pre-oviposition, the number of mosquitoes which died as a proportion of the total number of mosquitoes collected from the experimental huts. Post-oviposition, the number of mosquitoes which died as a proportion of the total number of mosquitoes placed into forced oviposition tubes.
- Oviposition: The number of mosquitoes which laid eggs as a proportion of the total number of mosquitoes placed into forced oviposition tubes.
- Egg number: The number of eggs laid per ovipositing female, or per dissected female for retained eggs.
- Hatching rate: The number of larvae hatched as a proportion of eggs laid.
- Larvae number: The number of larvae per ovipositing female.
- Ovary status: no visible eggs, normal, abnormal, or underdeveloped eggs (Table 4.2)

Mosquito mortality, oviposition, and ovary status were compared between net groups using Pearson Chi-Squared. To examine insecticide exposures effect on the number of eggs (laid and retained) number of larvae, and hatch rate, a Shapiro-Wilk test was used to assess if data were normally distributed. In tests with only two predictor groups, when data were normally distributed, a T-test was conducted to see if means were significantly different. When data were non-normally distributed a Mann-Whitney test was used. In tests with more than two predictor groups, when data were normally distributed a one-way ANOVA test was conducted with post-hoc Tukey's test if results were significant. For unequal groups, a Kruskal-Wallis test was used. All analysis was conducted in IBM SPSS Statistics 24.

4.3 Results

4.3.1 Blood-feeding assay

The effect of permitted sugar feeding on blood-feeding

Preliminary tests were conducted to determine the most suitable physiological state to test mosquitoes. Non-starved mosquitoes were exposed to untreated netting for 3-minutes in a

WHO cone test and then offered a blood meal as described above. Blood-feeding rates were lower than anticipated given nets were untreated and the mosquitoes were able to contact the host though the netting; 34.62% blood-fed after 1-hour, 50% within 8-hours, and 57.69% within 24-hours (42.31% unfed, 11.54% 24-hour mortality, N = 26 mosquitoes). Therefore, in subsequent tests, mosquitoes were fully water and sugar starved for ~24 hours prior to exposure and only provided with a sugar meal once they had blood-fed or at 24-hours post-exposure when the assay ended. On each experimental day at least 1 untreated netting replicate was conducted alongside test nets except for one-day when untreated controls were discarded due to potential contamination.

Knockdown, mortality and longevity

Following exposure to netting in 3-minute cone tests, combining unfed and blood-fed mosquitoes, knockdown and immediate mortality were low in all treatments, except for PermaNet 3.0 roof where mosquitoes showed rapid knockdown and 100% susceptibility to the netting (Figure 4.2). Immediate mortality was significantly different from untreated nets following exposure to Olyset Net ($P = 0.014$) and PermaNet 3.0 roof ($P = 0.000$), but not for PermaNet 3.0 sides ($P = 0.502$) or Interceptor G2 ($P = 0.79$).

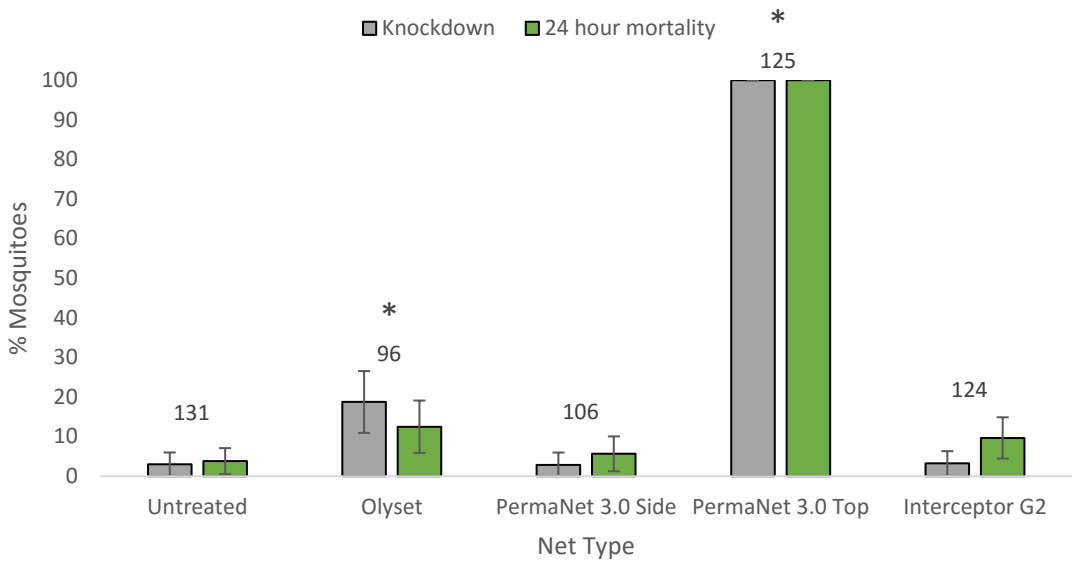


Figure 4.2. 1-hour knockdown and 24 hr mortality of Tengrela An. gambiae s.l. following a single exposure to test netting in a 3-minute WHO cone bioassay.

Error bars show 95% confidence intervals for the population proportion. Numbers above bars show the number of mosquitoes tested. Asterisks show when immediate (within 24-hours) mortality was statistically different (P < 0.05) from untreated control.

When just comparing blood-fed mosquitoes, no significant difference in mosquito longevity was seen following exposure to PermaNet 3.0 sides (P = 0.082), Olyset net (P = 0.514), or Interceptor G2 (P =792) compared to untreated net. No mosquitoes survived beyond 24-hours for PermaNet 3.0 roof, so longevity could not be assessed. Unfed mosquitoes were not analysed for longevity, as due to the nature of the blood-feeding assay very few mosquitoes were unfed.

Ability to blood-feed

When analysing blood-feeding, due to the way data was recorded, it was not possible to separate mosquitoes unable to feed due to being knocked down at the time a blood-meal was offered. Therefore, if a mosquito was unfed and knock downed it was classified as unfed in the analysis. Due to this classification, PermaNet 3.0 roof was found to have a significant effect on mosquito blood-feeding at all time points (Figure 4.2, P < 0.001), as mosquitoes were rapidly knocked down and unable to feed, but subsequently classified as unfed. In the regression model, PermaNet 3.0 roof was excluded from the model, due to

the fact all mosquitoes were all known to be knocked down prior being offered a blood meal. However, as it was not possible to remove knocked down mosquitoes from the other net types with the same certainty, these were included. In the other nets tested mosquito knockdown (< 19 %) and mortality (< 13%) were low, so this is unlikely to be a major confounder.

In initial analysis, at 1-hour post-exposure significantly fewer mosquitoes blood-fed following exposure to treated netting compared to untreated (Figure 4.3A, PermaNet 3.0 side P = 0.043, Olyset Net P = 0.050, Interceptor G2 P = 0.002). However, this effect was lost within 8-hours (Figure 4.3B; PermaNet 3.0 side P = 0.805, Olyset Net P = 0.726, Interceptor G2 P = 0.643) or 24-hours (Figure 4.3C PermaNet 3.0 side P = 0.929, Olyset Net P = 0.114, Interceptor G2 P = 0.482). Next, a binary logistic regression was conducted to investigate if this effect persisted when the effects of date, host presence, net type, temperature, and humidity on mosquito blood-feeding were also included. Host presence, temperature, and humidity did not significantly affect 1-hour blood-feeding and so were excluded from the final model. In the final model, the Nagelkerke R^2 indicated that the model accounted for 13.8% of the total variance in blood-feeding at 1-hours, with a correct prediction rate of 62.7%. Date (P = 0.000) and Net type (P = 0.004) both significantly affected blood-feeding at 1-hour. Only some days were significant. For net type, when controlling for the date, untreated net and Olyset net significantly affected 1-hour blood-feeding, with mosquitoes exposed to Olyset Net being 0.221 less likely to blood-feed compared to untreated net (95% CI 0.092 – 0.531, P = 0.001), or in other words, mosquitoes were 4.5 times more likely to feed at 1-hour after exposure to an untreated net compared to an Olyset Net. These results show that when controlling for effect of date the significant effect observed for other net types at 1-hour was lost.

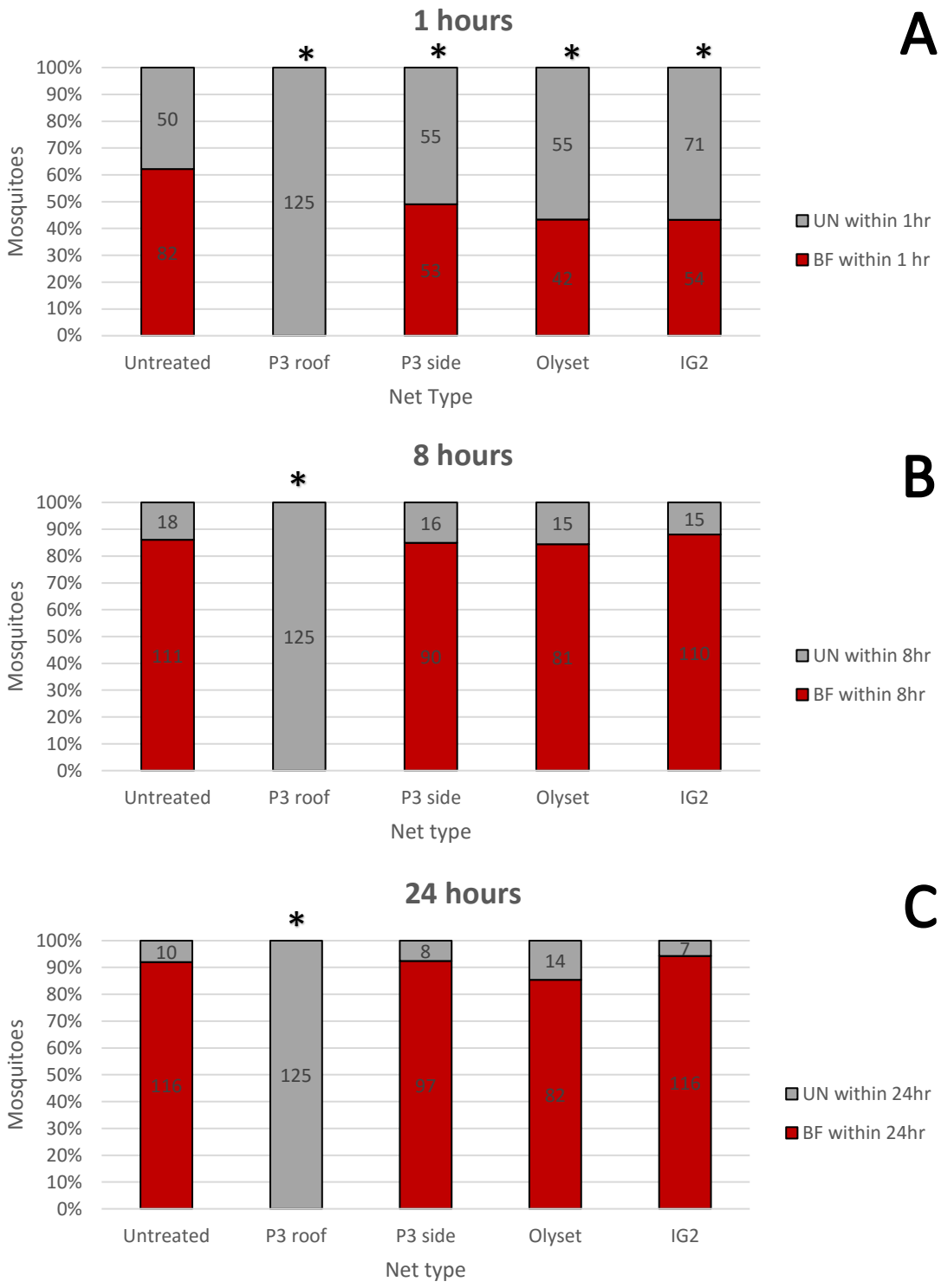


Figure 4.3. Cumulative percentage of mosquitoes unfed and blood-fed at 1-hour (A), within 8-hours (B), and within 24-hours (C) following exposure to netting in a 3-minute WHO cone assay.

Numbers in bars show the total number of mosquitoes. Asterisks show when blood-feeding was statistically different (P < 0.05) from untreated control. Abbreviations: UN = Unfed, BF = Blood-fed, P3 = PermaNet 2.0, IG2 = Interceptor G2.

4.3.2 Reproductive output hut trial: 2016

In the 2016 trial, 111 alive blood-fed females were collected in untreated huts, and 76 in PermaNet 2.0 huts. On day 1-3 days post collection, prior to forced oviposition, mortality was not significantly different between the two treatments (untreated: 20.72% mortality, PermaNet 2.0: 21.05% mortality, P = 0.956). Of the mosquitoes which underwent forced oviposition (untreated: n = 88 mosquitoes in tubes; PermaNet 2.0: n = 60 mosquitoes in tubes), no significant difference in mortality (untreated: 15.91%, PermaNet 2.0: 18.33%, P = 0.699), or oviposition (untreated: 44.32%, PermaNet: 2.0 36.67%, P = 0.353) was observed over the three days. Untreated huts mosquitoes laid 4077 eggs (from 38 females), which developed into 786 larvae (from 35 females, 3 missing data points). PermaNet 2.0 huts mosquitoes laid 1835 (from 22 females), which developed into 169 larvae (from 21 females, 1 missing data point). No difference was observed in the average number of eggs (Figure 4.4A. Untreated: 107 eggs/laying female, 95% CI 91 – 123; PermaNet 2.0: 83 eggs/laying female, 95% CI 64 – 103; P = 0.077) or larvae (Figure 4.4B: untreated 22 larvae/laying female, 95% CI 12 – 33; PermaNet 2.0 8 larvae/laying female, 95% CI 1 - 15; P = 0.138). There was no significant difference in hatching rates between the two groups (untreated 18.74% 95% CI 9.80 – 27.68, PermaNet 2.0 8.77% 95% CI 1.68 – 15.87, P = 0.184).

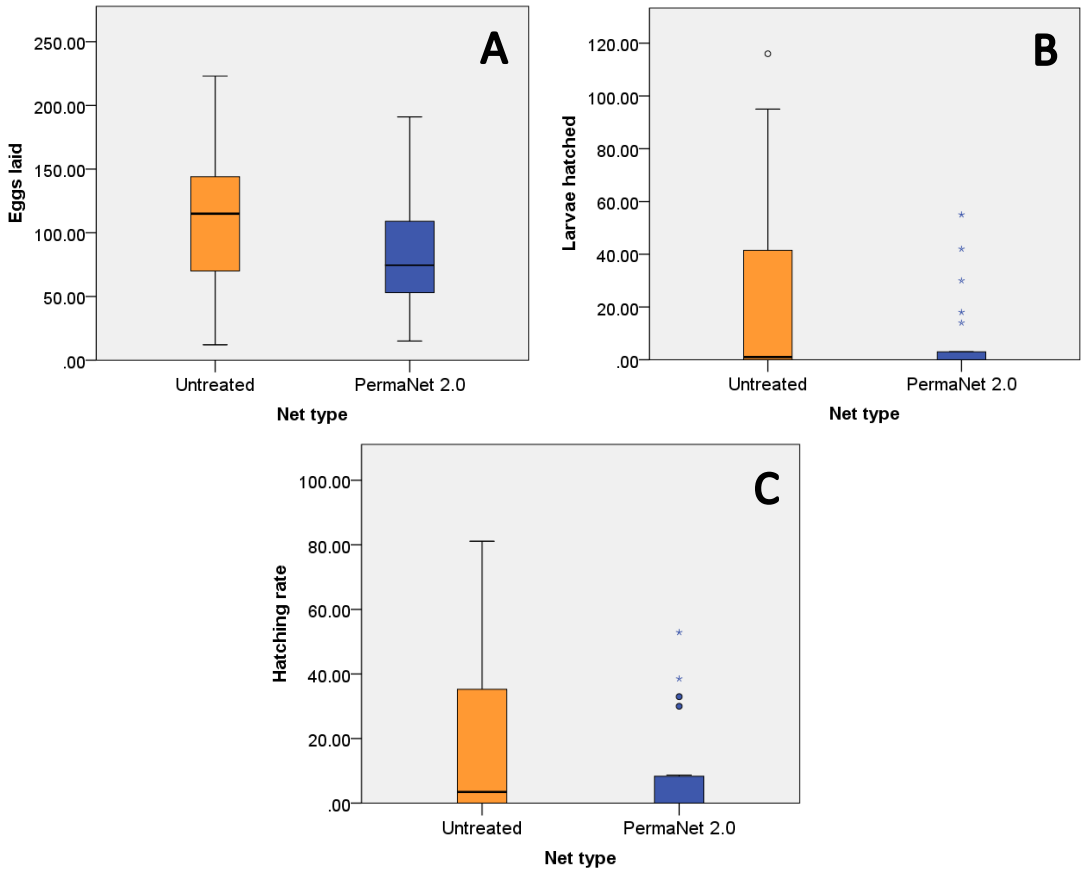


Figure 4.4. The reproductive output (egg number, larvae number and hatching rate) of mosquitoes collected from experimental huts containing untreated or PermaNet 2.0 nets in 2016.

Box and whisker plots of the number of eggs per laying female (A, untreated n = 38 mosquitoes, PermaNet 2.0 = 22 mosquitoes), the number of larvae per laying female (B, untreated n = 35 mosquitoes, PermaNet 2.0 = 21 mosquitoes), and the hatching rate per laying female (C, untreated n = 34 mosquitoes, PermaNet 2.0 = 21 mosquitoes) are shown. Missing data points were removed from the analysis.

Mosquitoes which had not died or laid eggs by day 6 had their ovaries dissected and eggs scored for normality (Figure 4.5). In the untreated group 35 mosquitoes were dissected and in the PermaNet 2.0 group 27 mosquitoes were dissected. In all mosquitoes which had no visible eggs, spermatheca and ovary dissections confirmed they were unmated and nulliparous, and these were removed from further analysis. Combining mosquitoes with abnormal or underdeveloped eggs there was no difference in ovary development between the untreated and PermaNet 2.0 groups (P = 0.311). In the mosquitoes with normal ovaries, no difference was observed in the average number of egg's retained (untreated: 100 eggs

retained/female with normal ovaries, 95% CI 83 – 117, PermaNet 2.0: 114 eggs retained/female with normal ovaries, 95% CI 97 – 131; P = 0.265).

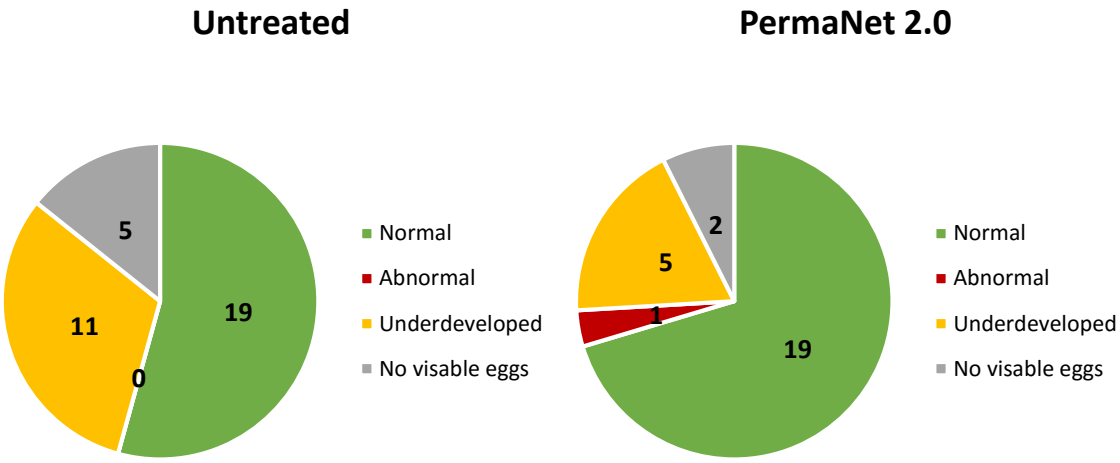


Figure 4.5. Pie charts of ovary status of dissected mosquitoes collected from untreated or PermaNet 2.0 huts in 2016.

4.3.3 Reproductive output hut trial: 2017

In the 2017 trial, 133 alive blood-fed females were collected in untreated huts, 74 in PermaNet 2.0 huts, and 89 in the Olyset Net huts. In Assay A mosquitoes were held in forced-oviposition tubes until they laid eggs or died. There was no difference in oviposition rate when comparing untreated huts (54.24% oviposition, n = 59 mosquitoes in tubes) to either PermaNet 2.0 (50.00% oviposition, n = 26 mosquitoes in tubes, P = 0.718) or Olyset Net (58.33% oviposition, n = 36 mosquitoes in tubes, P =0.697). Untreated huts mosquitoes laid 4119 eggs (from 32 females), which developed into 1454 larvae. PermaNet 2.0 huts mosquitoes laid 1140 (from 13 females), which developed into 246 larvae (from 12 females, 1 missing data point). Olyset Net huts mosquitoes laid 2303 (from 21 females)), which developed into 395 larvae. No difference was observed in the average number of eggs (Figure 4.6A: untreated 129 eggs/laying female, 95% CI 105 – 153; PermaNet 2.0: 88 eggs/laying female, 95% CI 50 – 126; Olyset Net: 110 eggs/laying female, 95% CI 83 – 136; P = 0.143) or larvae (Figure 4.6B: untreated 45 larvae/laying female, 95% CI 28 – 63; PermaNet 2.0: 21 larvae/laying female 95% CI 2 – 39; Olyset Net: 19 larvae/laying female, 95% CI 5 – 33; P = 0.087) between any of the groups. There was no significant difference in

hatching rates between the three groups (untreated 33.23% 95% CI 20.56 – 45.91, PermaNet 2.0 22.71% 95% CI 4.46 – 40.66, Olyset Net 15.21% 95% CI 4.49 – 25.93, P = 0.112).

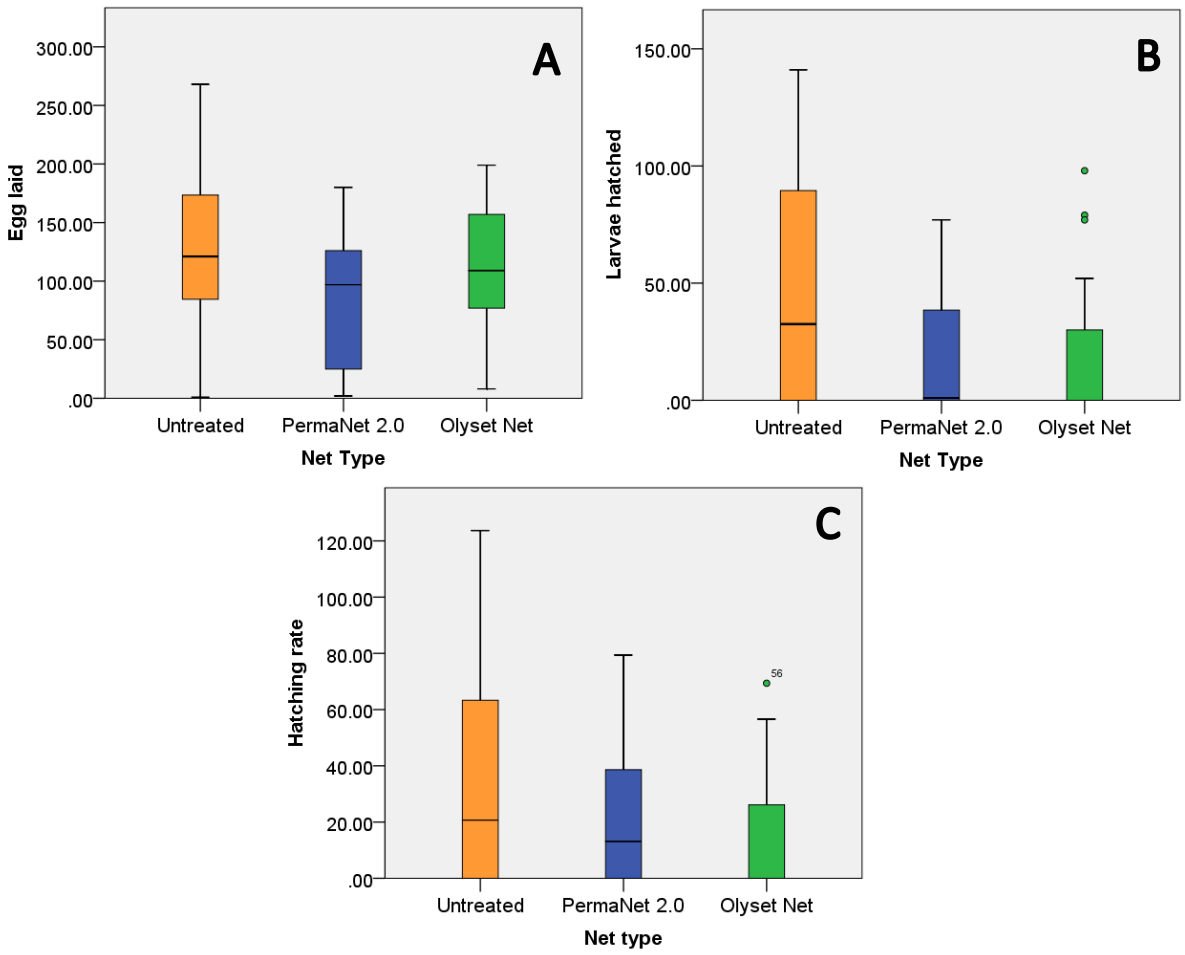


Figure 4.6. The reproductive output (egg number, larvae number and hatching rate) of mosquitoes collected from experimental huts containing untreated, PermaNet 2.0 or Olyset Nets in 2017.

Box and whisker plots of the number of eggs per laying female (A, untreated n = 32 mosquitoes, PermaNet 2.0 = 13 mosquitoes, Olyset Net = 21 mosquitoes), the number of larvae per laying female (B, untreated n = 32 mosquitoes, PermaNet 2.0 = 12 mosquitoes, Olyset Net = 21 mosquitoes), and the hatching rate per laying female (C, untreated n = 32 mosquitoes, PermaNet 2.0 = 12 mosquitoes, Olyset Net = 21 mosquitoes) are shown. Missing data points were removed from the analysis.

In Assay B, mosquitoes were dissected 3-days post-collection. In the untreated group 51 mosquitoes were dissected, in the PermaNet 2.0 group 37 mosquitoes were dissected, and

Olyset Net group 41 mosquitoes were dissected (Figure 4.7). In all mosquitoes which had no visible eggs, spermatheca and ovary dissections confirmed they were unmated and nulliparous, and these mosquitoes were not included in subsequent comparisons. Combining mosquitoes with abnormal or underdeveloped eggs there was no difference in ovary development between the untreated and PermaNet 2.0 groups (P = 0.462). However, significantly more mosquitoes in the Olyset Net group were found to have abnormal or underdeveloped eggs (P = 0.019) compared to untreated. In mosquitoes with normal ovaries, no difference in retained eggs was observed between untreated (untreated: 104 eggs retained/female with normal ovaries, 95% CI 104 – 127) and treated (PermaNet 2.0: 105 eggs retained/female with normal ovaries, 95% CI 93 – 117, Olyset Net: 127 eggs retained/female with normal ovaries, 95% CI 115 – 139) nets. However, post-hoc analysis showed an increased number of eggs retained in mosquitoes collected from Olyset Net huts compared to PermaNet 2.0 (P = 0.039).

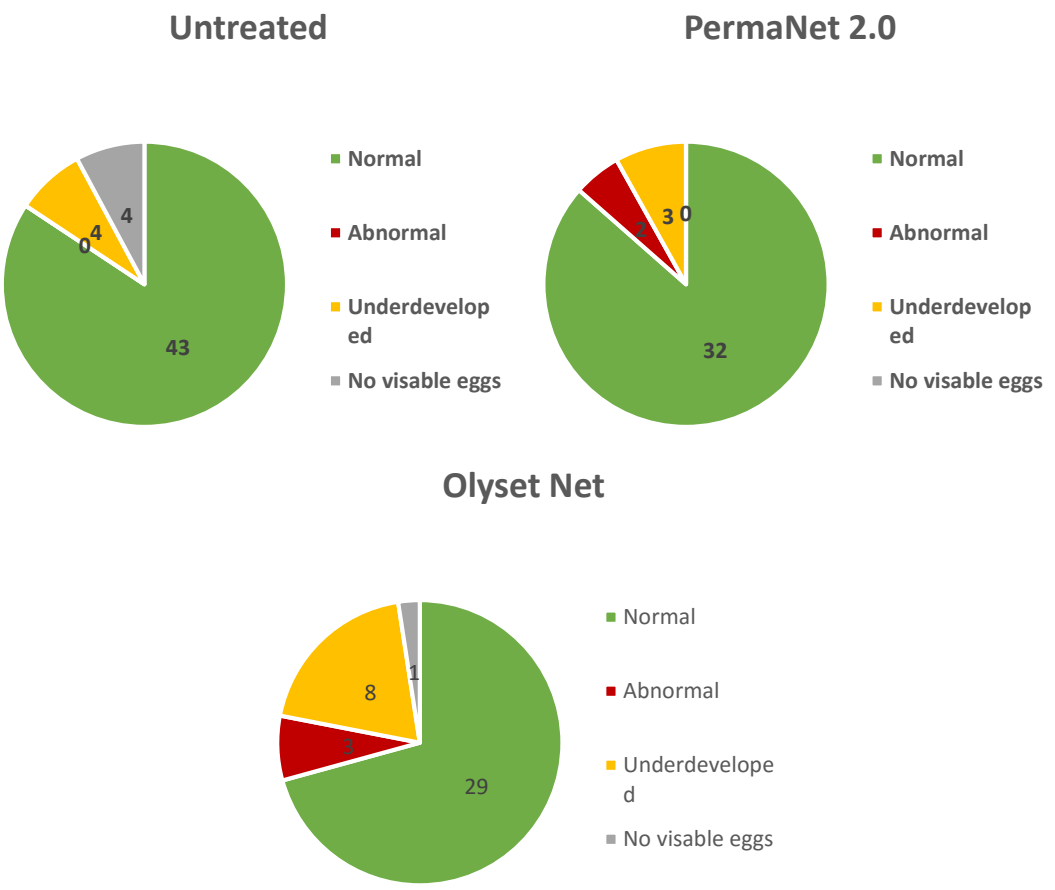


Figure 4.7. Pie charts of ovary status of dissected mosquitoes collected from untreated, PermaNet 2.0, or Olyset Net huts in 2017.

4.4 Discussion

The experiments reported in this chapter investigated if exposure to insecticidal nets affected mosquitoes' life-history traits: experiments were conducted to look at the effect of net exposure on mosquito ability to blood-feed, and mosquito reproductive output.

4.4.1 Effect of insecticidal net exposure on *An. gambiae* s.l. ability to blood-feed

Following exposure to first- and second-generation bednets, the preliminary analysis suggested a mosquito's ability to blood-feed was impacted by insecticidal nets at 1-hour post-exposure compared to the untreated net, and this effect was lost within 8 hours. However, further analysis of the 1-hour dataset revealed these results to be compounded by variations in the experimental day. Except for PermaNet 3.0 roof netting, which caused 100% knockdown and immediate mortality, only Olyset Net had a significant impact on mosquito blood-feeding at 1-hour compared to the untreated net when accounting for the day. Mosquito knockdown and immediate mortality were also significantly higher following Olyset Net exposure compared to untreated. Unfed mosquitoes were not separated by knockdown status (except for PermaNet 3.0 roof where it was clear all unfed mosquitoes were knocked-down). Therefore, it is not possible to distinguish if lower blood-feeding in Olyset Net's is as a result of the mosquito being knocked down at the time the blood-meal was offered, or unable to take a blood-meal due to other effects. Although the relative impact of being knocked down or feeding inhibition cannot be clarified here, cumulatively, this does illustrate the action being exerted by the net, in its ability to both knockdown and kill, or inhibit blood-feeding of the mosquito, thus impacting on their disease transmission potential. However, in the highly resistant population tested mortality was extremely low (<20%) following exposure to all insecticidal nets, except for PermaNet 3.0 roof.

The lack of blood-feeding inhibition at 1-hour in the other net treatments is surprising. Previous studies have observed blood-feeding inhibition shortly after LLIN exposure (Glunt *et al.*, 2018). In Glunt *et al.* (2018), *Anopheles* were exposed to PermaNet 2.0 or untreated netting in lined WHO tubes for 1 – 10 minutes (depending on their resistance level). Immediately following exposure mosquitoes were offered a human blood meal, and blood-feeding was observed to be significantly reduced in mosquitoes exposed to treated nets compared to untreated. Their study only observed blood-feeding at 1-hour post exposure,

but measured host-seeking (defined as a mosquito probing a mesh cage on the sides exhibiting host cues) in the same populations for up to 24-hours. At 1-hour, host-seeking was observed to be significantly reduced following PermaNet 2.0 exposure compared to untreated in all *Anopheles* strains, with this effect persisting for up to 24-hours in *An. arabiensis* (no significant difference was observed after this time point in a resistant *An. funestus* strain, or the wild *Anopheles* spp. from Mozambique). In their study, blood-feeding Inhibition was higher in those exposed for 5- and 10-minutes compared to 1 minute, suggesting duration of exposure could have influenced the results. As exposure time varied between the strains, it is not possible to ascertain if the mosquito's resistance level also affected blood-feeding inhibition. In the experiment reported in this chapter, mosquito responses were first tested at 1-hour post-exposure, not immediately. It is plausible that any feeding inhibition effects in this population are short-lived, or potentially non-existent at a "sub-lethal" i.e. unless the mosquito is incapacitated to the point of knock down, as is suggested by the PermaNet 3.0 roof result here, it is not deterred from feeding. Tchakounte *et al.* (2019) observed that LLIN-exposure in a 3-minute cone bioassay (PermaNet 2.0) did not affect the blood-feeding ability of F1 *An. funestus* and F7 *An. gambiae* s.l. in Cameroon. However, in this study blood-feeding ability was initially tested 3-days post-exposure, which does not approximate how insecticide contact and blood-feeding would likely occur under natural conditions. In nature, it is more likely that mosquitoes would encounter insecticides shortly prior to (LLINs) or after (IRS) obtaining a blood-meal. Additionally, the exposed mosquitoes seem to have been compared to a non-exposure control, and so it is not possible to determine how mosquitoes may have been affected by the handling procedure. The study design of the current trial meant only mosquito ability to feed was evaluated. It is not possible to establish if unfed mosquitoes were unwilling to feed (i.e. did not attempt to host seek) or were willing to feed but unable (i.e. attempted to probe but could not imbibe blood).

As the study design of most lab and field trials preclude the investigation of the duration of blood-feeding inhibition, the experiment discussed in this chapter aimed to establish if blood-feeding inhibition persisted over a 24-hour period. In this instance, the current experiment was limited by the post-exposure timepoints chosen. These were selected to provide a proxy for a mosquito obtaining a blood meal immediately after exposure (1 hour), within the same night (8 hours) or the following day (24 hours). These were not evenly distributed in time, which made the analysis of this data a challenge. An improved

method would be to allow immediate continuous access to a blood-meal following exposure, and "time to feeding" measured for each mosquito. This would also capture if blood-feeding inhibition does occur in this population prior to 1-hour post-exposure, to establish if any inhibition effects are seen. For such an assay a stopping point would need to be predetermined to allow a realistic cut-off for the experiment.

Mosquito numbers available for testing fluctuated during the field season due to productivity of identified breeding sites. To ensure ~100 mosquitoes were exposed to each net treatment, nets were not all tested on the same experimental days, and on these days different number of mosquitoes may have been exposed to each treatment (e.g. experimental day 2, 15 mosquitoes exposed to untreated net 21 to PermaNet 3.0 sides, 20 to PermaNet 3.0 roof). The logistical regression conducted on the 1-hour blood-feeding data showed date affected the mosquito's ability to take a blood-meal on some days when accounting for the effect of net type. As temperature and humidity were not significant predictor variables in the model, it is possible that this date-effect is related to the different cohorts of mosquitoes used for testing. Testing was conducted over a 2-month period. Mosquitoes were collected and reared using the same methods, however, it is difficult to rigorously standardised mosquito rearing in this field setting, and so micro-differences in mosquito growth and fitness may exist. Given the potential difference of the larval-reared population, future testing should consider equally splitting the mosquitoes available for testing across all treatment types each day in order to control for this variation.

Host-presence during exposure did not affect the probability of a mosquito blood-feeding at 1-hour post exposure. Previous studies have observed host presence significantly increases mosquito landings on a net compared to no host (Siegert, Walker and Miller, 2009). Therefore, host presence increases the duration of net contact. The Tengrela population used here are highly resistant (see Chapter 2. Section 2.4) so it is plausible that small differences in net contact within the 3-minute exposure time have a minor toxicological impact on this population. Host-presence is likely to be of greater importance in less-resistant populations where the duration of net contact may have a greater affect. Duration of insecticidal net exposure has been observed to effect *Anopheles* ability to blood-feed (although different mosquito strains with different resistant levels were tested) (Glunt *et al.*, 2018), and bed bugs willingness and ability to blood-feed (Jones, Bryant and Sivakoff, 2015). Any future tests should standardise this, preferably with a host present as

cue emanating from them will result in more natural host-seeking behaviour at the LLIN interface even when exposure conditions are artificial.

4.4.2 Effect of insecticidal net exposure on *An. gambiae* s.l. reproductive output

Exposure to insecticidal nets in WHO hut trials showed mostly no effects on delayed mortality, ovary development, oviposition, or larval development. The only significant result was in the 2017 trial when more mosquitoes were observed to have underdeveloped and abnormal ovaries following collection from Olyset Net huts, compared to untreated. Although, a significantly greater amount of abnormal/underdeveloped eggs were observed in the Olyset Net arm the majority (4/5) of abnormal eggs had a round morphology and were brown in colour, suggesting this irregularity may have been the result of bacterial infection and not insecticide exposure. Many eggs were found to be underdeveloped, which could be a result of blood-meal volume. Unfortunately, blood-meal volume was not measured in this experiment, due to the limited capacity of the field insectary.

These results support other trials which found no or minor effects of Olyset Net exposure compared to untreated nets on *An. gambiae* reproductive output (Ngufor *et al.*, 2014, 2016; Koffi *et al.*, 2015). A recent laboratory study also observed no effect on blood meal size, the number of eggs laid, the number of descendants produced, or emergence rate between resistant *An. gambiae* (KdrKis) exposed to permethrin-treated nets or controls after either a single or double net exposure (Mulatier *et al.*, 2019).

The mosquitoes examined in this trial are the natural host-seeking population, therefore their age and previous insecticide exposure history is unknown. Prior to the trial, dissections of the larval-collected mosquitoes reared to adults in the insectary showed low female insemination rates, so using a reared-released mosquito population for this experiment would have been unsuitable. Although this introduces unknown interactions into the experiment, it allows the effects of insecticidal net exposure to be examined with the natural malaria transmitting population. *Anopheles* mosquitoes are anautogenous so require a blood meal to produce eggs. Therefore, for this experiment, only blood-fed mosquitoes were suitable for measuring reproductive output. In order to take a blood-meal from the host inside the hut, the mosquitoes must either enter the net though a small (4 x 4 cm) holes cut in the fabric, or feed though the net while the host skin was in contact with

it. In both cases, it is likely that net contact would occur. As the huts are designed to target host-seeking mosquitoes attempting to obtain a blood-meal, it was assumed that blood-fed mosquitoes captured inside the hut had fed on the host under the net (thus contacting the net), and had not entered the hut already blood-fed.

Fecundity is closely linked to mosquito body size (Lyimo and Takken, 1993; Takken, Klowden and Chambers, 1998) and wing measurements are used as a proxy for body-size. However, this was not measured in the current trials. Although variations in mosquito size could be influencing the results, there is no reason to suspect mosquitoes of a certain size would be collected in huts containing one net type compared to the other. In a recent lab trial, when controlling for the effects of body size and blood-meal volume, no effect of Olyset Net exposure on the number of eggs laid was seen in an *An. gambiae* strain (Hauser, Thiévent and Koella, 2019). The authors observed haematin level (a proxy for blood-meal volume) to be positively correlated with egg number, but no interaction between net type and haematin was seen. The current study did not measure the effect of net exposure on blood meal volume, and it would be interesting to evaluate this on the field population.

The methodology for the reproductive output trial was altered between 2016 and 2017 in an attempted to improve oviposition rates. In the 2016 trial, oviposition was low in all groups (<45%) although dissections showed a large proportion of non-laying mosquitoes to have fully developed ovaries (i.e. Untreated net 54% of mosquitoes dissected had normal ovaries, n = 35). Subsequently, in the 2017 trial, the time permitted for oviposition was altered from 3-days to until the mosquito either laid eggs or died. Although oviposition increased in 2017, this was still low. It is documented that egg laying rates among wild mosquitoes are low under artificial conditions, and the rates observed in the current trial are comparable (or higher) than those seen in other hut trials (Ngufor *et al.*, 2014, 2016; Koffi *et al.*, 2015). Although *Anopheles* mosquitoes are largely considered to be anautogenous, several studies have documented gonotrophic discordance, where more than one blood-meal is needed to produce an egg batch, although research suggests this may be linked to body size (Lyimo and Takken, 1993; Takken, Klowden and Chambers, 1998). In this experiment mosquitoes were only able to blood-feed during exposure in experimental huts, therefore, this could provide a possible explanation for the low oviposition rates observed in all treatment arms. However, oviposition rates of <50% were

still observed in Ngufor *et al.* (2014) trials, despite mosquitoes acquiring a secondary blood meal.

Overall, the results suggest insecticide exposure from standard-LLINs has no physiological impact on the mosquito reproductive output. Exposure does not damage the structure of the ovaries, inhibit egg development or laying, or egg hatching. In this assay, hatched larvae were counted one week after eggs were floated (when larvae were in L2/3). Therefore, if insecticide exposure effects larval metamorphosis or developments into adults, this would not be captured by this test.

Determining how insecticide exposure effects mosquito fertility and fecundity is challenging, and no standardised methods of how to measure and analyse this information exists. Reproductive output is a chain of events from oogenesis, oviposition, egg hatching and larval development, which is additionally complicated by egg development being inextricably linked to blood-feeding in *Anopheles* mosquitoes. Small non-significant differences may exist at each stage, which independently may suggest no effect. In the results discussed above, no differences in many of these steps were observed between untreated and treated arms. Cumulatively, these steps may significantly affect reproductive output, however, the chain of events and external factors relating to this, make it difficult to assign any effect entirely to the physiological impact of insecticide exposure. To rigorously measure reproductive output, lab trials where net contact and blood-feeding are controlled would be beneficial. Large samples would be needed to overcome variation in blood meal volume and low oviposition rates encountered from forced egg-laying. Rearing environments (e.g. container size, number of larvae per container, amount of food provided) would need to be standardised, as larval development is density dependant and will be affected by over- or under-crowding. Such assays are challenging to conduct under semi-field conditions.

4.5 Conclusion

Combined with the lack of knockdown and mortality observed in this population, the absence of blood-feeding inhibition observed here is concerning. Although results from the experimental hut trial suggest insecticidal nets are still providing personal protection through blood-feeding inhibition in some instances (Chapter 3), this inhibition effect is not

observed when mosquitoes are subsequently offered a bloodmeal through untreated net. In reality, this could lead to mosquitoes being able to readily bite individuals not protected by a bednet, even if they have contacted an insecticidal net while host seeking the same night. The lack of effects on reproductive output are perhaps less surprising given the neuronal target site of pyrethroids, but still highlight that sub-lethal effects of insecticidal net exposure in this population appear to be non-existent. Encouragingly, however, is the ability of PermaNet 3.0 roof netting to rapidly incapacitate the highly resistant *An. gambiae* population.

Chapter 5. Exploring the feasibility of video benchtop tests to evaluate the effects of insecticidal net exposure on wild *Anopheles gambiae* behaviour

5.1 Introduction

Most international agencies and country control programmes will only purchase vector control products that have been 'pre-qualified' (evaluated for quality, safety and efficacy using pre-determined guidelines) by WHO. Currently, 20 LLINs have WHO PQ listing, of which 7 can be defined as next-generation nets, as they contain secondary compounds that are non-pyrethroid insecticides, synergists, or insect growth regulators (WHO, 2019a). Although the WHO process for evaluating vector control products has changed in the last few years (from WHOPES to PQ listing), the dossier requirements for entomological efficacy remain the same and are based on the assessment of phase I-III WHOPES trials (Table 1.2, page 30, WHO, 2013a). Except for "first in class" products, i.e. those with a novel mode of action that fall outside an established intervention class, which additionally require epidemiological evidence of public health impact. Currently, phase I trials comprise the WHO cone bioassay and tunnel test. These assess LLIN efficacy against standardised endpoints including 60-minute knockdown, 24-hour mortality, and blood-feeding success within 24-hours (tunnel test only). To proceed to phase II field testing, nets washed at least 20 times (a proxy for 3 years field use) must meet the efficacy criteria for cone bioassays (≥ 80% mortality or ≥ 95% knock-down) or tunnel tests (≥ 80% mortality or ≥ 90% blood-feeding inhibition). Tunnel tests are used when the efficacy thresholds in cone assays are not met. This is based on the premise that the cone assay may underestimate the true efficacy of a net if it has a high contact irritancy effect.

The existing WHO tests and efficacy thresholds were developed for pyrethroid-treated nets, the only insecticide class used on nets at that time. Pyrethroids were suitable for use in nets in part because of their fast-knockdown and lethality by tarsal contact (in susceptible mosquitoes), and their low toxicity to humans (Zaim, Aitio and Nakashima, 2000). Therefore, these test protocols, in general, do not measure any slow-acting or delayed impacts, or sub-lethal effects, and there is little guidance on how to assess these non-standard outcomes. Nor do the tests consider mosquito behaviour at the bednet

interface, or how this might be altered by insecticide presence. As a result, these assays may not be suitable for evaluating next-generation nets, some of which contain relatively slow-acting insecticides (e.g. Interceptor G2) or have modes of action other than killing (e.g. adult sterilisation). It has been suggested that current WHO guidelines may not be appropriate for screening certain non-neurotoxic insecticides (Oxborough *et al.*, 2015).

In Burkina Faso, where the fieldwork reported here was conducted, malaria is increasing despite widespread deployment of vector control tools. Since pyrethroid-resistance in vectors offers one possible explanation for this increase, next-generation nets were distributed during Burkina Faso's 2019 national net distribution campaign in the Western part of the country. In this region, insecticide-resistant mosquito populations are most prevalent. Given the concerns that the mode of action of some novel net treatments may not be captured using existing tests, identifying appropriate methods to replace or augment them is an immediate challenge.

Consequently, the primary aim of the work reported in this chapter was to explore the feasibility of using video benchtop tests for describing and quantifying behaviours of wild *Anopheles gambiae* at the bednet interface. The secondary aim was to use the same tests to evaluate the effects of exposure to standard and next-generation nets on the behaviour of wild *Anopheles gambiae*.

5.2 Methods

5.2.1 Study site, mosquito populations & net treatments

All laboratory tests were performed at the CNRFP insectaries in Banfora, Burkina Faso, using adult female F_0, reared from immature stages collected from Tengrela, Burkina Faso. Mosquitoes were collected, reared, and identified to species level using the methods described in Chapter 2 (Section 2.2). In both tests (video cone tests and baited box test) mosquitoes were exposed to untreated nets, PermaNet 3.0 sides, PermaNet 3.0 roof, and Interceptor G2. Olyset Net was tested in video cone tests, but not in baited box tests. Specifications of the nets tested are described in full in Chapter 2 (Section 2.5). All testing was conducted between August – October 2018.

5.2.2 Video cone test: experimental methods

The video cone test is a standard WHO cone test (WHO, 2013a) modified with the addition of a human host, and recorded using a smartphone, for subsequent behavioural analysis (Figure 5.1). It yields considerable additional information on mosquito interactions with the net, mimicking exposure to the insecticidal net during the response to a human host in a more realistic way.

Video cone test: Net exposure protocol

Mosquitoes were exposed to test netting using a WHO cone bioassay baited with a human host (Figure 5.1). Mosquitoes were 5-to-7-days-old, non-blood-fed females, which had been starved of sugar and water for a minimum of 24-hours prior to exposure. Test nets and mosquitoes were acclimatised to the testing room for >1 hour before experiments began. Testing began after 22:00 to coincide with peak *Anopheles* biting times in Burkina Faso (Dambach *et al.*, 2016; Epopa *et al.*, 2019).

Mosquitoes were aspirated in batches of 3-to-5 from a holding cup into a plastic cone. The operator then placed an uncovered forearm behind the test netting and exhaled naturally towards the cone board, emitting host cues during the exposure. The operator refrained from washing with scented substances for 8-hours before the test. After 3-minutes, mosquitoes were transferred back to holding cups. Tests were recorded individually under normal lighting using a smartphone (Apple iPhone SE, USA) at 30-60 frames per seconds, and videos were saved as MOV files. The recorded temperature in the testing room ranged from 27.8 – 28.3°C, and humidity between 59.3 – 99.6%.

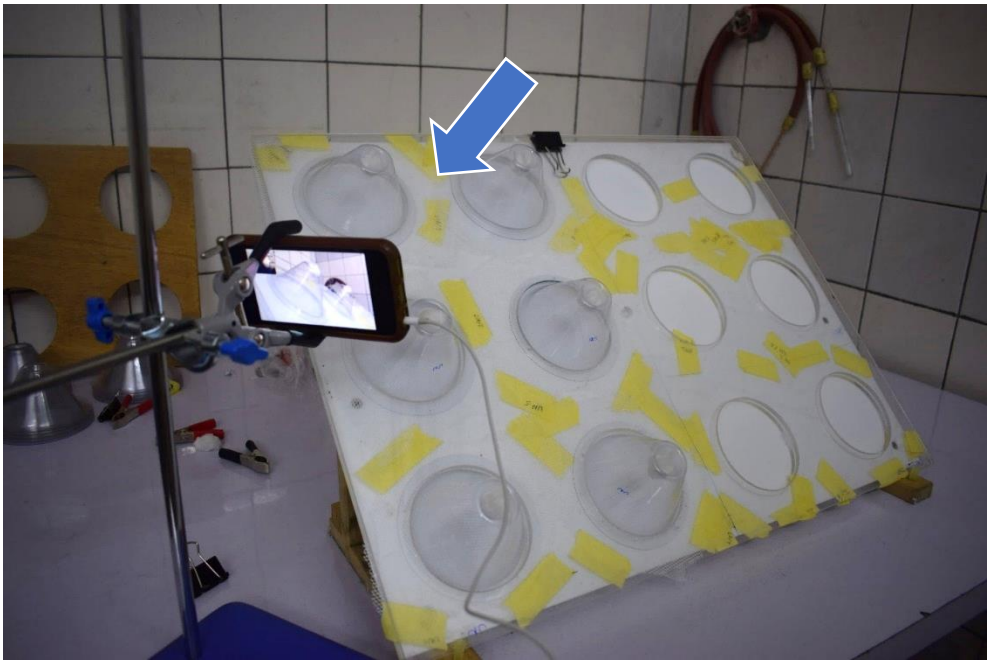


Figure 5.1. The video cone test apparatus used in the laboratory in Banfora, Burkina Faso. *The smartphone (Apple iPhone SE, USA) was held in place using a clamp stand. The phone was angled so that the recording arena captured the test net surface and cone (here the cone being recorded is marked with a blue arrow). The cone board was set up with test netting following the standard WHO guidelines* (WHO, 2013a).

The mosquitoes recorded in the video cone test were additionally used in another experiment which examined the effect of insecticidal net contact on mosquito blood-feeding ability (Chapter 4). Consequently, as dictated by the design of that experiment, post-exposure mosquitoes were not provided with sugar solution immediately; instead they were offered a human blood-meal from the test operator for 20-minutes at 1, 8 and 24-hours post-exposure. After they blood-fed or at 24-hours post-exposure mosquitoes were provided with 10% glucose solution soaked onto cotton wool. Mosquito mortality was recorded daily until all mosquitoes were dead, and dead mosquitoes were stored in silica. Blood-feeding and longevity data are reported in Chapter 4.

Video cone test: Data analysis

Videos were visually scanned by eye at 5-second intervals (scan sampling) and the number of mosquitoes resting on the net, resting on the cone, in flight, or obscured from view was recorded. Behaviours were classified using predetermined criteria (adapted from Hughes 2018, Table 5.1). The start time of the exposure was defined as when all mosquitoes had

exited the mouth aspirator into the cone arena. Videos were analysed in BORIS behavioural software version v. 7.4.4. (Friard and Gamba, 2016). Mosquito numbers varied between replicates (3-5 mosquitoes), and in some instances, mosquitoes escaped through test netting during the 3-minute test. To account for this variation, the proportion of mosquitoes displaying each behaviour at each time point was calculated (Figure 5.2). The mean proportion of mosquitoes exhibiting each behaviour at each time point was plotted onto scatter graphs, and 95% confidence intervals were calculated to show the variability between test replicates. Single composite images of each video cone recording replicate were created by overlaying the image frames every 0.1 seconds. This allowed visualisation of the mosquitoes location during the video cone exposure. Images were created using a bespoke behavioural video analysis software, VicTA (Video Cone Test Analyser), by Dr Jeff Jones *(Jones* et al*., Unpublished)*.

Table 5.1. Classifications used to define mosquito behaviours in video cone tests adapted from definitions used by Hughes (2018).

Behaviour	Description
Net	Mosquito is resting on the net or has landed on the net
Cone	Mosquito is resting on the cone or has landed on cone
Flight	Mosquito is in flight or has touched the net or cone at the sampling time point but during a bouncing flight where it does not rest on the net or cone
Obscured	Mosquito is not visible due to the recording angle or obscured by the cotton wool plug and its behaviour cannot be determined

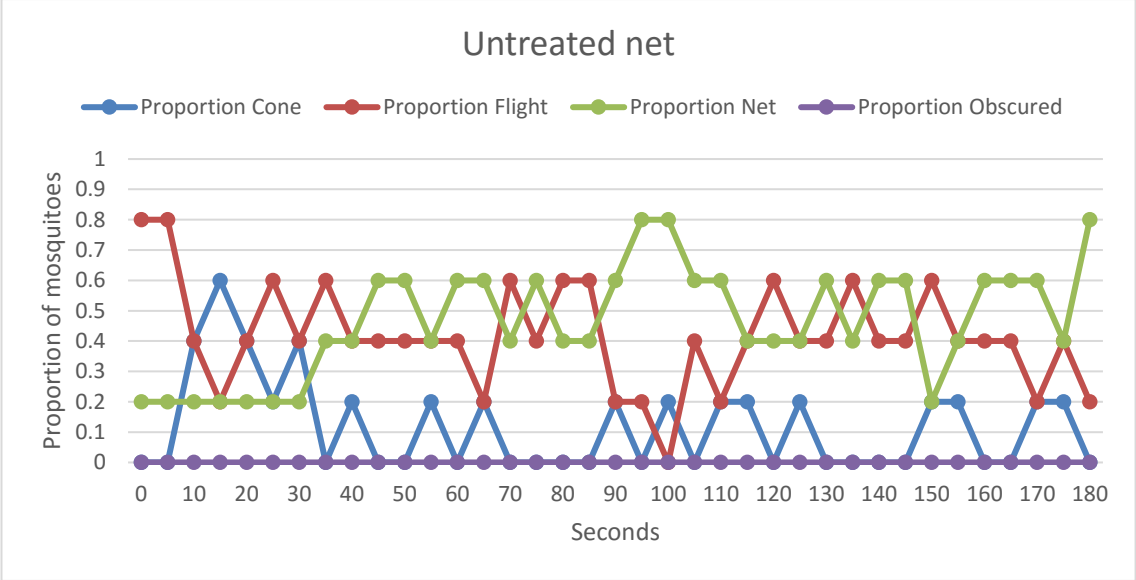


Figure 5.2. An example of the behavioural scan-sampling summary obtained for a single 3-minute video cone replicate.

The plot shows the proportion of mosquitoes resting on the cone, in-flight, resting on the test netting, or obscured from view during a 3-minute exposure to an untreated net in the video cone test (5 mosquitoes exposed).

5.2.3 Baited box test: experimental methods

The 'thumb test' (Hughes *et al.*, Unpublished) and 'baited box' test are variants of a laboratory benchtop test that records a video of mosquito activity at the bed net interface as the mosquitoes respond to a human host. The thumb test used individual mosquitoes and the baited box test used batches of mosquitoes. In the thumb test access to the host was possible only at a 2.5 cm diameter opening, covered by the test netting, situated opposite the mosquito entrance tube (Figure 5.3). This allowed all blood-feeding activity to be captured by a single camera focused on the net surface. The operator placed their thumb behind the test netting to act as a host. The mosquito was permitted to feed on the host's thumb or prevented from feeding by installing an additional untreated net barrier on the human side of the test net. Mosquitoes were aspirated individually into a gated holding tube, which was opened at the start of the test. Mosquitoes were classified as non-responders if they failed to exit the holding tube within 3-minutes, or if they fail to probe at the net interface within 10-minutes of exiting the holding tube (Hughes *et al.*, Unpublished).

Figure 5.3. The thumb test box setup (left) in position with LED and camera (middle) and with the operator's thumb in position (right).

Baited box test: equipment setup

The baited box method used here was an adaptation of the thumb test. The baited box test arena is a 10 cm^3 acrylic box (Retailacrylics, UK) with clear sides and base and a removable white roof (Figure 5.4). On one side it has four small ventilation holes (1.5 cm) and a gated mosquito entrance tube. The horizontal base and roof both have a central hole (7 cm). The base hole is a ventilation hole and the roof hole is the exposure hole where test netting is placed. The operator's forearm is positioned above the roof hole during exposure, which allows host cues to be present during the test. The ventilation holes are covered with polypropylene mesh. To set up the box, test netting is placed over the uncovered roof and taped across all four sides, taking care not to obscure the filming area when securing the netting. The white plastic roof is then placed over the netting.

The test is recorded using an infra-red camera (Ximea MQ013RG-E2 1.3 Megapixel near-infrared enhanced CMOS Camera, Ximea, Munster, Germany) with a 60 mm lens (F2.8 Nikon camera lenses used at F8). It is conducted in darkness and the setup is illuminated by an infra-red LED light (M850L2: wavelength spectrum from 790-885nm, ThorLabs Ltd, Ely, UK) passed through a paper or acrylic diffuser (16 x 16 cm, COMAR optics, Linton, Cambridge, UK). The setup is passively illuminated by light from the nearby computer screen. The box is placed on a ~5 cm platform, ~20 cm from the LED light and ~80 cm from the camera. The acrylic diffuser is positioned ~ 10 cm in front of the LED light. The LED light and diffuser are held in place with clamps stands and the camera with a tripod. The camera is connected directly to the recording computer by USB (Figure 5.4). Tests were recorded individually between 10 - 50 FPS using StreamPix recording software (StreamPix V.7, Norpix, Montreal, Canada) and recordings were saved as AVI or MP4 files.

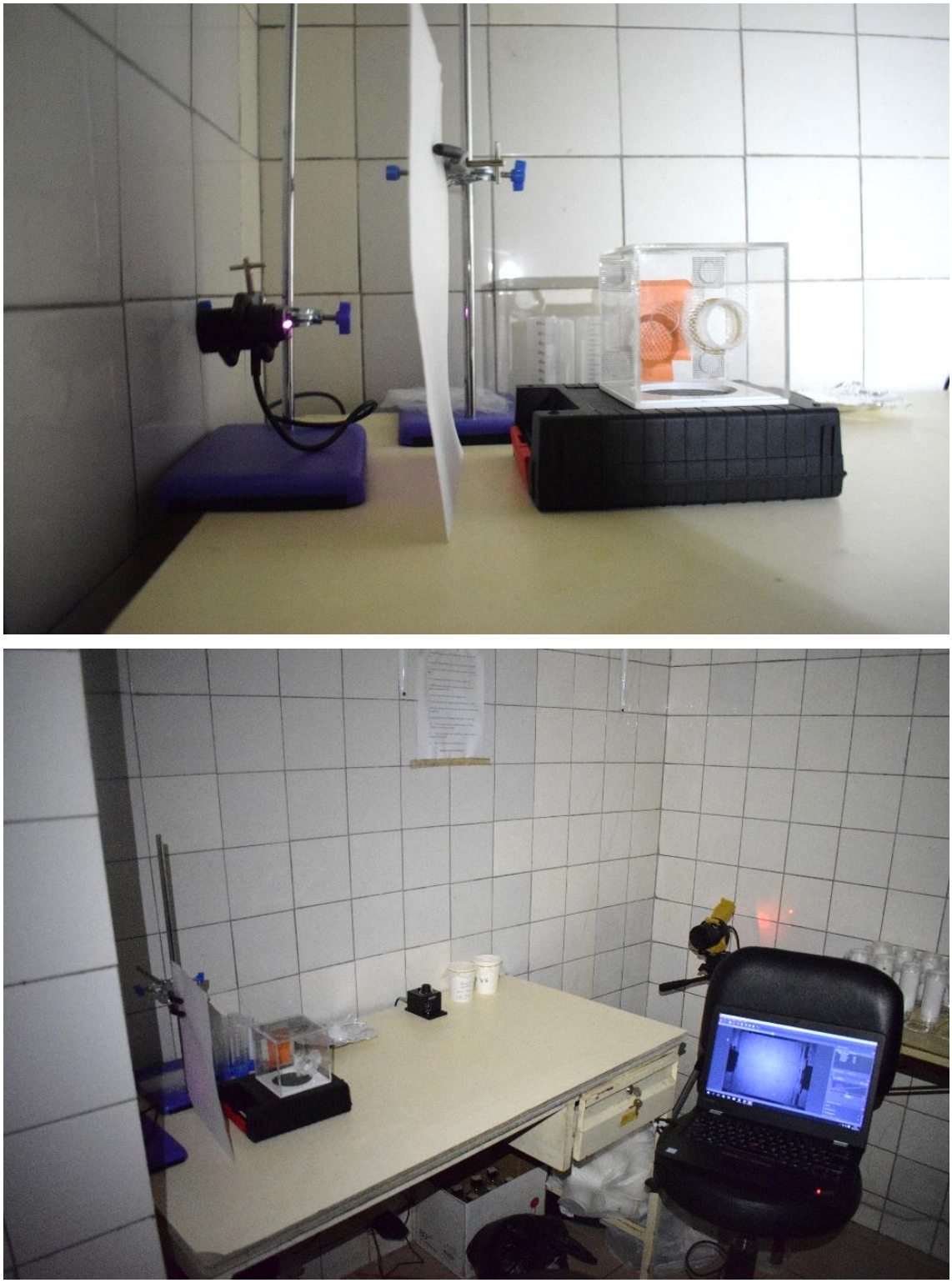


Figure 5.4. The baited box test apparatus in the laboratory in Banfora, Burkina Faso. *Images show the position of the infra-red LED and diffuser (top) and camera and laptop (bottom) in relation to the baited box test arena. The camera is held in position using a tripod, and the infra-red LED and diffuser using clamp stands. All videos were recorded directly onto the laptop.*

Baited box test: Net exposure protocol

Mosquitoes were exposed to test netting using a baited box setup with a human host present. Mosquitoes were 3-to-6-days-old, non-blood-fed females, which had been starved of sugar and water for a minimum of 24-hours prior to testing. Boxes with test netting and mosquitoes were acclimatised to the testing room for >1 hour before experiments began. The test netting was not changed between replicates, so mosquitoes were exposed to the same net piece per treatment. Testing began after 19:00 to coincide with peak indoor *Anopheles* biting times in Burkina Faso (Dambach *et al.*, 2018; Epopa *et al.*, 2019).

Mosquitoes (~5) were aspirated from a holding cup into a holding tube and the recording was initiated. Within 1-minute mosquitoes were aspirated via the gated entrance tube directly into the box. The operator exhaled through the test net/exposure hole on the roof of the box for 30-seconds at the start of the exposure, before placing an uncovered forearm above the box. The operator refrained from using scented substances for ~8-hours before the test. A gap between the test netting and operators arm inhibited mosquito blood-feeding during exposure. The operator exhaled naturally towards the box during the exposure.

Mosquitoes were video recorded for 20-minutes and then transferred back into holding cups. After the test, they were provided with 10% glucose solution soaked onto cotton wool. Mortality was recorded daily for 7-days, and dead mosquitoes were stored in silica. The recorded temperature in the testing room ranged from 27.8 – 28.1°C, and humidity between 52.3 – 99.9%.

Baited box test: Data collection and analysis

Videos were visually scan sampled at 10-second intervals and the number of mosquitoes exhibiting each behaviour (flying, box resting, base resting, mesh resting, mesh probing, mesh contact, net resting and net probing) was recorded. Behaviours were classified using predetermined criteria (adapted from Hughes *et al.*, Unpublished, Table 5.2). The start time of the exposure was defined as when the gate to the entrance tube was closed after mosquitoes were aspirated into the box. Videos were analysed in BORIS behavioural software version v. 7.4.4. (Friard and Gamba, 2016). Mosquito numbers varied between

replicates (3-6 mosquitoes), and in some instance's mosquitoes escaped through test netting during the 20-minute test. To account for this variation, the proportion of mosquitoes displaying each behaviour at each time point was calculated. During data analysis, some behaviours were combined as described in Table 5.2 (e.g. mesh contact is a combination of mesh resting and mesh probing behaviours). The mean proportion of mosquitoes exhibiting each behaviour at each time point were plotted onto scatter graphs. 95% confidence intervals were calculated to show the variability between test replicates. To visualise mosquito location in baited boxes during the exposure composite images were created by overlaying the recorded image every 0.1 seconds. They were created using ViCTA by Dr Jeff Jones *(Jones* et al*., Unpublished)*.

Table 5.2. Classifications used to define mosquito behaviours in baited box tests adapted from definitions used by Hughes et al. (Unpublished).

Behaviour	Description
Flight	Mosquito is in flight or has touched the net or box at the sampling time point but during a bouncing flight where it does not rest on the net or box
Box resting	Mosquito is resting on the box sides or has landed on the box sides
Base resting	Mosquito is resting on the base of the box or has landed on the base of the box
Mesh resting	Mosquito is resting on the untreated mesh covering the airholes or has landed on the untreated mesh but is not probing
Mesh probing	Mosquito is probing on the untreated mesh covering the air holes
Mesh contact	Mosquito is contacting mesh by 'mesh resting' or 'mesh probing'
Net resting	Mosquito is resting on the treatment netting or has landed on the treatment netting but is not probing
Net probing	Mosquito is probing on the treatment netting
Net contact	Mosquito is contacting treatment net by 'net resting' or 'net probing'

To quantify mosquito activity within the box, videos were examined using ViCTA, a bespoke behavioural image analysis software (Jones *et al.*, Unpublished). The box arena was divided into 100 regions (Figure 5.5), and mosquito movement within these regions was analysed by sampling video frames at 0.1 second intervals. Moving mosquitoes were detected using a Mixture of Gaussians (MOG) background model and the detected outline contours of moving objects were assessed to ensure they lay within a minimum and maximum threshold size range (previously determined empirically to correspond to the size of a

mosquito). To summarise movement activity, the moving mosquito detection counts at every 0.1 second frame intervals were aggregated at every 5-seconds and summed to give discrete 5-second time periods of movement for each of the 100 regions. To improve image quality, and therefore movement activity detection, gamma correction, brightness, and contrast enhancement were applied as required to enhance the signal range of the video footage before sampling. This was to compensate for variations in video quality (for example low exposure rates, noise contamination and speckling caused by the infra-red LED passing though the diffuser) caused by the challenging experimental field conditions.

Total activity detected within the whole box, and within the top row of the box only (Figure 5.5), was then summarised for each net type. When mosquito numbers deviated away from 5 mosquitoes (e.g. mosquito escaped during the exposure) activity was weighted using the following equation:

$$Tw = \frac{T}{(n/5)}$$

Where Tw is the weighted activity level, T is the total activity level, and *n* is the number of mosquitoes in the replicate. When mosquito numbers are <5 the weighting function increased the total activity, and when mosquito numbers are >5 it decreased the total activity. Activity over time was also calculated, however, this was unweighted as results were strongly affected by weighting. The effect of treatment on the number of detected mosquito movements was compared using GLMMs with a negative binomial distribution to account for overdispersion. The experimental day was included as a random effect in the model. The analysis was conducted within R statistical software version 3.6.2 (2019-12-12) (R Core Team, 2017) using the glmmADMB (Fournier *et al.*, 2012) package.

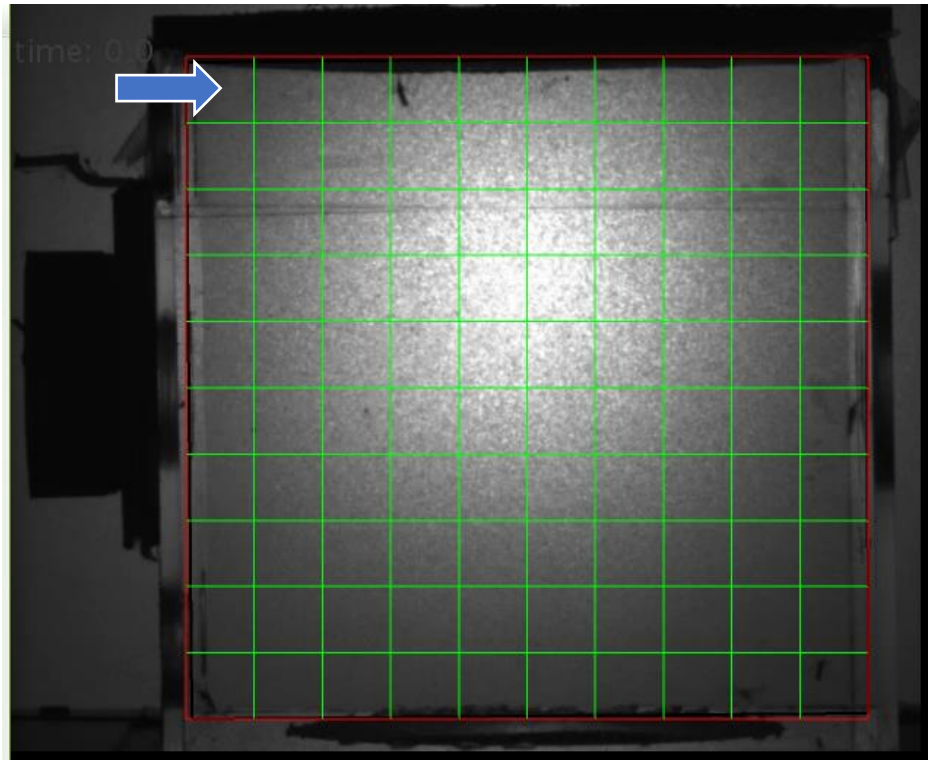


Figure 5.5. Image showing the regional division of the baited box for ViCTA analysis. *The baited box is divided into 100 (10 x 10) regions and mosquito movement within each of these regions is recorded every 0.1 seconds by the ViCTA software* (Jones *et al.*, Unpublished). *Total activity within the whole box, or within the top row of the box only (indicated with a blue arrow) was then summarised for each net type.*

5.3 Results

5.3.1 Video cone test

Video cone tests were conducted over 8 non-consecutive days between August – October 2018. On each experimental day at least 1 untreated netting replicate was conducted alongside test nets, therefore the number of days tested, and replicates vary between net treatments (Table 5.3). Immediate mortality (within 24-hours) was less than 25% for all insecticidal nets, except for PermaNet 3.0 roof where mortality was 100% (Table 5.3).

Video cone tests were analysed using two methods. In the first method, the video recordings were scan-sampled every 5-seconds and behaviours were documented (Figure 5.7). In the second method, composite images of each video replicate were created by overlaying the recorded frames every 0.1s (Figure 5.6).

Table 5.3. Summary of video cone tests performed and mortality post-testing.

Number of testing days and replicates varied between net treatments. The total number of mosquitoes exposed in video cone tests and the total number recorded for mortality differ due to accidental loss of mosquito during the test.

Net type	No. days testing	No. replicate tests	Total no. mosquitoes exposed	Mortality	
				Total no. mosquitoes	% 24-hour mortality (95% CI)
Untreated	8	14	65	61	3.28 (-1.19 – 7.75)
Olyset Net	2	10	47	43	0.00
PermaNet 3.0 sides	3	10	49	47	2.13 (-2.00 – 6.25)
PermaNet 3.0 roof	5	10	50	50	100
Interceptor G2	2	10	50	47	23.40 (11.30 – 35.51)

In all video cones, regardless of test netting type, the proportion of mosquitoes obscured from view during exposure was low (less than 0.2). Composite images, as exemplified by Figure 5.6, show no patterns in mosquito location in the cones in response to any net type (all composite images can be found in Appendix 1).

The scan sampling plots, summarised in Figure 5.7, suggest that the behaviour of the Tengrela population was similar during exposure to untreated and pyrethroid-only netting (Olyset Net and PermaNet 3.0 sides). In response to these net types, mosquitoes quickly changed between the different behaviours (resting on the net, resting on the cone, in-flight) at each sampling point, causing the behaviours to converge. No behavioural patterns were observed over time and no behaviours were dominant during the exposure (Figure 5.7 A-C). At most time points, 95% confidence intervals overlapped for the three behaviours as a result of variability between the testing replicates.

The mosquitos response to the next-generation nets was different depending on the net type. During exposure to PermaNet 3.0 roof (deltamethrin + PBO net), mosquito flight was dominant for the first 90-seconds of the test (Figure 5.7D), suggesting contact irritancy. At this timepoint, flight declined as mosquito resting on the net increased, resulting in all behaviours converging towards the end of the exposure. In Interceptor G2 (alphacypermethrin + chlorfenapyr) exposure, mosquito resting on the net was the high and resting on the cone was low throughout the exposure (Figure 5.7E). This suggests a lack of contact irritancy or repellence and a potential reduction in mosquito activity.

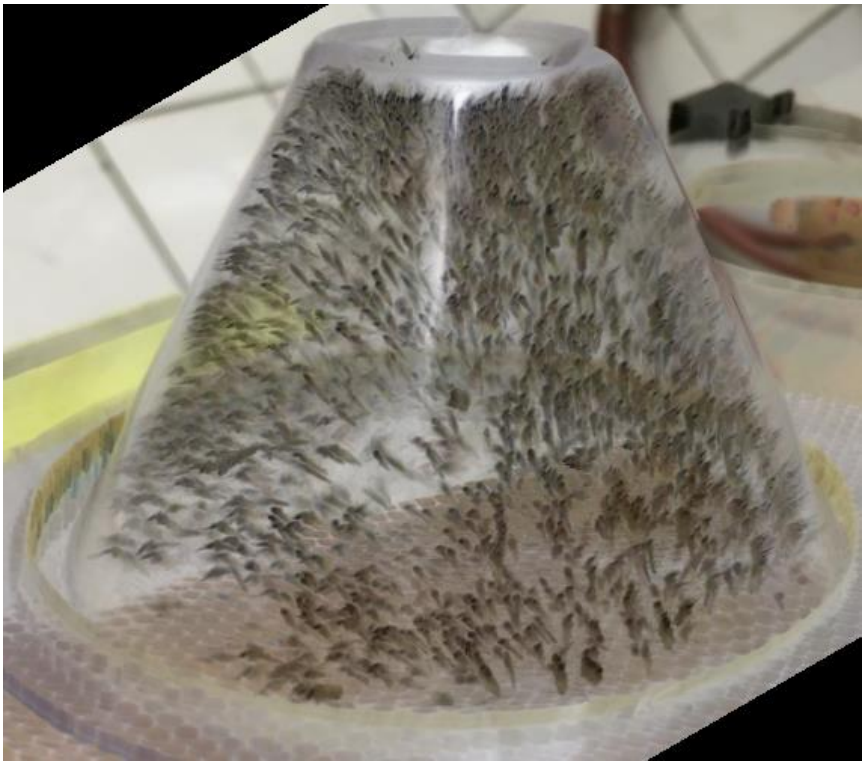
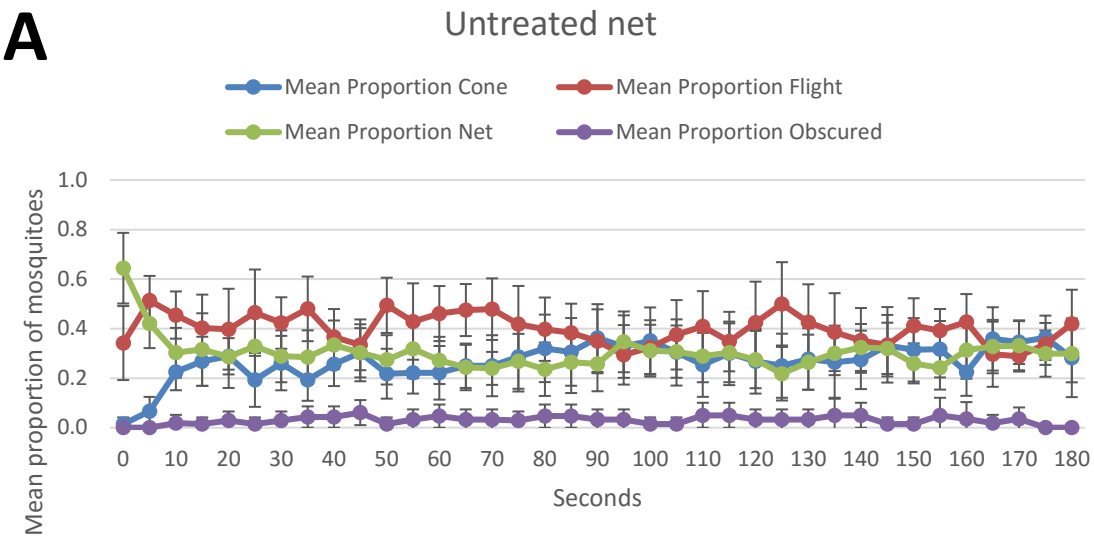


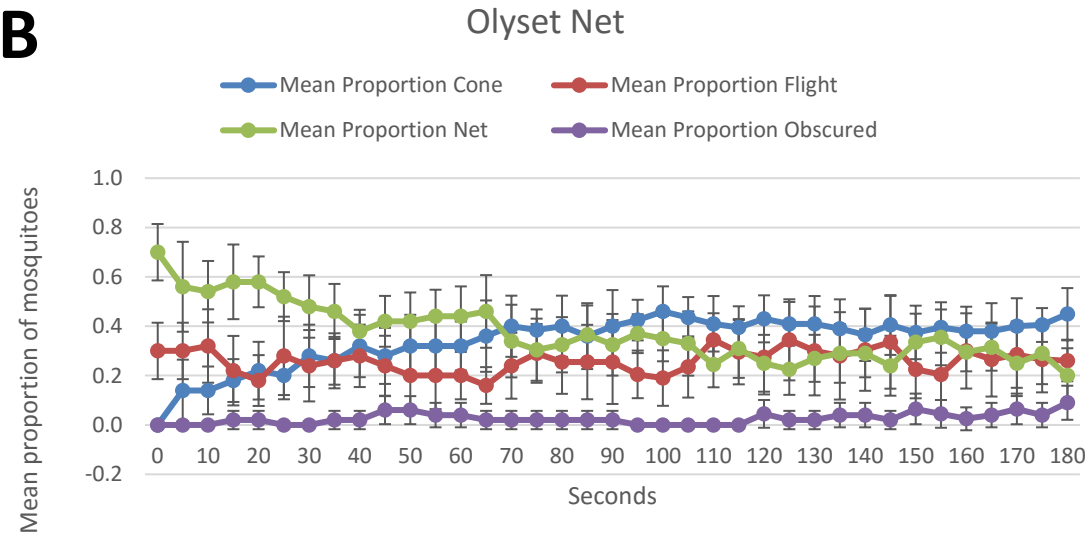
Figure 5.6. Example composite image showing mosquito location during exposure to an untreated net in the 3-minute video cone test.

Composite images were created by overlaying each recorded video frame every 0.1 seconds. Images were created using ViCTA by Dr Jeff Jones (Jones et al., Unpublished). All composite images can be found in Appendix 1.

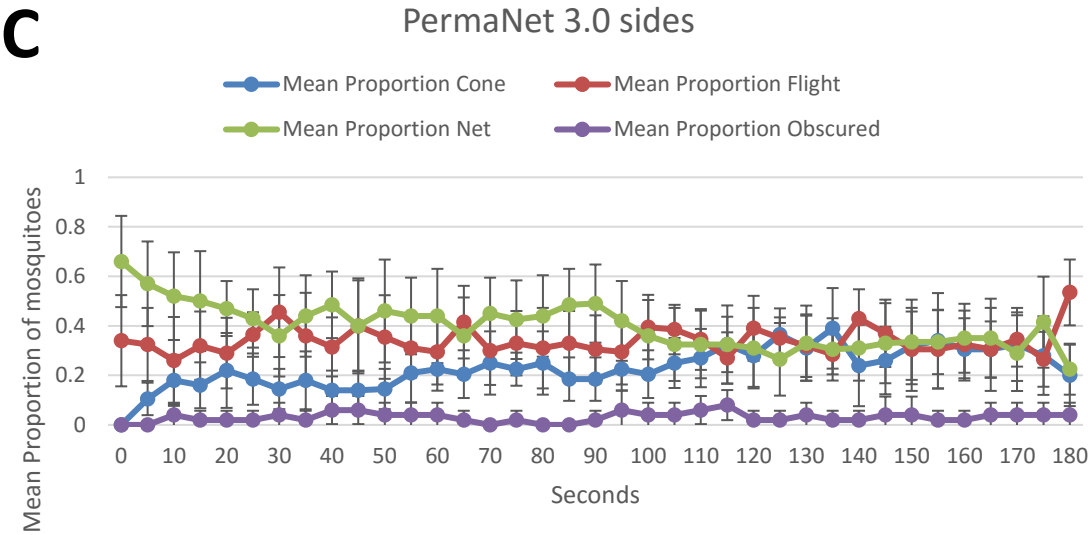
A


Untreated net
Mean Proportion Cone
Mean Proportion Flight
Mean Proportion Net
Mean Proportion Obscured
Mean proportion of mosquitoes
1.0
0.8
0.6
0.4
0.2
0.0
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
Seconds

B


Olyset Net
Mean Proportion Cone
Mean Proportion Flight
Mean Proportion Net
Mean Proportion Obscured
Mean proportion of mosquitoes
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
Seconds

C


PermaNet 3.0 sides
Mean Proportion Cone
Mean Proportion Flight
Mean Proportion Net
Mean Proportion Obscured
Mean Proportion of mosquitoes
1
0.8
0.6
0.4
0.2
0
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
Seconds

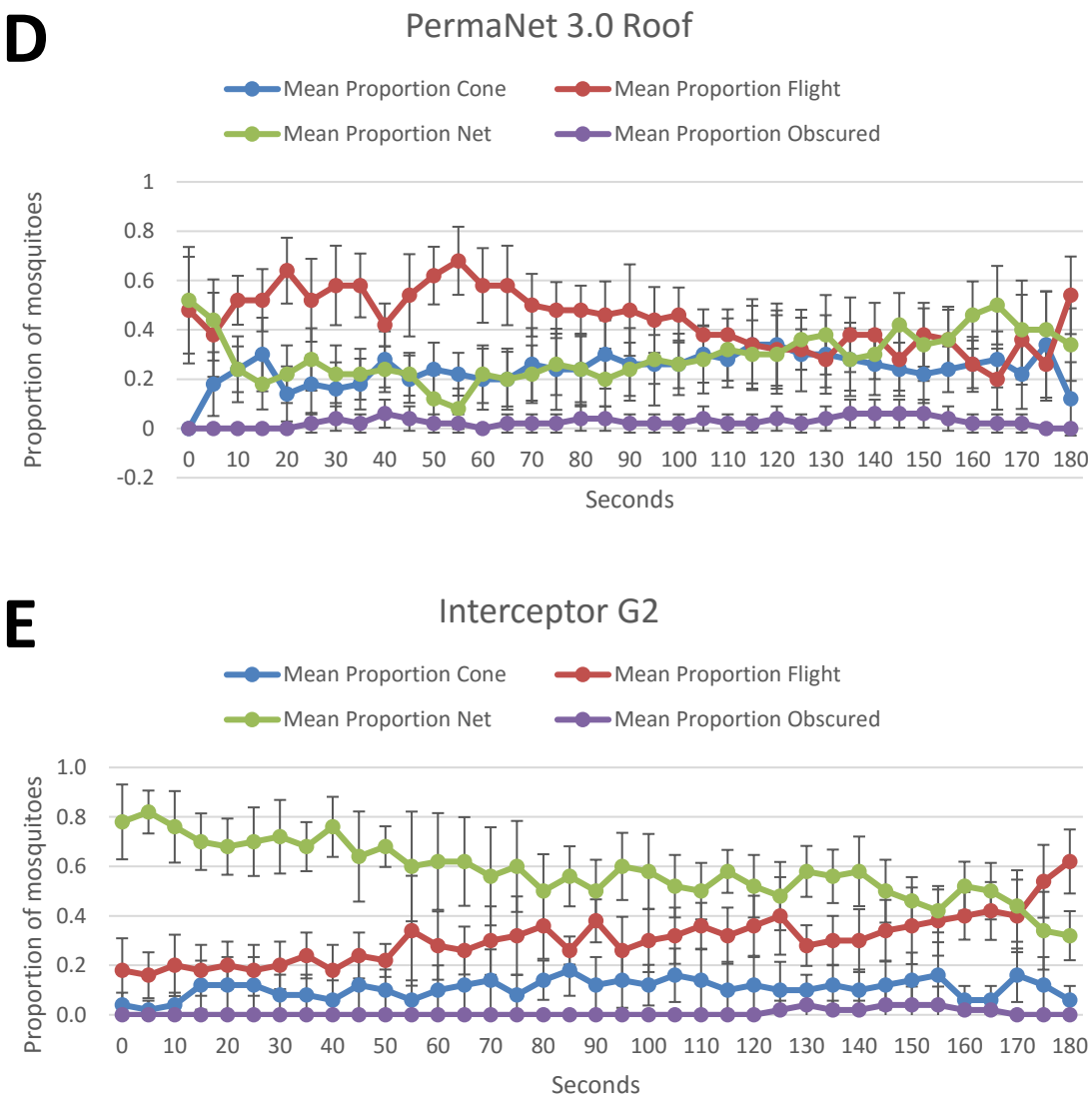


Figure 5.7. Scan sampling analysis of mosquito behaviour during exposure to test netting in 3-minute video cone test.

Mosquitoes were exposed to test netting and the mean proportion of mosquitoes resting on the cone, in-flight, resting on the test netting, or obscured from view is shown. Error bars show 95% confidence intervals. (A) Untreated net: 14 replicates; (B) Olyset Net: 10 replicates; (C) PermaNet 3.0 sides: 10 replicates; (D) PermaNet 3.0 roof: 10 replicates; (E) Interceptor G2: 10 replicates.

5.3.2 A preliminary study of the thumb test for field use

Previous studies with the thumb test used laboratory colonised mosquito strains. This preliminary study was conducted to investigate the feasibility of using the thumb test for field, use using a wild larval-reared population of *Anopheles.*

The original thumb test setup and several variations were trialled, as follows:

1. Original thumb test setup and response classification (Section 5.2.3, Page 123). Results:
    a. Untreated netting: 5-7-day-old non-blood-fed non-starved mosquitoes, 19% response rate (n = 21 mosquitoes tested), of the non-responders 61% (n = 11 mosquitoes) failed to leave the holding tube within 3 minutes.
    b. PermaNet 2.0 netting: 6-day-old non-blood-fed non-starved mosquitoes, 12% response rate (n = 17 mosquitoes tested).
2. 24-hours starvation: Mosquitoes were sugar and water-starved for 24-hours prior to testing. 7-day-old non-blood-fed mosquitoes were exposed to untreated netting using the original setup and response classification. Result: 0% response rate (n = 5 mosquitoes), 60% (n = 3 mosquitoes) failed to leave the tube within 3 minutes.
3. Inverted box: The thumb box arena was inverted so that test netting could be placed on the large air hole normally found on the base of the box. Result: It was not possible to visualise mosquito probing in this version as the thicker acrylic wall obscured the view.
4. Netted roof: The test setup was altered so that mosquitoes were exposed to netting by draping it over the roof of the thumb box. This increased the host surface area available to mosquitoes. The response classification was altered to 5-minutes for mosquitoes to exit the holding tube. Result: 20% response rate (n = 25 mosquitoes), 65% of non-responders exited the tube within 5-minutes but failed to probe within 10-minutes.
5. 48-hours starvation: Mosquitoes were fully sugar and water-starved for 48-hours prior to testing. Mosquitoes were exposed to untreated netting using the netted roof setup and 5-minute responder classification. Result: 0 responders (n = 5 mosquitoes), 60% (n = 3 mosquitoes) left the tube within 5-minutes but failed to probe within 10-minutes.
6. Forced entry: Mosquitoes who failed to exit the holding tube within 3-minutes were manually pushed into the box using a modified plunger system added to the entry tube. Mosquitoes were classified as non-responders if they failed to probe within 10-

minutes of entering the box. 3-5-day old unfed starved mosquitoes were exposed to untreated netting. Result: 29% response rate (n = 17 mosquitoes).

7. *An. gambiae* Kisumu control: 7-day-old *An. gambiae* Kisumu starved on the day of testing were exposed to untreated netting using the netted roof setup with 5-minute responder classification. Additionally, the ventilation holes in the box were blocked. Result: 100% response rate and blood-feeding (n = 5 mosquitoes).

The original thumb test setup achieved low response rates when using individual wild larval-reared *Anopheles*, this contrasted with results from the lab susceptible strain where a 100% response rate was obtained. The low response rate coupled with the length of time required to complete each individual thumb test (25 minutes) clearly demonstrated that it was not practical to continue to pursue the use of the original methodology. It was therefore decided to abandon the thumb test variant and revert to a baited box, in which mosquitoes were exposed to a 10 x 10 cm area of netting in batches of ~5 mosquitoes per replicate. The baited box test does not give information on individual mosquito's responses, or duration of behaviours, but it is able to describe how behaviours of a group of mosquitoes change over time during insecticidal net exposure. The initial responder classification, used in the thumb test, defined mosquitoes as responding only when they probed through the test netting. This was originally characterised when testing pyrethroid-only netting and would be unsuitable for testing nets which repelled, caused contract irritancy, or affected mosquito biting behaviour. Therefore, in the baited box test reported here, no responder classification was used. Mosquitoes were aspirated directly into the box arena and all behaviours were recorded for 20-minutes.

5.3.3 Baited box test

Baited box tests were conducted over 3 non-consecutive days in October 2018. On each experimental day at least 1 untreated box replicate was conducted alongside test boxes, therefore the number of days tested, and replicates vary between net treatment (Table 5.4). In baited box tests, immediate mortality was 100% following exposure to PermaNet 3.0 roof. All other net types showed mortality levels of less than 10% (Table 5.4).

Baited box tests were analysed using three methods. In the first method, 10-second scan sampling was used to record mosquito behaviour. The results of the scan sampled activity

are summarised in Figure 5.8. In the second and third method, bespoke ViCTA image analysis was used to (1) detect and count mosquito movements, in the whole box or top row of the box only, and (2) create composite images of each video replicate to show location of detected movement within the baited box (Figure 5.9, Figure 5.10 and Table 5.5).

Table 5.4. Summary of baited box test performed and mortality post-testing.

Number of testing days and replicates varied between net treatments. The total number of mosquitoes exposed in baited box tests and the total number recorded for mortality differ due to accidental mosquito release during the transfer from boxes to holding cups.

Net type	No. days testing	No. replicate tests	Total no. mosquitoes exposed	Mortality		
				Total no. mosquitoes	% 24-hour mortality (95% CI)	% 7-day mortality (95% CI)
Untreated	3	8	38	33	0	9.09 (-0.72 – 18.90)
PermaNet 3.0 sides	3	8	39	36	0	5.56 (-1.93 – 13.04)
PermaNet 3.0 roof	2	6	26	26	100	100
Interceptor G2	3	10	51	51	1.96 (-1.84 – 5.77)	7.84 (0.46 – 15.22)

The scan sampled activity plots suggest that the behaviour of the Tengrela mosquito population was similar during exposure to all net types in 20-minute baited box (Figure 5.8). The exception to this was PermaNet 3.0 roof netting, where mosquitoes showed increased resting on the base of the box (analogous to knockdown), compared to other behaviours. In all the other net types, mosquitoes quickly moved between the different behaviour at each sampling point with no behavioural patterns or dominant behaviours

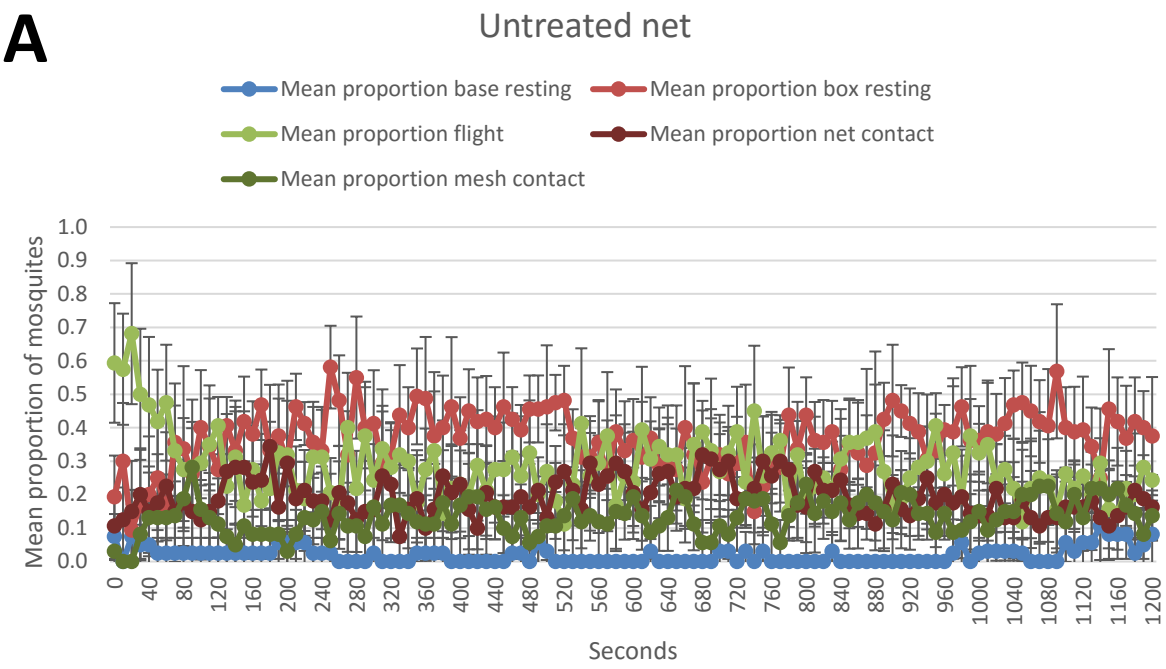
observed. At most time points 95% confidence intervals overlapped indicating variability between replicates.

During exposure with untreated netting (Figure 5.8A), few mosquitoes rested on the base of the box, instead showing higher proportions resting on the box sides. In the entire box, ViCTA analysis detected 17,653 mosquito movements, with 42% (12 – 65%) of this activity occurring in the top row of the box closest to the human host (Figure 5.9, Table 5.5). Detected mosquito movement oscillated over time but remained constant in both the entire box and top row (Figure 5.10).

When exposed to PermaNet 3.0 sides (deltamethrin-only netting), contact with the netting was higher within the first 2-minutes of exposure but then declined to relatively low levels. This suggests mosquitoes were initially attracted to land on the test netting in response to host cues but deterred from continuously resting on the net. Mosquito movement detected in the entire box, or in the top row only, were not significantly different from untreated (Figure 5.9, Table 5.5). On average, 35% (13 - 73%) of detected activity was observed in the top row. Mosquito movement detected in the entire box and the top row oscillated over time, tailing off towards the end of the exposure (Figure 5.10).

When exposed to PermaNet 3.0 (deltamethrin + PBO) roof netting (Figure 5.8C), flight was the dominant behaviour initially, however, this rapidly declined within the first minute, and fell to extremely low levels at around 10-minutes. Mosquito resting on the base of the box steadily increased over time and was the dominant behaviour after the first 3-minutes. Compared to all other netting types tested, activity was significantly reduced in both the entire box and top row following PermaNet 3.0 roof exposure (Figure 5.9, Table 5.5). However, the proportion of this activity spent in the top row (35% average, 17 – 54%) was similar to levels seen in PermaNet 3.0 sides and untreated. Detected movement dramatically declined to extremely low levels within the first 5-minutes of the exposure (Figure 5.10).

A


Untreated net
Mean proportion base resting
Mean proportion box resting
Mean proportion flight
Mean proportion net contact
Mean proportion mesh contact
Mean proportion of mosquites
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
40
80
120
160
200
240
280
320
360
400
440
480
520
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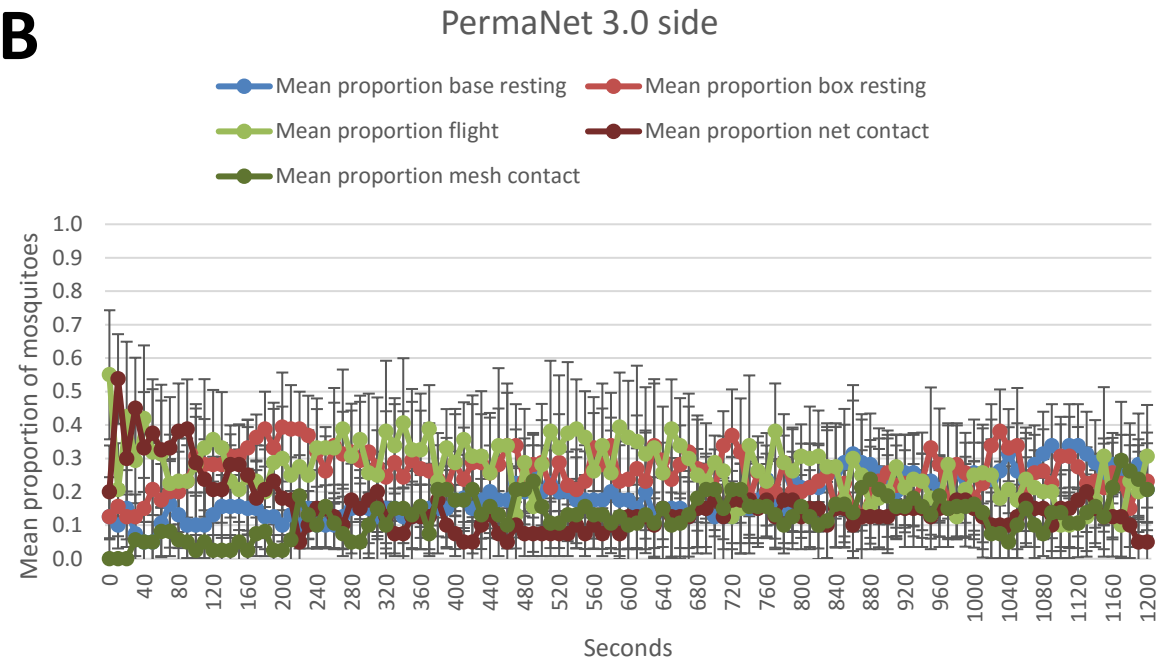
960
1000
1040
1080
1120
1160
1200
Seconds

B


PermaNet 3.0 side
Mean proportion base resting
Mean proportion box resting
Mean proportion flight
Mean proportion net contact
Mean proportion mesh contact
Mean proportion of mosquitoes
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
40
80
120
160
200
240
280
320
360
400
440
480
520
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920
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1000
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1160
1200
Seconds

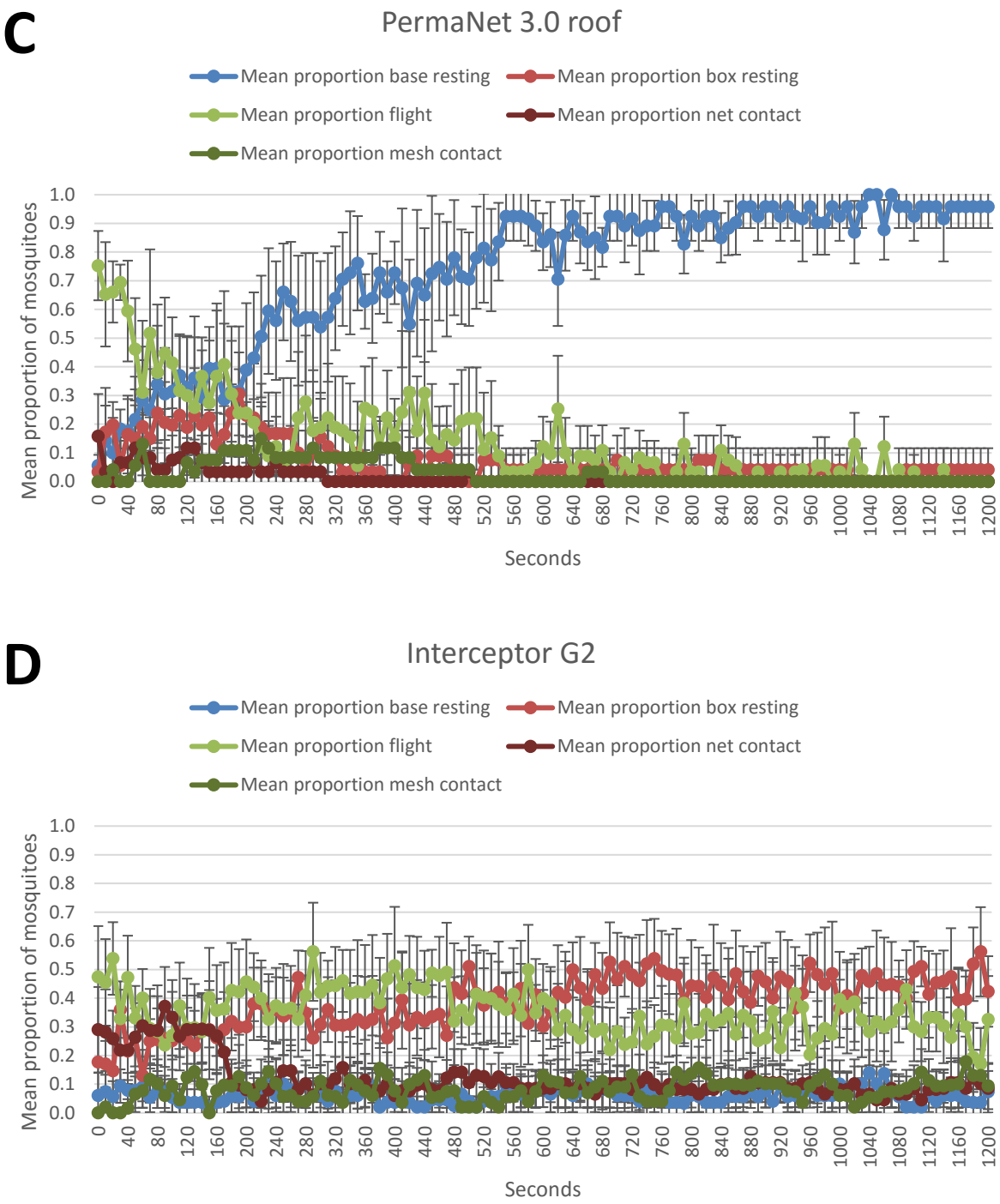

Figure 5.8. Scan sampling analysis of mosquito behaviour during exposure to test netting in 20-minute baited box test.

Mosquitoes were exposed to test netting and the mean proportion of mosquitoes resting on the base, resting on the box, in-flight, in contact with the test netting (resting or probing) or in contact with the untreated mesh (resting or probing) is shown. Error bars show 95% confidence intervals. (A) Untreated net: 8 video replicates; (B) PermaNet 3.0 side: 8 video replicates; (C) PermaNet 3.0 roof: 6 video replicates, (D) Interceptor G2: 10 video replicates.

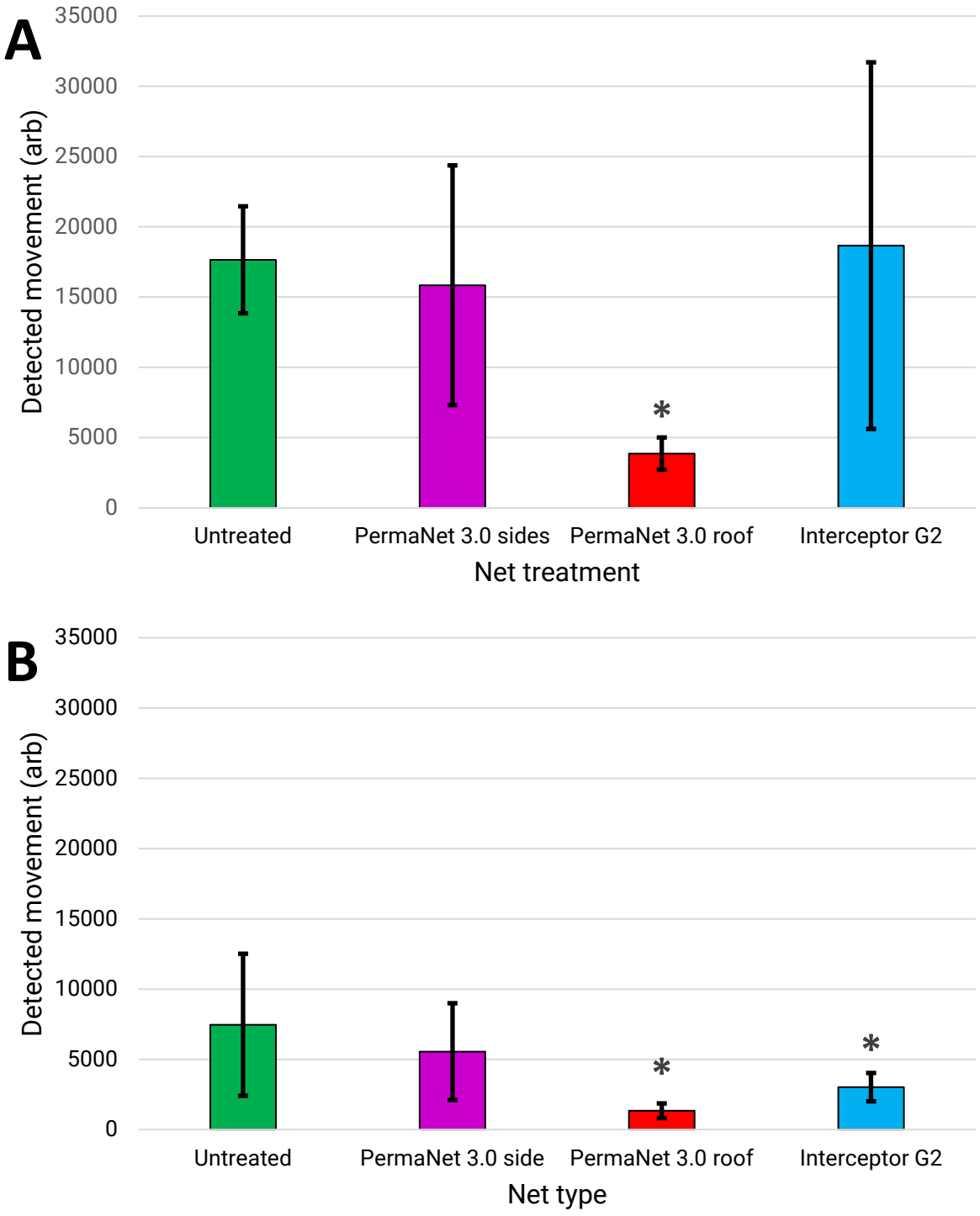

Figure 5.9. Mean total weighted mosquito activity detected in the entire box (A), or in the top row of the box only (B) during 20-minute exposure to test netting in baited boxes. *Activity levels were detected using ViCTA software. Error bars show standard deviation. Asterisks show when activity was significantly different compared to untreated control (P > 0.05, GLMMs). Untreated net: 8 video replicates; PermaNet 3.0 side: 8 video replicates; PermaNet 3.0 roof: 6 video replicates, Interceptor G2: 10 video replicates.*

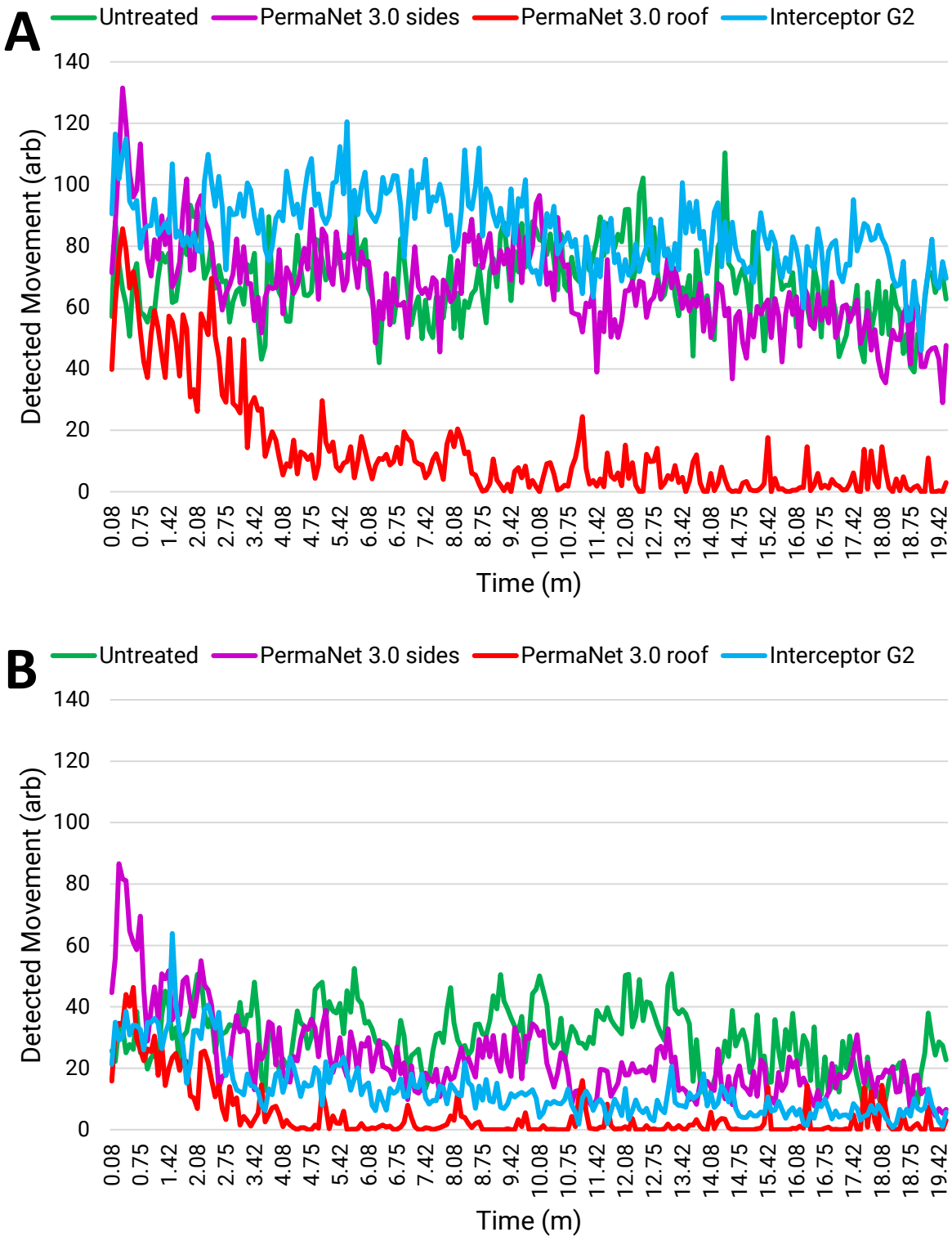

Figure 5.10. Mean unweighted mosquito activity over time detected in the entire box (A), or top row of the box only (B) during 20-minute exposure to test netting in baited boxes. *Activity levels were detected using ViCTA software. Untreated net: 8 video replicates; PermaNet 3.0 side: 8 video replicates; PermaNet 3.0 roof: 6 video replicates, Interceptor G2: 10 video replicates.*

Table 5.5. The number of mosquito movements detected by ViCTA in 20-minute baited boxes in the top row only, or whole box.

Movements have been weighted when mosquito numbers deviate from 5. Values in the same row sharing a letter superscript do not differ significantly (P > 0.05, GLMMs: Appendix 1, ***Error! Reference source not found.****).*

	Untreated	**PermaNet 3.0 sides**	**PermaNet 3.0 roof**	**Interceptor G2**
Top row	7455[a]	5542[a]	1339	3017
Whole box	17653[a]	15843[a, b]	3861	18664[a, b]

In Interceptor G2, flight and resting on the box were more common than other behaviours (Figure 5.8D). Flight appears to be more dominant within the first 10-minutes of the test and box resting towards the end of the test. Net contact rapidly declines after the first 2 ½ minutes to low levels. Movement detected in the whole box was similar to the untreated net and PermaNet 3.0 sides (Figure 5.9A, Table 5.5). Movement detected in the top row with Interceptor G2 was significantly different to all other net types, this was reduced compared to untreated and PermaNet 3.0 sides and increased compared to PermaNet 3.0 roof (Figure 5.9B, Table 5.5). The proportion of activity which occurred in the top row (16% average, 5 – 63%) was reduced compared to other net types. Detected movement in the entire box and the top row oscillated over time, tailing off towards the end of the exposure (Figure 5.10).

Composite images showed activity levels to vary between replicates, and this appeared to be associated with the date tested (Appendix 1, Figure A1.6 - 9). The date was included as a random effect in GLMM analysis of detected activity. In untreated boxes, composite images show mosquitoes movement was largely detected on the box roof in contact with the netting or on the left-hand side of the box towards the host (Figure 5.11; Appendix 1; Figure A1.6). In PermaNet 3.0 sides and Interceptor G2, composite images show the location and amount of detected activity vary dramatically between replicates (Figure 5.11; Appendix 1 : Figure A1.7, Figure A1.9). In PermaNet 3.0 roof detected activity is shown to be markedly reduced (Figure 5.11, Appendix 1, Figure A1.8).

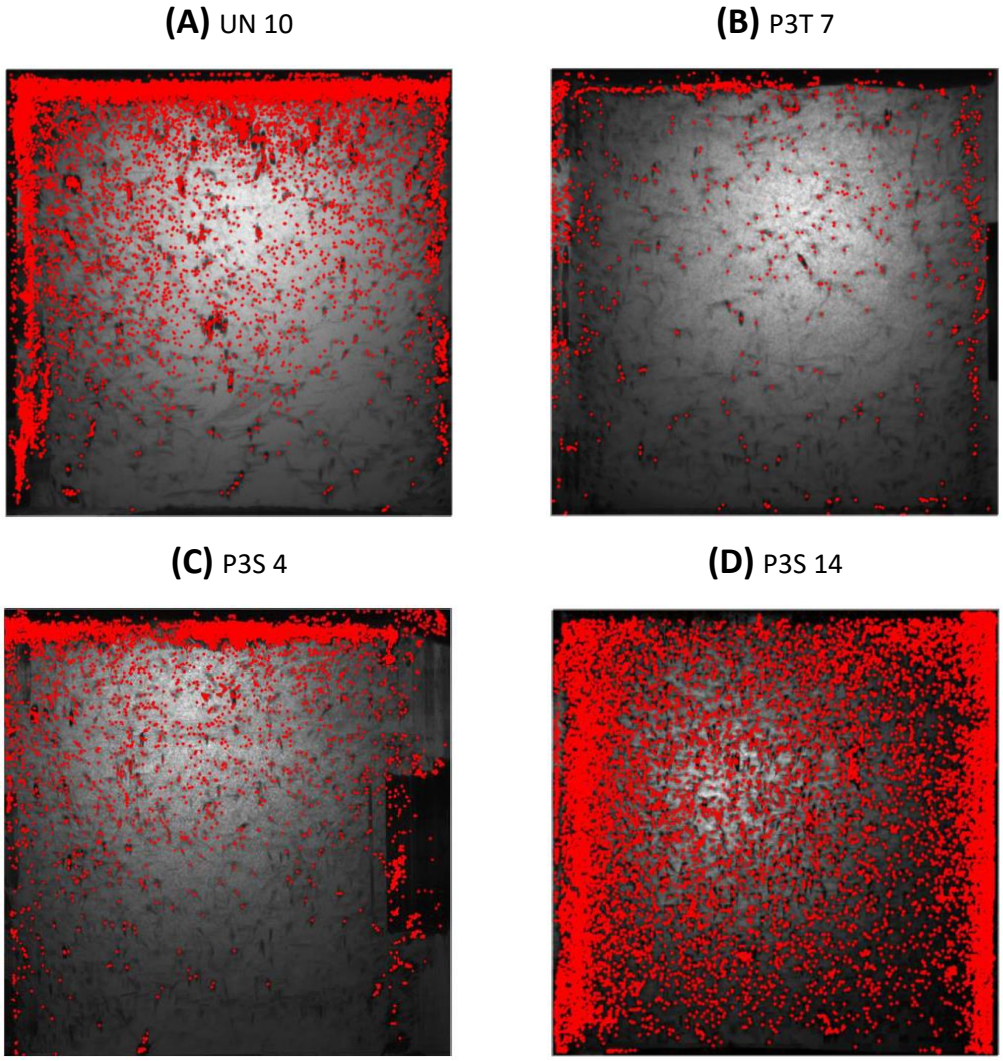

Figure 5.11. Examples of composite images showing detected mosquito activity during exposure to untreated (A), PermaNet 3.0 roof (B), and PermaNet 3.0 Sides (C & D) in 20-minute baited boxes.

Red dots show when mosquito activity was detected by ViCTA automated analysis. All composite images can be found in Appendix 1.

5.4 Discussion

5.4.1 Exploring the feasibility of using video benchtop tests for field use

The primary aim of the work discussed in this chapter was to explore the feasibility of using video benchtop tests as a method for describing and quantifying behaviours of wild *Anopheles* during interactions with different insecticidal nets. Behavioural responses of

wild larval-reared mosquitoes during exposure to insecticidal netting were investigated using three methods: video cone tests, thumb tests, and baited box tests. These tests differ in their duration of exposure, the size of the test arena, the time required to complete a series of tests and appropriate controls and the types of behaviour they can record for quantification.

The video cone test is a standard WHO cone bioassay (WHO, 2013a) modified with the addition of a human host and recorded using a smartphone. As with the standard cone test setup, the test is rapid, simple and reproducible to perform under field conditions and required minimal adaptation, additional training, or cost. It is a simple way of observing mosquito behaviour under more naturalistic (human host present) conditions than the standard cone bioassay and collected simple behavioural data on several net types. By monitoring the proportion of mosquitoes in contact with the net or cone over time, the test provides observational information on a net's contact irritancy, and the proportion of mosquitoes in flight provides insight into mosquito activity following net exposure. In addition to outcomes usually recorded (i.e. mosquito knockdown and mortality), the test can be used as a method of insecticidal net exposure when examining sub-lethal effects, such as blood-feeding or longevity (reported in Chapter 4). In the iteration reported in this chapter, using wild-larval reared mosquitoes in a field setting, the video cone test was able to detect flying and resting behaviours and distinguish resting on the net from resting on the cone.

The thumb test and baited box test also record mosquito behaviour at the bednet interface in the presence of a human host. The original thumb test setup was deemed unsuitable for use in this current project as mosquito response rates were extremely low and time available for testing was limited. In the baited box test variation (Hughes *et al.*, Unpublished), mosquitoes are aspirated directly into the box, and exposure to the net is at a larger aperture. Compared to the video cone test, the larger recording/test arena of the box, combined with the higher resolution camera, altered netting area, and longer exposure time, allowed additional information on mosquito behaviour to be observed. Additionally, by filming in infra-red light the visual environment is more natural for this nocturnal species (Gibson, 1995). However, it requires more complex bespoke equipment and longer training for its use. In the baited boxes, mosquitoes could rest on the treated netting, sides, or base of the box. Their location within the box allowed further information

on net contact irritancy or killing efficacy to be collected. Using ViCTA to detect mosquito movement in the whole box or limited to the top row (closest to the host), allowed evaluation of how mosquito activity was altered (e.g. is it increased or decreased) by the presence of test netting.

Aspirating mosquitoes directly into boxes and analysing them as a group using scan sampling at 10-second intervals overcame the issue with response rates that were encountered with the thumb test. However, it reduced the level of behavioural detail obtained from each test. Individual mosquito recordings would allow data to be collected which showed sequence and duration of behaviours in response to insecticidal net exposure, which was not possible with the grouped scan sampling. Subsequently, when time is not a limiting factor, the original thumb test setup could be used to collect more detailed information on mosquito behaviour.

In the baited box test, sensory cues emanating from the host probably enter the test arena through the untreated meshed airholes on the side of the box as well as the large exposure hole on the roof of the box. Mosquitoes were often observed probing though and resting on the untreated mesh air holes on the box side. These behaviours, mesh probing and mesh resting, were originally classified separately, but during analysis were pooled into "mesh contact" (Table 5.2). When resting on the untreated mesh mosquitoes could be responding to host cues that enter through the mesh, so it was important to distinguish this behaviour from box resting. Additionally, mesh probing, which is suggestive of host-seeking behaviour, was separated from net probing as although mesh probing provided an insight into mosquito host-seeking behaviour, it was important to differentiate this from the host-seeking activity which occurred while being simultaneously exposed to the insecticidal net. During analysis net resting and net probing were also combined into a single behaviour ("net contact", Table 5.2), to provide insight into any net repellent or contract irritant effects the net may have. This highlights that pre-defining behaviours prior to testing require consideration. This may be especially important when assessing next-generation nets which have novel active ingredients or modes of action. When classifying these behaviours, it is important to be mindful of what these could mean for insecticidal net contact or mosquito host seeking. For example, in the baited box test mesh probing and net probing could have been combined into "probing" behaviour, or mesh resting could have been included with box resting. Each of these behaviours tells us something

different about the mosquito's interaction and response to the test netting, so it is important for them to be discrete, while at the same time not being so specific that no meaningful conclusions can be described from the data.

The ViCTA movement detection and composite image analysis allowed mosquito location within the box to be visualised, and activity levels to be quantified using GLMMs. The ViCTA composite images (overlaid every 0.1 seconds) can detect nuances in mosquito location within the box which may not be captured using scan sampling if they change behaviours in quick succession. By focusing on movement detection in different areas, ViCTA can quantify behavioural responses. In the tests reported in this chapter, movement detection was focused on the top row of the box to provide insight into net contact and responses to host cues, but similarly, detection could focus on movement in the lower half of the box to investigate repellent or contact irritant effects of the netting. ViCTA Composite image analysis of baited boxes showed day of testing noticeably affected mosquito behaviour, and this this appeared to be consist across treatments. For example, mosquito activity was higher in both PermaNet 3.0 side and Interceptor G2 boxes when visually comparing composite images from the 19th October 2018 with other days (Appendix 1, Figure A1.6 - 9). This effect did not appear to be related to temperature and humidity and may be as a result of different cohorts of mosquitoes tested. Subsequently, date was included as a random effect in all models.

ViCTA analysis augments the scan sampling analysis, which is better able to describe specific behaviours such as probing, and if feeding was permitted, blood-feeding. Additionally, visually scanning the videos over their duration allows the operator to identify behaviours or activities which may merit further investigation with the ViCTA software.

5.4.2 Defining and measuring the effect of insecticidal net exposure on the behaviour of a wild population of *Anopheles gambiae* behaviour at the net interface

The second aim of this study was to use the video cone and baited box tests to describe the effects of exposure to standard and next-generation nets on the behaviour of wild *An. gambiae* at the net interface.

The video cone test has been previously used to evaluate the behaviour of laboratory reared susceptible and resistant *Anopheles* at the LLIN interface (Hughes, 2018; McCall, Personal communication). In the field tests reported here, behaviour between individual test replicates was highly variable, in contrast with what was observed by Hughes (2018), who found behaviour to be more consistent. When mosquitoes are colonised, genetic diversity quickly decreases as a result of inbreeding (Ng'habi *et al.*, 2015), and several short-range behavioural studies have previously reported colonisation to affect mosquito behaviours such as repellence, attraction, and blood-feeding (Chadee and Beier, 1997; Chadee, Beier and Mohammed, 2002; Thanispong *et al.*, 2009; Clark *et al.*, 2011). The genetic diversity of the wild population could partially explain the behavioural variation observed between test replicates.

In the previous lab studies, mosquitoes spent more time resting on the net than in flight or resting on the cone (Hughes, 2018), or did not rest on the cone at all (McCall, Personal communication), regardless of net type. In the video cone tests and baited box tests reported in this chapter, except for PermaNet 3.0 roof, no behaviour dominated across the duration of the test following exposure to all other net types. Mosquitoes moved between the behavioural categories in quick succession, which resulted in behaviours converging. The changing behaviour of the mosquito could reflect them being attracted to the host but stimulated to fly when the physical net barrier inhibits host contact, thus causing the mosquito to fluctuate between flying and resting on the net.

Following exposure to pyrethroid-only netting mortality was comparable to untreated (24-hour mortality >5% in all tests; 7-day mortality >10% in baited box tests). No significant difference in detected activity between PermaNet 3.0 sides or untreated was observed, when comparing mosquito movements in the whole baited box or top row only.

PermaNet 3.0 roof netting (deltamethrin + PBO) caused 100% 24-hour mortality in all tests. Following exposure to PermaNet 3.0 roof flight declined quickly as resting increased in both video tests. This occurred after 60-seconds in video cone tests and 180-seconds in baited box tests. This decline in activity is likely as a result of the combined effect of the pyrethroid and PBO. The PBO inhibits enzymes which usually detoxify the pyrethroid in this resistant strain (Williams *et al.*, 2019), allowing the neurotoxic effect of the pyrethroid to immobilises the mosquito. In the baited box test, resting on the base of the box was the

dominant behaviour. Although this behaviour is defined as “resting”, observationally it was clear that mosquitoes were rapidly knocked down by the PermaNet 3.0 roof netting following aspiration into the box. This occurred after ~3 minutes, the time at which the video cone test exposure stops, which potentially explains why knockdown was not observed during the video cone test.

Composite image analysis suggests that contact with the treated netting in baited boxes was very brief, however, this was enough to rapidly knockdown and kill the highly pyrethroid-resistant *Anopheles*. Mosquito activity in the box quickly declined to a low baseline following exposure and overall was significantly reduced in comparison to all other net types, both in the whole box and in the top row only. The proportion of activity detected in the top row of the box (closest to the host) was similar to untreated and PermaNet 3.0 sides, suggesting that the PermaNet 3.0 roof is no more repellent than other test nets. The mortality levels seen here are supported by additional field data: An experimental hut trials conducted in the same locality found that wild *Anopheles* mortality rates were 1.69-1.78-fold greater in huts with PBO-nets compared to pyrethroid-only LLINs (Toe *et al.*, 2018). PBO by itself is not insecticidal, and it works by inhibiting metabolic enzymes within the mosquito that detoxify pyrethroid. Additionally, a Cochrane review has shown that in areas of high-pyrethroid resistance PBO-nets increase mosquito mortality and reduce blood-feeding rates (Gleave *et al.*, 2018).

PBO-bed nets were the first next-generation nets to receive WHO PQ listing (WHO, 2019a) and reach the market. Understanding how pyrethroid-susceptible and resistant field mosquitoes interact with these nets and documenting mosquito behaviour to see how this might change with changes in susceptibility to these nets is essential. The efficacy of PBO-bed nets depends on the mosquitoes resistance level and mechanisms (Churcher *et al.*, 2016; Gleave *et al.*, 2018). Subsequently, it is important that the behavioural effects of these nets be examined using field populations with varying susceptibility and resistance mechanisms to establish how behaviour might be altered, as any repellent properties of the net, for example, will reduce their efficacy. In some pyrethroid-PBO nets, PBO is only present on the net roof, and so a resistant mosquito population targeted by the PBO component of the net must contact this section for the net to be effective. Although responses to a complete human-occupied bednet have not yet been determined, the

results presented here indicate that the proportions of mosquitoes arriving at the PermaNet 3.0 roof should not be compromised by the presence of PBO.

Following exposure to Interceptor G2 netting, 24-hour mosquito mortality was greater in video cone tests (~25%) than in baited box tests (>2%). In the baited box tests, composite image of baited boxes shows behaviour to be extremely variable depending on day tested. Accounting for the date effect, no difference in mosquito activity in the whole box was seen in Interceptor G2 compared to untreated or PermaNet 3.0 sides, however activity in the top row (closest to the host) was significantly reduced. When examining the proportion of total activity that occurred in the top row *i.e.* closest to the test netting/host, this was reduced compared to other net treatments suggesting repellence. Interceptor G2 is a dual insecticide LLIN which is coated with alpha-cypermethrin and chlorfenapyr. Alphacypermethrin is known to have repellent properties, which is suggested by the reduction in contact with the top row seen following Interceptor G2 exposure compared to other net types

Interceptor G2 has been reported to have improved efficacy compared to Interceptor LN (alphacypermethrin-only LLIN) against pyrethroid-resistant mosquitoes in several experimental hut trials (N'Guessan *et al.*, 2016b; Bayili *et al.*, 2017; WHO, 2017d; Camara *et al.*, 2018). However, reproducing the levels of mortality observed in huts in benchtop tests has been challenging. In a standard 3-minute cone bioassay several studies have documented low mortality (24 to 72-hour) following exposure to unwashed Interceptor G2 against resistant, and more concerningly, pyrethroid-susceptible mosquitoes (N'Guessan *et al.*, 2016b; Camara *et al.*, 2018). Higher mortalities have been observed when Interceptor G2 is used in overnight tunnel tests leading several authors to suggest that cone bioassays are not suitable for predicting the performance of the net (N'Guessan *et al.*, 2016a; Bayili *et al.*, 2017). This is sometime attributed to the higher activity of chlorfenapyr during the night (Oxborough *et al.*, 2015), when as a result of the *Anopheles* circadian rhythm, flight is increased, and subsequently cellular respiration and oxidative metabolism which the chlorfenapyr targets (Balmert *et al.*, 2014), is at its peak. In these studies, the authors did not compare the same types of assay (e.g. tunnel tests) during the day and overnight. However, in a previous study of chlorfenapyr only net Oxborough *et al.* (2015) observed increased mortality when 30-minute cylinder bioassays were conducted during the night compared to the day.

In the current tests, video cone test (> 22:00) and baited box tests (>19:00) were conducted after dusk to coincide with peak *Anopheles* biting times in Burkina Faso, and to imitate the light: dark rearing cycle, and therefore the circadian rhythm, of the test mosquitos. The extremely low mortality observed in these tests suggest that Interceptor G2 does not work effectively at night using the employed benchtop tests to measure efficacy. However, as mortalities cannot be compared to tests conducted during the day, it is not possible to establish if the net performs better at night as suggested by N'Guessan *et al.* (2016) and Camara *et al.* (2018).

5.5 Conclusion

The work reported in this chapter suggest that the video cone test can be conducted using wild population under field conditions. The thumb-test was unsuitable in this setting due to low mosquitoes response rates coupled with the time required to perform the test, and limited time in the field. The baited-box test was able to collect behavioural information on groups of wild-*Anopheles*. Automated bespoke video analysis (ViCTA) of baited boxes tests allowed mosquito movement to be detected and activity levels to be quantified in response to net exposure, providing insight into the actions of the net.

It is important that these results are verified by further evaluation of the tests in other field mosquito populations, to establish if the results reported here are characteristic of field populations in general, or specific to the Tengrela populations tested here, which exhibit some of the highest levels of pyrethroid resistance reported in *An. gambiae*.

Despite this high level of resistance, brief exposure with pyrethroid-PBO netting was sufficient to rapidly knockdown and kill this population.

Chapter 6. General discussion

Changes to mosquito density, lifespan or blood-feeding ability may dramatically influence their vectorial capacity and subsequent malaria transmission potential. Little is known about how these parameters are affected by insecticide exposure, particularly in wild insecticide-resistant populations. Consequently, the first part of this thesis investigated how insecticide exposure, mainly from insecticidal nets, impacted on wild pyrethroid-resistant *Anopheles* life-history traits and behaviour. In response to increasing insecticide resistance, new vector control products with novel modes of action are being developed and deployed. Efficacy test to assess these products should be able to evaluate new intervention classes initially, but also, monitor these products operationally. Current standard tests were developed for evaluating pyrethroid-only nets, which cause rapid knockdown and death in susceptible mosquitoes. These tests will not be suitable in instances where modes of action differ from simply lethality alone, or where lethality is delayed. Consequently, if current tests are used, we risk underestimating the efficacy of a product or disregarding its potential effectiveness in early stage testing. Thus, the second part of this thesis investigated the suitability of two novel benchtop bioassays for describing and quantifying behaviours of *Anopheles* at the LLIN bednet interface.

Overall, there was little evidence that exposure to either pyrethroid-only or next-generation nets led to detectable impacts on longevity, blood-feeding or reproductive output. No impacts on mosquito longevity were observed following exposure to PermaNet 2.0, Olyset Net (Chapter 3), PermaNet 3.0 or Interceptor G2 (Chapter 4), regardless of exposure method/bioassay (i.e. WHO cone tests or experimental hut trials). Immediate mortality (within 24-hours) was also extremely low in the mosquito population studied. In experimental hut trials with wild-*Anopheles*, immediate mortality did not exceed 20% in pyrethroid-net huts. This level is similar to another hut trial conducted in the same study area in 2014 (Toe *et al.*, 2018), but lower than mortality recorded in other countries at that time (Malima *et al.*, 2017; Oumbouke *et al.*, 2019). In recent years, mortality from hut trials using pyrethroid-only nets has dramatically declined to similar low levels (Bayili *et al.*, 2019; Furnival-Adams *et al.*, 2020). In the present study, mortality was higher in hut trials with reared-release mosquitoes, but this was still less than 50%. Concerningly, in the lab-studies, mortality was still below the level defining insecticide susceptibility (>80% mortality) following multiple net exposures. After up to five exposure, mortality was less than 10% in

young mosquitoes (<10-days-old) and did not exceed 65% in older mosquitoes (16-day-old). The low immediate mortality and absence of any impact on longevity following LLIN exposure indicates there could be a loss of community protection here and is a major concern for malaria control in this region.

Blood-feeding inhibition was observed in the experimental hut trials, with lower blood-feeding rates in pyrethroid-net huts comparted to controls (Chapter 3). Following collection from pyrethroid-net huts, blood-feeding levels were ~20% in trials where mosquitoes were reared and released, and 40-50% in trials where mosquitoes naturally entered huts. Blood-feeding levels in naturally-entering *Anopheles* are similar to those reported in the same study site in 2014 (Toe *et al.*, 2018), but reduced compared to trials conducted in Burkina Faso in recent years, which documented higher blood-feeding rates (Bayili *et al.*, 2019). In other countries, blood-feeding rates reported recently are comparatively low (e.g. Côte d'Ivoire, ~35% blood-feeding, Furnival-Adams *et al.*, 2020). These results suggest that in this area pyrethroid-only nets provide some personal protection that extends beyond simply the nets physical barrier. The results from the blood-feeding lab experiment reported here suggest any blood-feeding inhibition effect, however, may not be long-lasting (Chapter 4). In this study, blood-feeding rate was reduced at 1-hour post-exposure in Olyset Net arms compared to untreated, but this effect was lost within 8-hours post-exposure. A high proportion of mosquitoes obtained a blood meal within 8-hours (~85% mosquitoes' blood-fed), regardless of exposure-net type. Due to the experimental design of this assay, the Olyset Net effect reported could be as a result of lethality in this arm, rather than the mosquitoes being unwilling or unable to blood-feed due to a sub-lethal effect. In this experiment, mosquitoes were presented with a host to feed on, so only their ability to blood-feed, and not their ability to orientate towards and locate a host, were investigated. It would be beneficial to establish if blood-feeding inhibition occurs in scenarios where the effects on long-range host-seeking are also evaluated. If blood-feeding inhibition is such a short-lasting effect, this could mean mosquitoes initially inhibited from feeding on an individual protected by an insecticidal net may readily feed on an unprotected individual the same night. Consequently, further studies examining how mosquitoes respond to and interact with protected and unprotected individuals within the same sleeping space are needed. Room-scale mosquito video tracking technology (Parker *et al.*, 2015; Angarita-Jaimes *et al.*, 2016) for example, could provide a means to investigate this using wild-population in a controlled but more naturalistic setting.

Regarding reproductive output, the results reported in Chapter 4 suggest pyrethroid-net exposure does not impact mosquito fertility or fecundity. This supports two previous studies which also describe an absence of effect (Hauser, Thiévent and Koella, 2019; Mulatier *et al.*, 2019). Given the neuronal target site of the pyrethroids, this may not be surprising. Methods for measuring *Anopheles* reproductive output following bednet exposure are not standardised. Insecticidal nets containing pyrethroids plus juvenile hormone analogues (*e.g.* Royal Guard), which affect mosquito reproduction, have received PQ-listing from WHO (WHO, 2019a). Consequently, normative guidelines on how the efficacy of such products are evaluated are needed. Without detailed guidance, studies may use different methodology or measure different outcomes. This will complicate or prevent comparisons between trials and potentially result in difficulties confirming the entomological efficacy of such novel net types. This may be more important for "second-in-class" nets evaluated after Royal Guard, which will not require epidemiological evidence of impact, but will required to show non-inferiority to the "standard net" (Royal Guard) using entomological data from several hut trials.

The lack of effect of insecticidal net exposure on longevity could be as a result of high-pyrethroid resistance (Appendix 2, Hughes *et al.*, 2020). The Tengrela population of mosquitoes studied here exhibit some of the highest levels of pyrethroid resistance reported in *An. gambiae*. This resistance is driven by several mechanisms, some previously undescribed in other populations (Ingham *et al.*, 2019; Williams *et al.*, 2019). Delayed mortality has been reported in other studies in less resistant strains (Viana *et al.*, 2016), and was observed in this thesis when mosquitoes were exposed to extremely high pyrethroid concentration in WHO tube bioassays. Similarly, the absence of an effect seen in other life history traits evaluated here could be related to the intensity of resistance eroding any sub-lethal impacts, hence further research is needed to conclude that these nets do not induce sublethal effects. Burkina Faso is one of the top-10 high burden countries for malaria in Africa (WHO, 2019b), and the intense pyrethroid resistance in their mosquito populations is likely a contributing factor to why malaria is persistently high here despite several mass-LLIN distribution campaigns. The lack of sub-lethal effects detected following net exposure may also be contributing to this. Field populations are inherently behaviourally and genetically diverse. It is possible that sub-lethal effects will differ between species and within species. Given this diversity and the challenge of accounting

for random effects in the field, the tests reported here may be inadequately powered or not sensitive enough to detect sub-lethal impacts. Consequently, it would be irresponsible to extrapolate the absence of effects observed here to other populations and countries. Additional studies investigating sub-lethal effect in less resistant field population or population with different insecticide resistance mechanisms are required.

This thesis sought evidence for sub-lethal effects following insecticidal net exposure, so by design only focused on adult host-seeking female mosquitoes. Insecticide exposure from a range of sources has the capacity to affect mosquitoes at each stage of their life cycle. Mosquitoes may also encounter sub-lethal concentrations of insecticide in their juvenile stages, either directly through targeted larviciding, or indirectly from insecticidal run-off into breeding sites from agricultural or domestic insecticide use. Several studies in *Aedes* have observed sub-lethal pyrethroid concentrations to reduce locomotor activity of larvae when compared to unexposed controls (Tomé *et al.*, 2014; Marriel *et al.*, 2016; Sampaio *et al.*, 2017; Costa *et al.*, 2018). This could have subsequent effects on their foraging and predator avoidance ability. Additionally, insecticide exposure could impact on male mosquito behaviours, such as swarm formation or mating ability. In other insects, insecticide exposure has been observed to increase reproductive male fitness (Haddi *et al.*, 2016). As they do not transmit malaria, males are often disregarded when it comes to studying the impact of vector control tools. However, although not the primary target of the intervention, effects on reproduction could affect mosquito density, and possibly susceptibility to parasites (Dahalan *et al.*, 2019), with resultant impacts on transmission.

This thesis focused on how exposure to insecticidal nets impacted mosquitoes and did not consider other interactions which may have a role in malaria transmission. For example, studies have shown that ivermectin ingestion, and exposure to bendiocarb and DDT affect *Plasmodium* development in *Anopheles* (Alout *et al.*, 2014; Kobylinski, Escobedo-Vargas, *et al.*, 2017; Kobylinski, Ubalee, *et al.*, 2017). If insecticidal net exposure impacts on *Plasmodium* development this will have a significant impact on disease transmission. Furthermore, in several insects, beneficial symbiosis with endosymbiont bacteria which degrade insecticides have been observed to confer insecticide-resistance (Kikuchi *et al.*, 2012; Tago *et al.*, 2015; Itoh *et al.*, 2018). Recently, artificial manipulation of *An. arabiensis* gut microbiota was observed to both improve and decreases insecticide-resistance in a strain specific way (Barnard *et al.*, 2019), with evidence from whole genome sequencing

suggesting an association between gut microbiota and insecticide resistance (Dada *et al.*, 2018). Furthermore, *Wolbachia* (an intracellular bacterium) has been documented to occur naturally in *Anopheles* (Baldini *et al.*, 2014), and interfere with fecundity and *Plasmodium* development (Shaw *et al.*, 2016) in populations from Burkina Faso. Other studies have shown pesticide exposure to reduce diversity in extracellular bacteria in mosquito larval habitats (Muturi, Orindi and Kim, 2013; Muturi *et al.*, 2017). But, despite the indication of symbiont-mediated resistance in *Anopheles* and their effects on *Plasmodium* development, investigations of how insecticide exposure affects intracellular bacteria are limited to one study (Dada *et al.*, 2019). This investigation reported differences in the bacterial composition of *An. albimanus* exposed to pyrethroids compared to unexposed, and found some insecticide-degrading bacterial species in greater abundance in exposed mosquitoes. The study also reported that the cuticle surface of both larval and adult mosquitoes had more diverse microbiota than their gut, which could have implications for cuticular resistance, if these bacteria also degrade insecticides.

To transmit malaria, a mosquito must take an infectious bloodmeal, survive the parasite's intrinsic incubation period (9-16 days; Beier, 1998; Vaughan, 2007; Paaijmans *et al.*, 2010), and take a secondary blood-meal. Consequently, in terms of age, mosquitoes used in efficacy tests are generally not characteristic of the disease transmitting population. In standard tests, mosquitoes are exposed to insecticides at 2-5-days-old (WHO, 2013a). If a product is more efficacious in older mosquitoes we may be under estimating the efficacy of interventions with currently used tests (Alout *et al.*, 2017). In the tests reported in this thesis, sub-lethal effects were evaluated in mosquitoes < 11-days-old (predominately 3-to-5-days-old). In general, the age range used for monitoring studies (2-5-days-old) allows programmes to be able to collect and rear mosquitoes in adequate numbers for testing and allows comparability of results globally and overtime. Although this is understandable due to its convenience for routine monitoring, the evaluation of commercial products could be improved, if efficacy was also evaluated on mosquitoes of disease transmitting age (i.e. > 14-days-old). In phase III testing, mosquito mortality and blood-feeding are evaluated (WHO, 2013a), however collecting information on how these products affect mosquito population age structure would provide information on if/how such tools were selectively impacting on older mosquitoes in a natural environment. Therefore, adding estimations of mosquito age as outcomes in phase III trials would be beneficial.

As tested here against the highly resistant vector population in south west Burkina Faso, the efficacy of next-generation nets was highly variable. PermaNet 3.0 was able to rapidly incapacitate the vector, causing complete blood-feeding inhibition as a result of death soon after exposure, even when net contact was brief. This net has also been observed to be more effective than standard nets in experimental hut trials against the same mosquito population (Toe *et al.*, 2018). Barrier bed-nets were shown to target and kill the wild population with greater efficacy that standard pyrethroid-only nets (Chapter 3, Murray *et al.*, 2020). Conversely, Interceptor G2 performed relatively poorly, with no evidence of blood-feeding inhibition and low mortality in laboratory experiments, although this net was not evaluated in experimental huts in this study. Interceptor G2 was distributed in Burkina Faso's 2019 national net distribution campaign and is being operationally monitored for efficacy.

In the face of widespread insecticide resistance, the success of next-generation control tools will be fundamental to reverse current trends in malaria, which have seen case reductions stagnate and increase in some instances (WHO, 2019b). There has been a switched from a "one size fits all" to a targeted approach for malaria control, and the community has pushed for locally tailored vector control tools (WHO, 2017b; Wilson *et al.*, 2020). In the same way, on a micro-scale, this ideology should be adopted when evaluating novel vector control tools. Evaluation methods should vary depending on their primary objective. Tests which aim to evaluate the efficacy of products initially (e.g. screening and phase I testing) should be flexible to a range of products and focus on to the mode of action of the product being evaluated. In this situation there is no one size fits all approach. Monitoring products operationally, however, will require tests with less flexibility and greater field utility to allow the study outputs to be compared over time and space. The video test used in the studies reported in this thesis (Chapter 5) were able to quantify mosquito behaviour at the bed-net interface and provide information on activity levels following exposure. In a field setting, they provided information on how the nets were impacting on the wild pyrethroid-resistant population, which assisted in answering research questions, however, with the analysis method reported here, their applicability as a tool for monitoring efficacy is questionable, due to the time associated analysing the results of the tests.

Current tests do not capture the full action of vector control tools. By not measuring sub-lethal or behavioural effects during product evaluations and routine testing we may be overlooking the protective mode of action being exerted by both existing and novel products. More troublingly, however, by not measuring such effects we may fail to notice promptly when control tools become less effective, which worryingly appears to be the case in Burkina Faso. Incorporating measurement of sub-lethal effects into standard efficacy tests are needed. If an AI in development is found to irreversibly inhibit blood-feeding with little or no killing effects, for example, the implications of such a chemistry would be comparable to complete lethality from a transmission perspective. Similarly, if a product does not kill a mosquito immediately but can decrease its lifespan to under 14-days-old (the age at which a mosquito is likely to be infectious), this may still impact transmission. By focusing on lethality, we may be overlooking other areas where the disease transmitting population could be controlled. There is a need for the vector control community to move away from products that purely cause mosquito death to investigate other areas which could provide protection (e.g. bed nets which inhibit parasite development, Paton *et al.*, 2019) and complement existing interventions.

6.1 Priorities for further research

This thesis found sub-lethal exposure to insecticidal nets had little impact on the life-history traits and behaviour of the wild pyrethroid-resistant *Anopheles gambiae* s.l. population tested. Further studies, such as those suggested below, would be beneficial to collect additional information in this area:

- Studies on the sub-lethal effects of insecticidal net exposure using wild-*Anopheles* populations with lower insecticide resistance levels, or different insecticide resistance mechanisms.
- Tests looking at the sub-lethal effects of exposure following interactions with other insecticidal vector control interventions, such as IRS, where the duration of insecticide contact is likely to be longer.
- The impact of insecticidal net exposure on the longevity of *Anopheles* mosquitoes that are more characteristic of the likely infectious population (*i.e.* mosquitoes that have been previously blood-fed and are greater than 14-days-old).

- The impact of insecticidal net exposure on the development of *Plasmodium* and subsequently the longevity of *Plasmodium*-infected *Anopheles* following net exposure.
- Room-scale video tracking tests which investigate how mosquitoes respond to and interact with individuals protected with a bednet and unprotected individuals within the same sleeping space. Additionally, such tests could be complemented with video bench-top assays that measure the mosquitoes' time-to-blood-feed following insecticidal net exposure.
- The development of standardised test protocols for measuring longevity and reproductive output following exposure to vector control interventions.

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Appendix 1

Additional tables and figures

Chapter 3

Table A1.1. Summary of longevity analysis of An. gambiae s.l. from multiple study sites following exposure to increasing multiple insecticides in a WHO tube assay.

*Survival was compared between treatment and control using Cox regression. Immediate mortality (within 24-hours) was excluded from the analysis, * indicate statistically statistical significance (P < 0.05).*

Mosquito population	Insecticide	Total mosquitoes	P-value
Mangodara	Control	44	0.262
	Bendiocarb	3	
Sitiena	Control	42	0.853
	Deltamethrin 0.50%	11	
	Control	17	0.658
	Deltamethrin 0.75%	3	
Tengrela	Control	46	0.778
	Bendiocarb	9	
	Control	29	0.081
	Propoxur	4	
Toumousenni	Control	24	0.993
	Bendiocarb	12	
	Control	70	0.733
	Deltamethrin 0.25%	56	
	Control	70	0.09
	Deltamethrin 0.50%	15	
	Control	49	0.905
	Deltamethrin 0.75%	1	
	Control	37	0.329
	Deltamethrin 1.00%	7	
	Control	45	0.006*

	Malathion	2	
	Control	69	0.845
	Propoxur	38	
Toundoura	Control	25	0.947
	Deltamethrin 0.25%	4	
	Control	25	0.499
	Deltamethrin 0.50%	1	

Table A1.2. Summary of number of nights volunteer sleepers and net treatment spent in each hut during 'reared-release', 'wild-entry' and 'barrier bednet' experimental hut trials in 2016 and 2017.

Shaded cells show hut not in use. Abbreviations: SH, OY, BA, SO SA IS, YA MO, AM (anonymised volunteer names); PN2 (PermaNet 2.0); OP (organophosphate fenitrothion); NPI (non-pyrethroid insecticide).

Year	Trial	Volunteer/net treatment	Hut					
			1	2	3	4	5	6
2016	Reared-release	Sleeper SH					4	2
		Sleeper OY					2	4
		Untreated Net					5	1
		PermaNet 2.0					1	5
	Wild-entry	Sleeper SH					6	4
		Sleeper OY					4	6
		Untreated Net					5	5
		PermaNet 2.0					5	5
	Barrier bednet	Sleeper BA	4	4	4	4		
		Sleeper SO	4	4	4	4		
		Sleeper SA	4	4	4	4		
		Sleeper IS	4	4	4	4		
		Untreated Net	4	4	4	4		
		PermaNet 2.0	4	4	4	4		
		PermaNet 2.0 + PN2 Barrier	4	4	4	4		
		PermaNet 2.0 + OP Barrier	4	4	4	4		
2017	Reared-release	Sleeper SH		4	2			

		Sleeper OY		2	4			
		Untreated Net		5	1			
		PermaNet 2.0		1	5			
	Wild-entry	Sleeper BA	2	2	2	2	2	2
		Sleeper SO	2	2	2	2	2	2
		Sleeper SA	2	2	2	2	2	2
		Sleeper IS	2	2	2	2	2	2
		Sleeper YA	2	2	2	2	2	2
		Sleeper MO	2	2	2	2	2	1
		Sleeper AM						1
		Untreated Net	6	6		6	6	
		PermaNet 2.0		6	6		6	6
		Olyset Net	6		6	6		6
	Barrier bednet	Sleeper BA	6	6	6	6	6	6
		Sleeper SO	6	6	6	6	6	6
		Sleeper SA	6	6	6	6	6	6
		Sleeper IS	6	6	6	6	6	6
		Sleeper YA	6	6	6	6	6	6
		Sleeper MO	6	6	6	6	6	6
		Untreated Net	6	6	6	6	6	6
		PermaNet 2.0	6	6	6	6	6	6
		PermaNet 2.0 + PN2 Barrier	6	6	6	6	6	6
		PermaNet 2.0 + OP Barrier	6	6	6	6	6	6
		PermaNet 2.0 + NPI Barrier	6	6	6	6	6	6
		Untreated Net + PN2 Barrier	6	6	6	6	6	6

Table A1.3. Volunteer sleeper and net treatment rotation for 2016 reared-release experimental hut trial.

Abbreviations: UN (untreated net); PN2 (PermaNet 2.0); SH, OY (anonymised volunteer names).

Week	Day	Hut 5	Hut 6	Date
		UN	PN2	
1	1	SH	OY	26-Sep
1	2	OY	SH	27-Sep
1	3	SH	OY	28-Sep
1	4	OY	SH	29-Sep
1	5	SH	OY	30-Sep
		PN2	UN	
2	6	SH	OY	03-Oct

Table A1.4. Volunteer sleeper and net treatment rotation for 2016 wild-entry experimental trial.

Abbreviations: PN2 (PermaNet 2.0); UN (untreated net); SH, OY (anonymised volunteer names).

Week	Day	Hut 5	Hut 6	Date
		PN2	UN	
1	1	SH	OY	10-Oct
1	2	OY	SH	11-Oct
1	3	SH	OY	12-Oct
1	4	OY	SH	13-Oct
1	5	SH	OY	14-Oct
		UN	PN2	
2	6	SH	OY	17-Oct
2	7	OY	SH	18-Oct
2	8	SH	OY	19-Oct
2	9	OY	SH	20-Oct
2	10	SH	OY	21-Oct

Table A1.5. Volunteer sleeper and net treatment rotation for 2016 barrier bednet experimental hut trial.

Abbreviations: PN2 (PermaNet 2.0); PN2B (PermaNet 2.0 barrier); OPB (organophosphate fenitrothion barrier), UT (untreated net); BA, SO SA IS (anonymised volunteer names).

Week	Day	Hut 1	Hut 2	Hut 3	Hut 4	Date
		PN2	PN2+PN2B	PN2+OPB	UT	
1	1	BA	SO	SA	IS	26-Sep
1	2	IS	BA	SO	SA	27-Sep
1	3	SA	IS	BA	SO	28-Sep
1	4	SO	SA	IS	BA	29-Sep
		UT	PN2	PN2+PN2B	PN2+OPB	
2	5	BA	SO	SA	IS	03-Oct
2	6	IS	BA	SO	SA	04-Oct
2	7	SA	IS	BA	SO	05-Oct
2	8	SO	SA	IS	BA	06-Oct
		PN2+OPB	UT	PN2	PN2+P2B	
3	9	BA	SO	SA	IS	10-Oct
3	10	IS	BA	SO	SA	11-Oct
3	11	SA	IS	BA	SO	12-Oct
3	12	SO	SA	IS	BA	13-Oct
		PN2+P2B	PN2+OPB	UT	PN2	
4	13	BA	SO	SA	IS	17-Oct
4	14	IS	BA	SO	SA	18-Oct
4	15	SA	IS	BA	SO	19-Oct
4	16	SO	SA	IS	BA	20-Oct

Table A1.6. Volunteer sleepers and net treatment rotation for 2017 reared-release experimental hut trial.

Abbreviations: UN (untreated net); PN2 (PermaNet 2.0); IS, SE (anonymised volunteer names).

Week	Day	Hut 2	Hut 3	Date
		UN	PN2	
1	1	IS	SE	11-Sep
1	2	SE	IS	12-Sep
1	3	IS	SE	13-Sep
1	4	SE	IS	14-Sep
		PN2	UN	
2	5	IS	SE	18-Sep
2	6	SE	IS	19-Sep
2	7	IS	SE	20-Sep
2	8	SE	IS	21-Sep
2	9	IS	SE	22-Sep
2	10	SE	IS	23-Sep

Table A1.7. Volunteer sleepers and net treatment rotation for 2017 wild-entry experimental trial.

Abbreviations: UN (untreated net); PN2 (PermaNet 2.0); OLY (Olyset Net); BA, SO, SA, IS, YA, AM, MO (anonymised volunteer names).

Week	Day	Hut 1	Hut 2	Hut 3	Hut 4	Hut 5	Hut 6	Date
		UN	PN2	OLY	UN	PN2	OLY	
1	1	BA	SO	SA	IS	YA	AM	02-Jul
1	2	MO	BA	SO	SA	IS	YA	03-Jul
1	3	YA	MO	BA	SO	SA	IS	04-Jul
1	4	IS	YA	MO	BA	SO	SA	05-Jul
1	5	SA	IS	YA	MO	BA	SO	06-Jul
1	6	SO	SA	IS	YA	MO	BA	07-Jul
		OLY	UN	PN2	OLY	UN	PN2	
2	7	BA	SO	SA	IS	YA	MO	09-Jul
2	8	MO	BA	SO	SA	IS	YA	10-Jul
2	9	YA	MO	BA	SO	SA	IS	11-Jul
2	10	IS	YA	MO	BA	SO	SA	12-Jul
2	11	SA	IS	YA	MO	BA	SO	13-Jul
2	12	SO	SA	IS	YA	MO	BA	14-Jul

Table A1.8. Volunteer sleepers and net treatment rotation for 2017 barrier bednet experimental hut trial.

Abbreviations: PN2 (PermaNet 2.0); PN2B (PermaNet 2.0 barrier); NPIB (non-pyrethroid insecticide barrier); UN (untreated net); OPB (organophosphate fenitrothion BARRIER); BA, SO, SA, IS, YA, MO (anonymised volunteer names).

Week	Day	Hut 1	Hut 2	Hut 3	Hut 4	Hut 5	Hut 6	Date
		PN2+P2B	PN2	PN2+NPIB	UT	PN2+OPB	UT+OPB	
1	1	BA	SO	SA	IS	YA	MO	16-Jul
1	2	MO	BA	SO	SA	IS	YA	17-Jul
1	3	YA	MO	BA	SO	SA	IS	18-Jul
1	4	IS	YA	MO	BA	SO	SA	19-Jul
1	5	SA	IS	YA	MO	BA	SO	20-Jul
1	6	SO	SA	IS	YA	MO	BA	21-Jul
		UT+OPB	PN2+PNB	PN2	PN2+NPB	UT	PN2+OPB	
2	7	BA	SO	SA	IS	YA	MO	23-Jul
2	8	MO	BA	SO	SA	IS	YA	24-Jul
2	9	YA	MO	BA	SO	SA	IS	25-Jul
2	10	IS	YA	MO	BA	SO	SA	26-Jul
2	11	SA	IS	YA	MO	BA	SO	27-Jul
2	12	SO	SA	IS	YA	MO	BA	28-Jul
		PN2+OPB	UT+OPB	PN2+PNB	PN2	PN2+NPIB	UT	
3	13	BA	SO	SA	IS	YA	MO	30-Jul
3	14	MO	BA	SO	SA	IS	YA	31-Jul
3	15	YA	MO	BA	SO	SA	IS	01-Aug
3	16	IS	YA	MO	BA	SO	SA	02-Aug
3	17	SA	IS	YA	MO	BA	SO	03-Aug

3	18	SO	SA	IS	YA	MO	BA	04-Aug
		UT	PN2+OPB	UT+OPB	PN2+PNB	PN2	PN2+NPIB	
4	19	BA	SO	SA	IS	YA	MO	06-Aug
4	20	MO	BA	SO	SA	IS	YA	07-Aug
4	21	YA	MO	BA	SO	SA	IS	08-Aug
4	22	IS	YA	MO	BA	SO	SA	09-Aug
4	23	SA	IS	YA	MO	BA	SO	10-Aug
4	24	SO	SA	IS	YA	MO	BA	11-Aug
		PN2+NPIB	UT	PN2+OPB	UT+OPB	PN2+PN2B	PN2	
5	25	BA	SO	SA	IS	YA	MO	13-Aug
5	26	MO	BA	SO	SA	IS	YA	14-Aug
5	27	YA	MO	BA	SO	SA	IS	15-Aug
5	28	IS	YA	MO	BA	SO	SA	16-Aug
5	29	SA	IS	YA	MO	BA	SO	17-Aug
5	30	SO	SA	IS	YA	MO	BA	18-Aug
		PN2	PN2+NPIB	UT	PN2+OPB	UT+OPB	PN2+PN2B	
6	31	BA	SO	SA	IS	YA	MO	20-Aug

6	32	MO	BA	SO	SA	IS	YA	21-Aug
6	33	YA	MO	BA	SO	SA	IS	22-Aug
6	34	IS	YA	MO	BA	SO	SA	23-Aug
6	35	SA	IS	YA	MO	BA	SO	24-Aug
6	36	SO	SA	IS	YA	MO	BA	25-Aug

Table A1.9. Parameters for GLMMs run on experimental hut trials.

Treated nets were compared to untreated controls. Table lists response, fixed effects and random effects variables, and the statistical distribution used. Trial ID; 1 = 2016 longevity hut trial, 2 = 2017 longevity hut trial, 3 = 2016 reproductive output hut trial, 4 = 2017 reproductive hut trial, 5 = 2016 barrier net hut trial, 6 = 2017 barrier net hut trial.

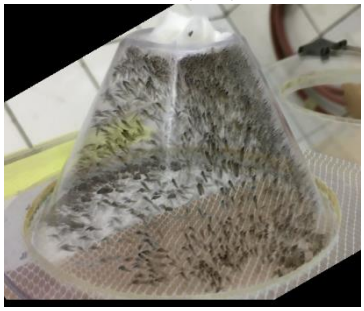
ID	Response	Fixed effects	Random effects	Distribution	Comparison	P-value
1	Mortality	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2	0.002
1	Blood-feeding	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2	0.000
1	Exophily	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2	0.003
2	Mortality	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2	0.000
2	Blood-feeding	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2	0.000
2	Exophily	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2	0.075
3	Mortality	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2	0.181
3	Blood-feeding	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2	0.183

3	Exophily	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2	0.993
3	Deterrence	Net treatment	Date, Sleeper, Hut	Negative Binomial	UN vs PN2	0.816
4	Mortality	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2	0.015
4	Blood-feeding	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2	0.028
4	Exophily	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2	0.604
4	Deterrence	Net treatment	Date, Sleeper, Hut	Negative Binomial	UN vs PN2	0.015
4	Mortality	Net treatment	Date, Sleeper, Hut	Binomial	UN vs OLY	0.001
4	Blood-feeding	Net treatment	Date, Sleeper, Hut	Binomial	UN vs OLY	0.052
4	Exophily	Net treatment	Date, Sleeper, Hut	Binomial	UN vs OLY	0.559
4	Deterrence	Net treatment, Date	Sleeper, Hut	Binomial	UN vs OLY	0.585
4	Deterrence	Net treatment	Date, Sleeper, Hut	Poisson	UN vs OLY	0.639
5	Mortality	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2	0.700
5	Blood-feeding	Net treatment, Date	Sleeper, Hut	Binomial	UN vs PN2	0.032
5	Exophily	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2	0.040
5	Deterrence	Net treatment	Date, Sleeper, Hut	Negative Binomial	UN vs PN2	0.197
5	Mortality	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2+PN2B	0.079

5	Blood-feeding	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2+PN2B	0.003
5	Exophily	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2+PN2B	0.000
5	Deterrence	Net treatment	Date, Sleeper, Hut	Negative Binomial	UN vs PN2+PN2B	0.162
5	Mortality	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2+OPB	0.002
5	Blood-feeding	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2+OPB	0.002
5	Exophily	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2+OPB	0.012
5	Deterrence	Net treatment	Date, Sleeper, Hut	Poisson	UN vs PN2+OPB	0.222
6	Mortality	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2+PN2B	0.037
6	Blood-feeding	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2+PN2B	0.000
6	Exophily	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2+PN2B	0.000
6	Deterrence	Net treatment	Date, Sleeper, Hut	Negative Binomial	UN vs PN2+PN2B	0.125
6	Mortality	Net treatment, Date, Hut	Sleeper	Binomial	UN vs PN2	0.152
6	Blood-feeding	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2	0.000
6	Exophily	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2	0.001
6	Deterrence	Net treatment	Date, Sleeper, Hut	Poisson	UN vs PN2	0.041
6	Mortality	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2+NPI	0.000

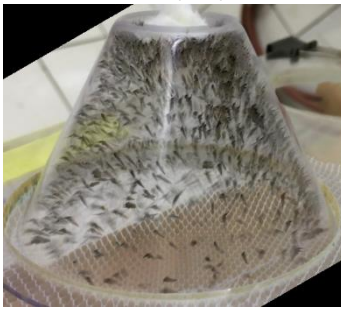
6	Blood-feeding	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2+NPI	0.000
6	Exophily	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2+NPI	0.001
6	Deterrence	Net treatment	Date, Sleeper, Hut	Negative Binomial	UN vs PN2+NPI	0.121
6	Mortality	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2+OPB	0.000
6	Blood-feeding	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2+OPB	0.000
6	Exophily	Net treatment	Date, Sleeper, Hut	Binomial	UN vs PN2+OPB	0.238
6	Deterrence	Net treatment	Date, Sleeper, Hut	Negative Binomial	UN vs PN2+OPB	0.005
6	Mortality	Net treatment	Date, Sleeper, Hut	Binomial	UN vs UN+OPB	0.000
6	Blood-feeding	Net treatment	Date, Sleeper, Hut	Binomial	UN vs UN+OPB	0.000
6	Exophily	Net treatment	Date, Sleeper, Hut	Binomial	UN vs UN+OPB	0.700
6	Deterrence	Net treatment	Date, Sleeper, Hut	Negative Binomial	UN vs UN+OPB	0.006

UN1 – 12/08/18



UN2 – 12/08/18

UN3 – 12/08/18



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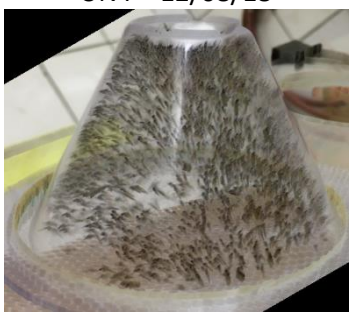
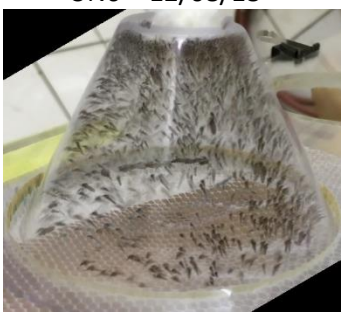
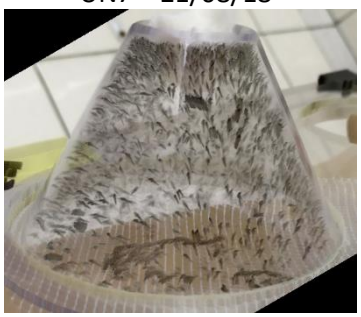


UN5 – 12/08/18

UN6 – 12/08/18

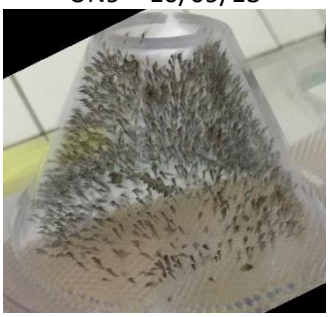


UN7 – 21/08/18



UN8 – 24/08/18

UN9 – 10/09/18



UN10 – 12/09/18

UN11 – 13/09/18

UN12 – 04.10.18

UN13 – 07/10/18

UN14 – 07/10/18

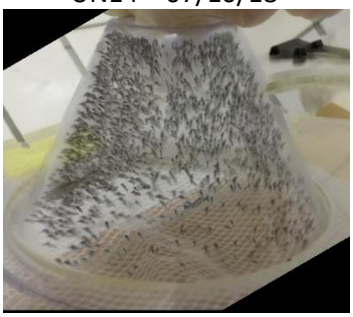


Figure A1.1. Composite images showing mosquito location during exposure to untreated netting in the 3-minute video cone test.

Text above image shows video replicate ID and date of testing. Composite images were created by overlaying each recorded video frame every 0.1 second. Images were created using ViCTA by Dr Jeff Jones (Jones et al., Unpublished).

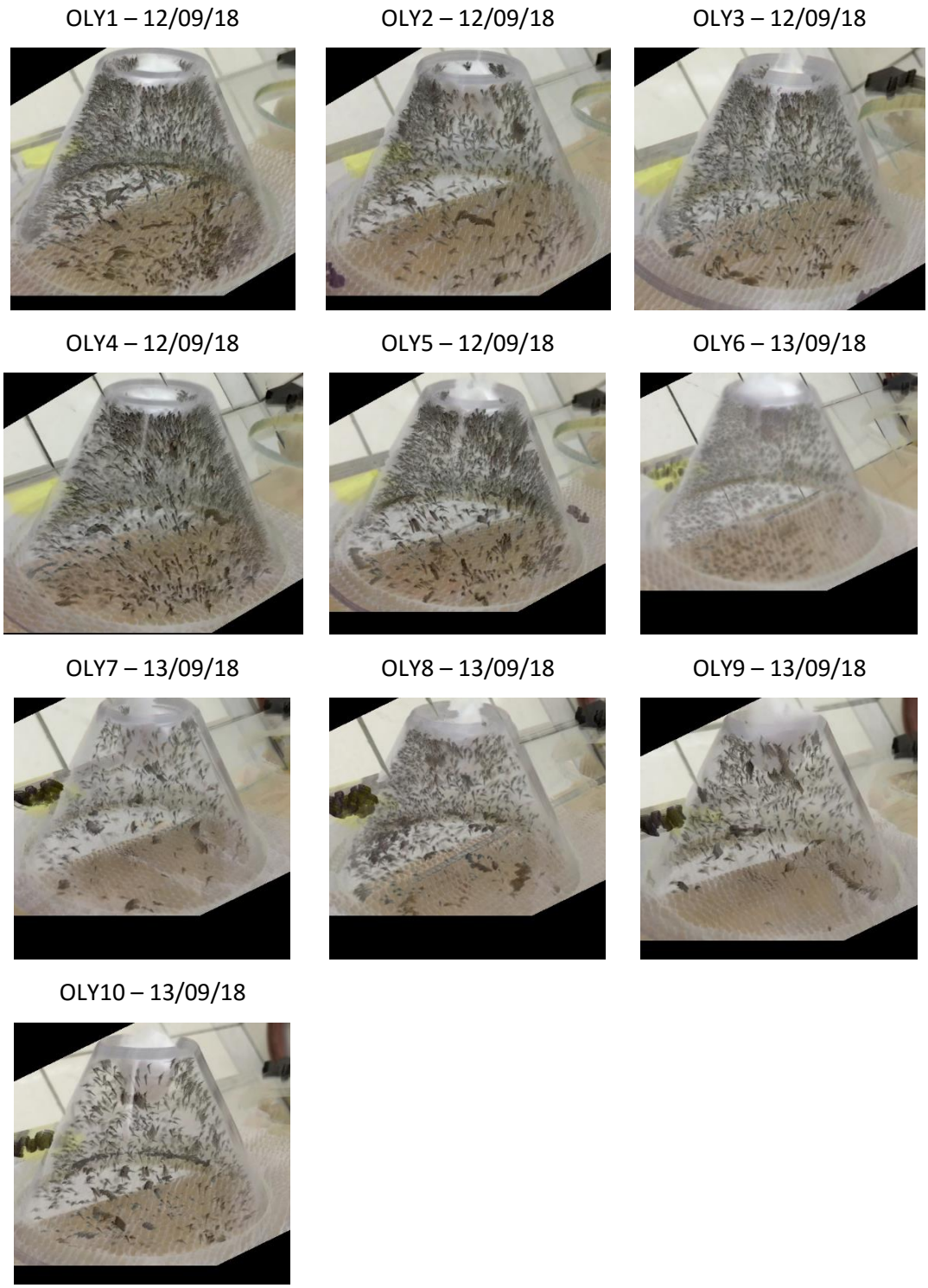


Figure A1.2. Composite images showing mosquito location during exposure to Olyset Net netting in the 3-minute video cone test.

Text above image shows video replicate ID and date of testing. Composite images were created by overlaying each recorded video frame every 0.1 second. Images were created using ViCTA by Dr Jeff Jones (Jones et al., Unpublished).

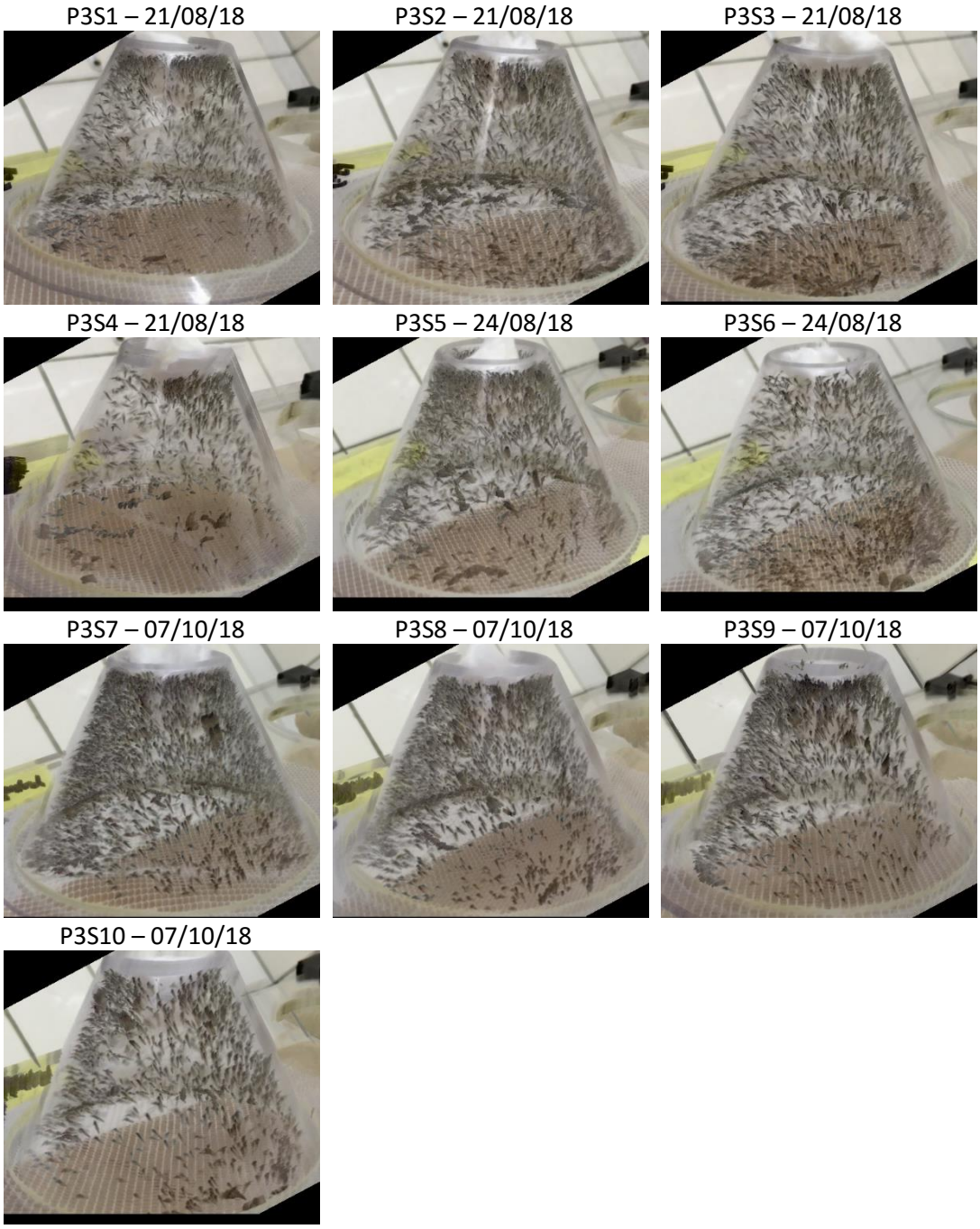


Figure A1.3. Composite images showing mosquito location during exposure to PermaNet 3.0 side netting in the 3-minute video cone test.

Text above image shows video replicate ID and date of testing. Composite images were created by overlaying each recorded video frame every 0.1 second. Images were created using ViCTA by Dr Jeff Jones (Jones et al., Unpublished).

P3T1 – 21/08/18 P3T2 – 21/08/18 P3T3 – 21/08/18

P3T5 – 24/08/18 P3T6 – 24/08/18 P3T7 – 04/10/18

P3T8 – 04/10/18 P3T9 – 07/10/18 P3T10 – 07/10/18

P3T11 – 07/10/18

Figure A1.4 Composite images showing mosquito location during exposure to PermaNet 3.0 roof netting in the 3-minute video cone test.

Text above image shows video replicate ID and date of testing. Composite images were created by overlaying each recorded video frame every 0.1 second. Images were created using ViCTA by Dr Jeff Jones (Jones et al., Unpublished).

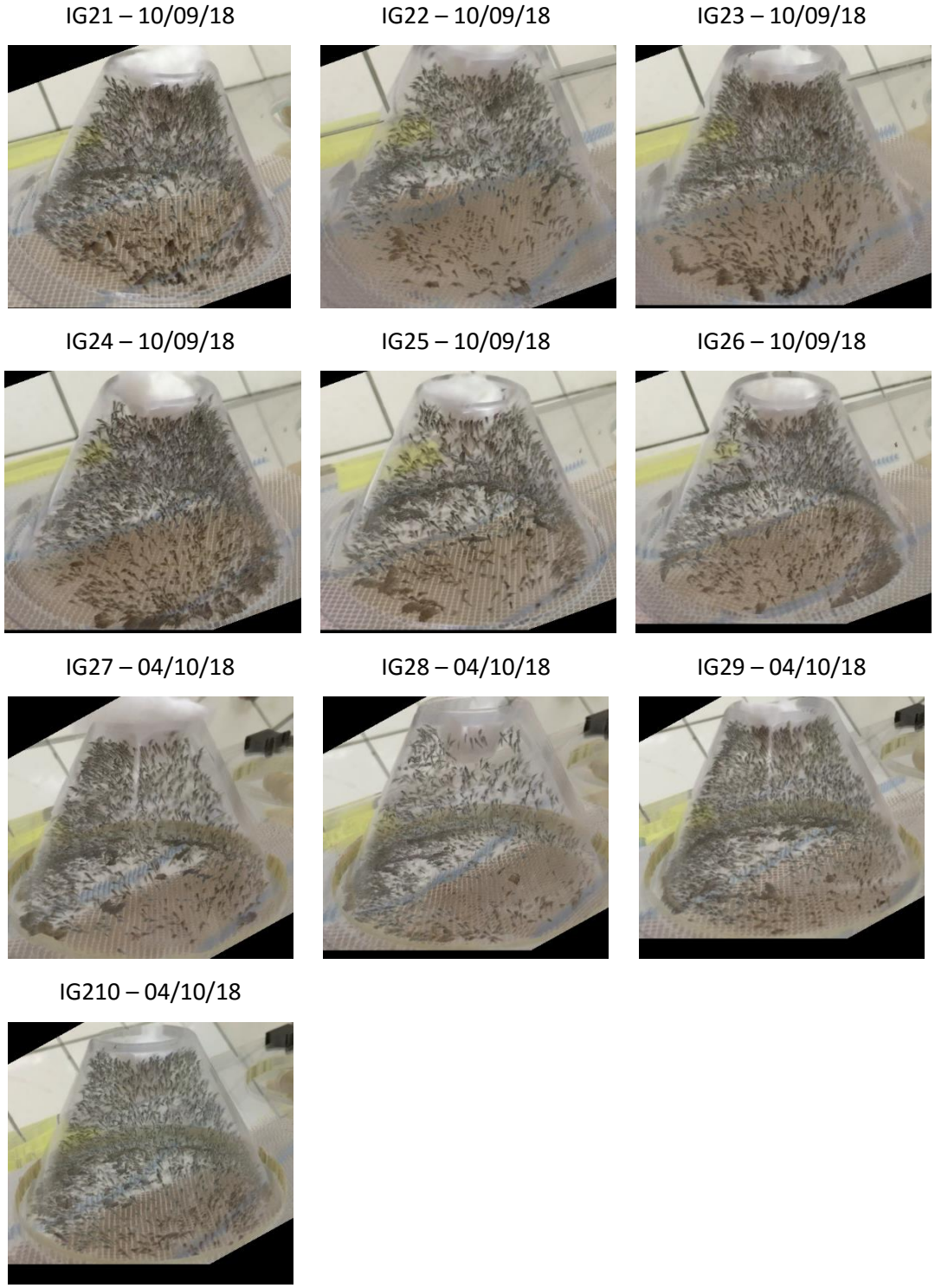


Figure A1.5. Composite images showing mosquito location during exposure to Interceptor G2 netting in the 3-minute video cone test.

Text above image shows video replicate ID and date of testing. Composite images were created by overlaying each recorded video frame every 0.1 second. Images were created using ViCTA by Dr Jeff Jones (Jones et al., Unpublished).

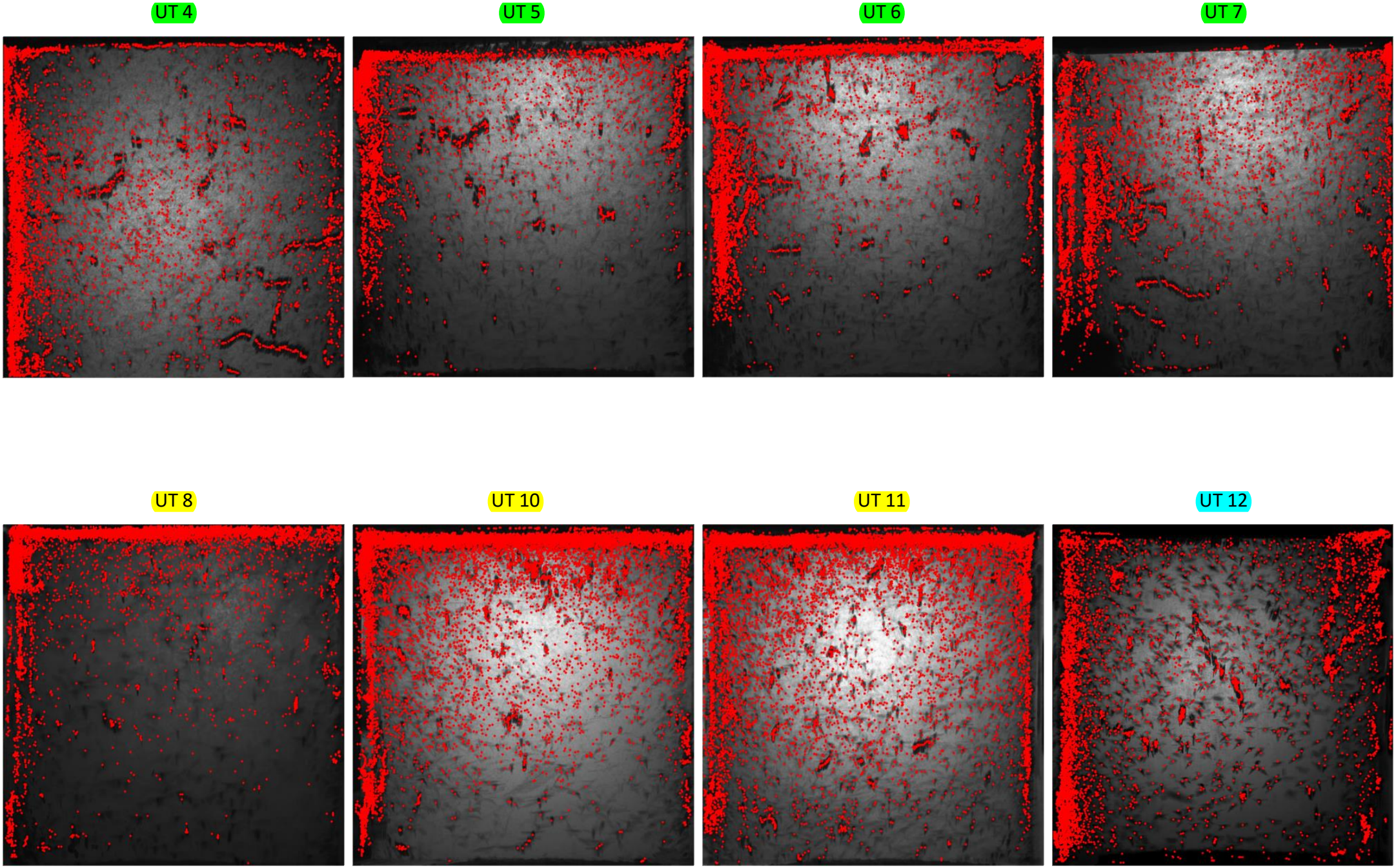


Figure A1.6 Composite images showing mosquito activity detected during exposure to untreated (UT) net in 20-minute baited box tests. Red dots show the movement of mosquitoes detected by ViCTA software. Numbers above images indicate replicate ID, and the highlighted colour represents the date of testing on 12th (green), 15th (yellow) or 19th (blue) October 2018. Replicate ID numbers are not sequential as some videos failed to record.

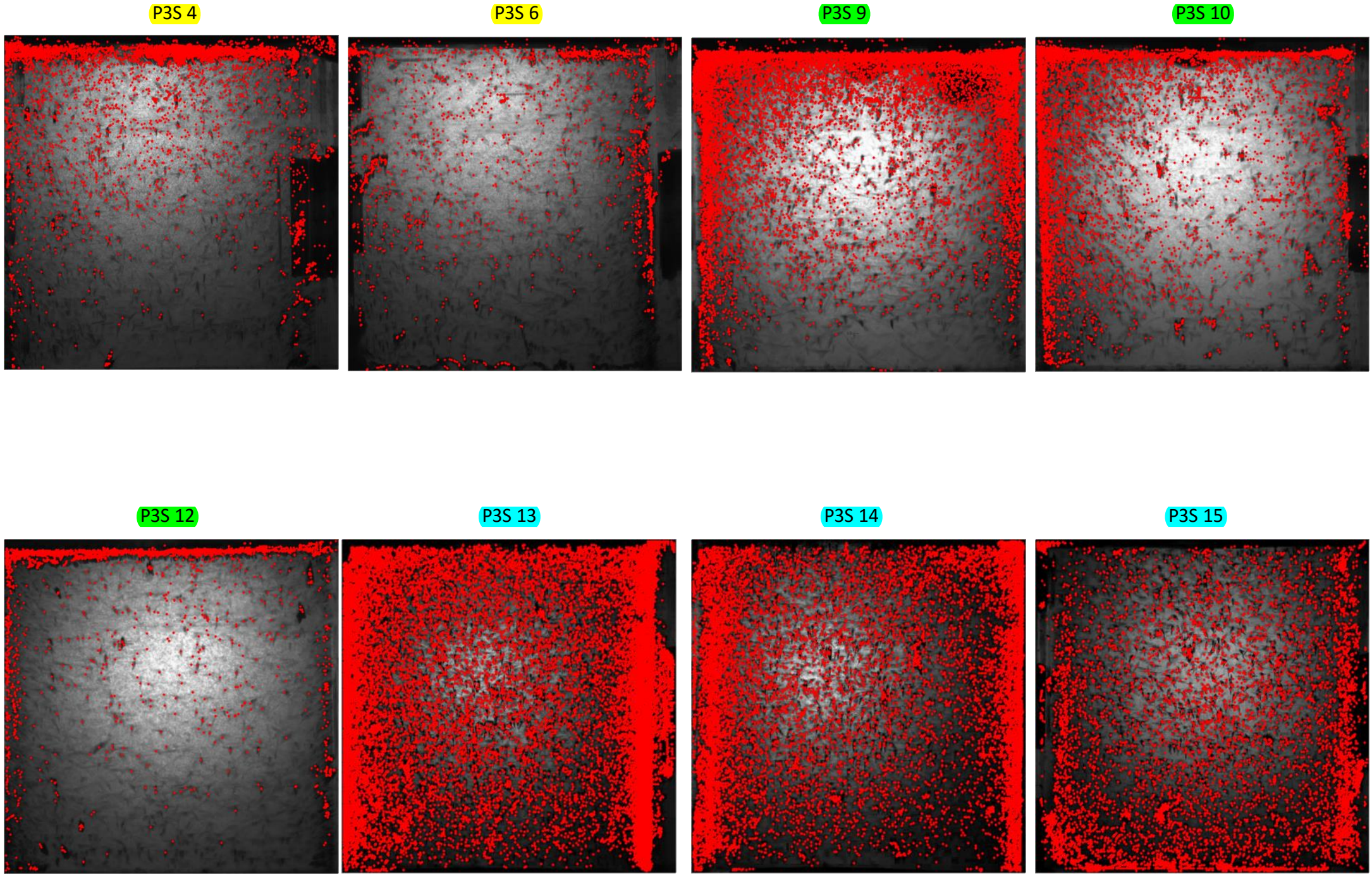


Figure A1.7. Composite images showing mosquito activity detected during exposure to PermaNet 3.0 sides (P3S) net in 20-minute baited box tests. Red dots show the movement of mosquitoes detected by ViCTA software.Numbers above images indicate replicate ID, and the highlighted colour represents the date of testing on 12th (green), 15th (yellow) or 19th (blue) October 2018. Replicate ID numbers are not sequential as some videos failed to record.

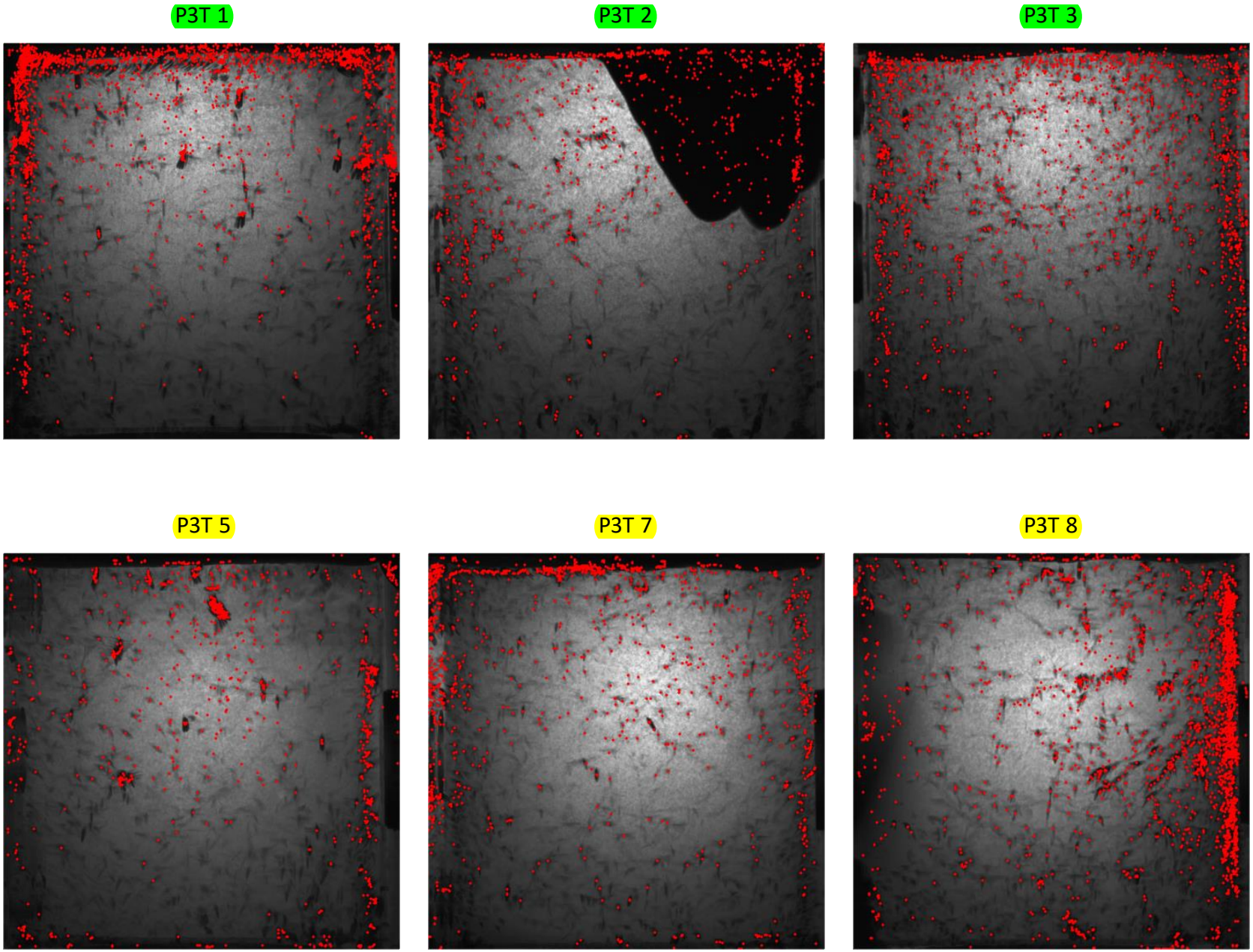


Figure A1.8. Composite images showing mosquito activity detected during exposure to PermaNet 3.0 roof (P3T) net in 20-minute baited box tests. Red dots show the movement of mosquitoes detected by ViCTA software. Numbers above images indicate replicate ID, and the highlighted colour represents the date of testing on 12th (green) or 15th (yellow) October 2018. Replicate ID numbers are not sequential as some videos failed to record.

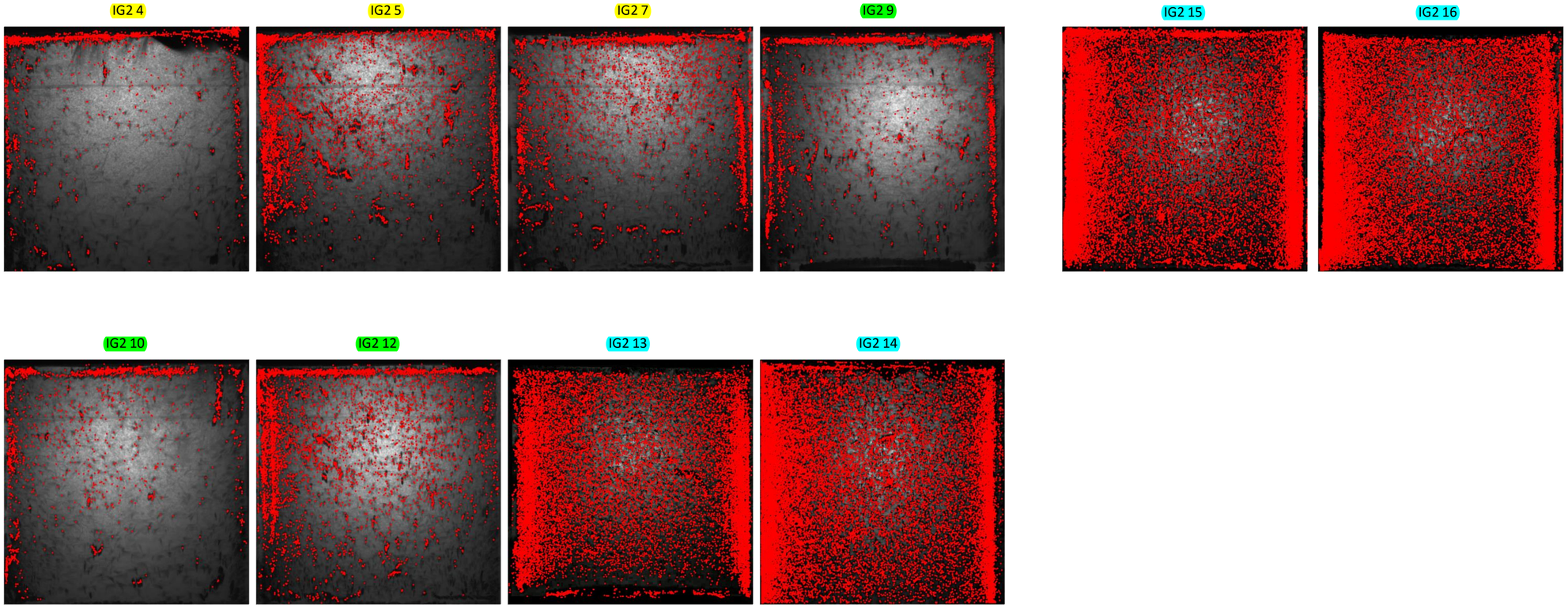


Figure A1.9. Composite images showing mosquito activity detected during exposure to Interceptor G2 (IG2) net in 20-minute baited box tests. Red dots show the movement of mosquitoes detected by ViCTA software. Numbers above images indicate replicate ID, and the highlighted colour represents the date of testing on 12th (green), 15th (yellow) or 19th (blue) October 2018. Replicate ID numbers are not sequential as some videos failed to record.

Figure A1.10. The Video Cone Test Analyser (ViCTA) rig apparatus*. The ViCTA rig immobilises the cone and camera in the same position for every video cone test. This reduces background noise in the video and allows for automated analysis. Image courtesy of Dr Jeff Jones.*

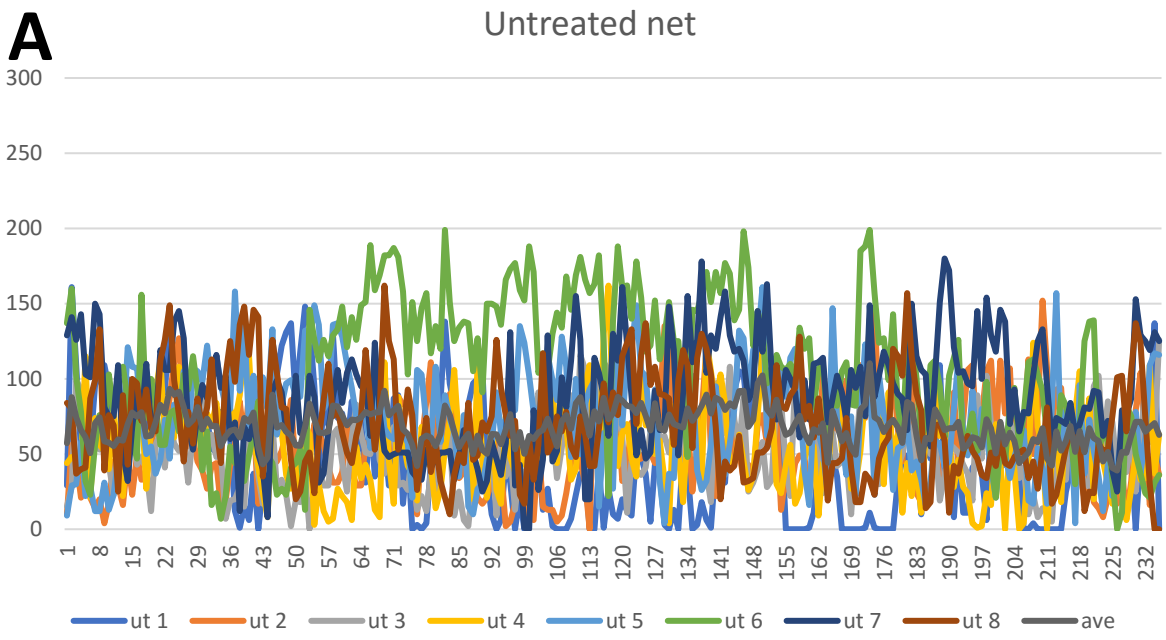
Table A1.1. Weighted mosquito movements detected by ViCTA in 20-minute baited boxes in the top row only, or whole box. *Movements have been weighted when mosquito numbers deviate from 5.*

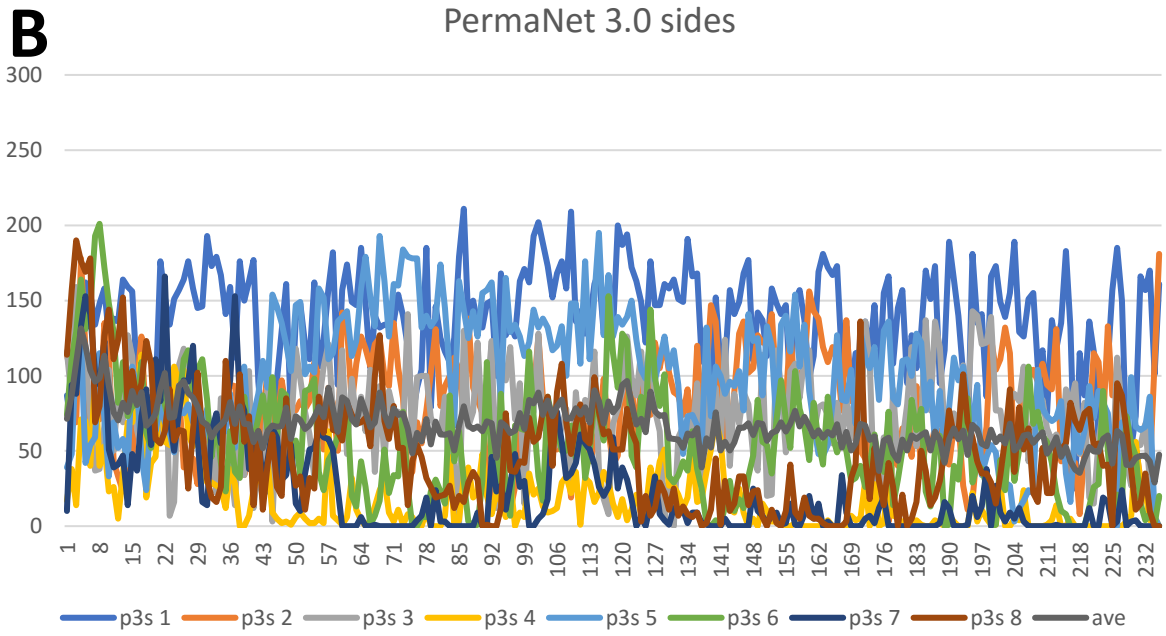
Net	Date	Rep	Detected movement top row	Detected movement whole box
Untreated	12th October 2018	4	1,548	12,872
		5	5,130	15,345
		6	9,008	20,314
		7	2,499	15,961
	15th October 2018	8	11,138	17,141
		10	16,116	24,922
		11	12,017	20,839
	19th October 2018	12	2,181	13,826

PermaNet 3.0 sides	12th October 2018	4	8,223	11,248
		6	1,973	5,331
	15th October 2018	9	13,094	23,211
		10	6,456	12,936
		12	4,288	5,670
	19th October 2018	13	4,447	32,400
		14	3,591	18,787
		15	2,263	17,162
PermaNet 3.0 roof	12th October 2018	1	2,197	4,822
		2	1,731	3,233
		3	991	2,994
	15th October 2018	5	637	3,017
		7	1,471	3,108
		8	1,005	5,995
Interceptor G2	12th October 2018	4	4,310	6,889
		5	2,833	11,100
		7	3,263	9,645
	15th October 2018	9	2,543	7,338
		10	1,904	4,242
		12	2,977	10,506
	19th October 2018	13	1,651	31,013
		14	3,295	30,714
		15	5,123	39,543
		16	2,272	35,652

Table A1.2. Comparisons of the number of detected mosquito movements in the top row and whole baited box. *Net treatments were compared using GLMMs with a negative binominal distribution and date included as a random effect.*

Net Comparison	Detected movement top row	Detected movement whole box
Untreated vs PermaNet 3.0 side	0.589	0.613
Untreated vs PermaNet 3.0 roof	< 0.000	< 0.000
Untreated vs Interceptor G2	0.005	0.057
PermaNet 3.0 side vs PermaNet 3.0 roof	< 0.000	< 0.000
PermaNet 3.0 side vs Interceptor G2	0.010	0.950
PermaNet 3.0 roof vs Interceptor G2	< 0.000	< 0.000


A
Untreated net
300
250
200
150
100
50
0
1 8 15 22 29 36 43 50 57 64 71 78 85 92 99 106 113 120 127 134 141 148 155 162 169 176 183 190 197 204 211 218 225 232
ut 1
ut 2
ut 3
ut 4
ut 5
ut 6
ut 7
ut 8
ave


B
PermaNet 3.0 sides
300
250
200
150
100
50
0
1 8 15 22 29 36 43 50 57 64 71 78 85 92 99 106 113 120 127 134 141 148 155 162 169 176 183 190 197 204 211 218 225 232
p3s 1
p3s 2
p3s 3
p3s 4
p3s 5
p3s 6
p3s 7
p3s 8
ave

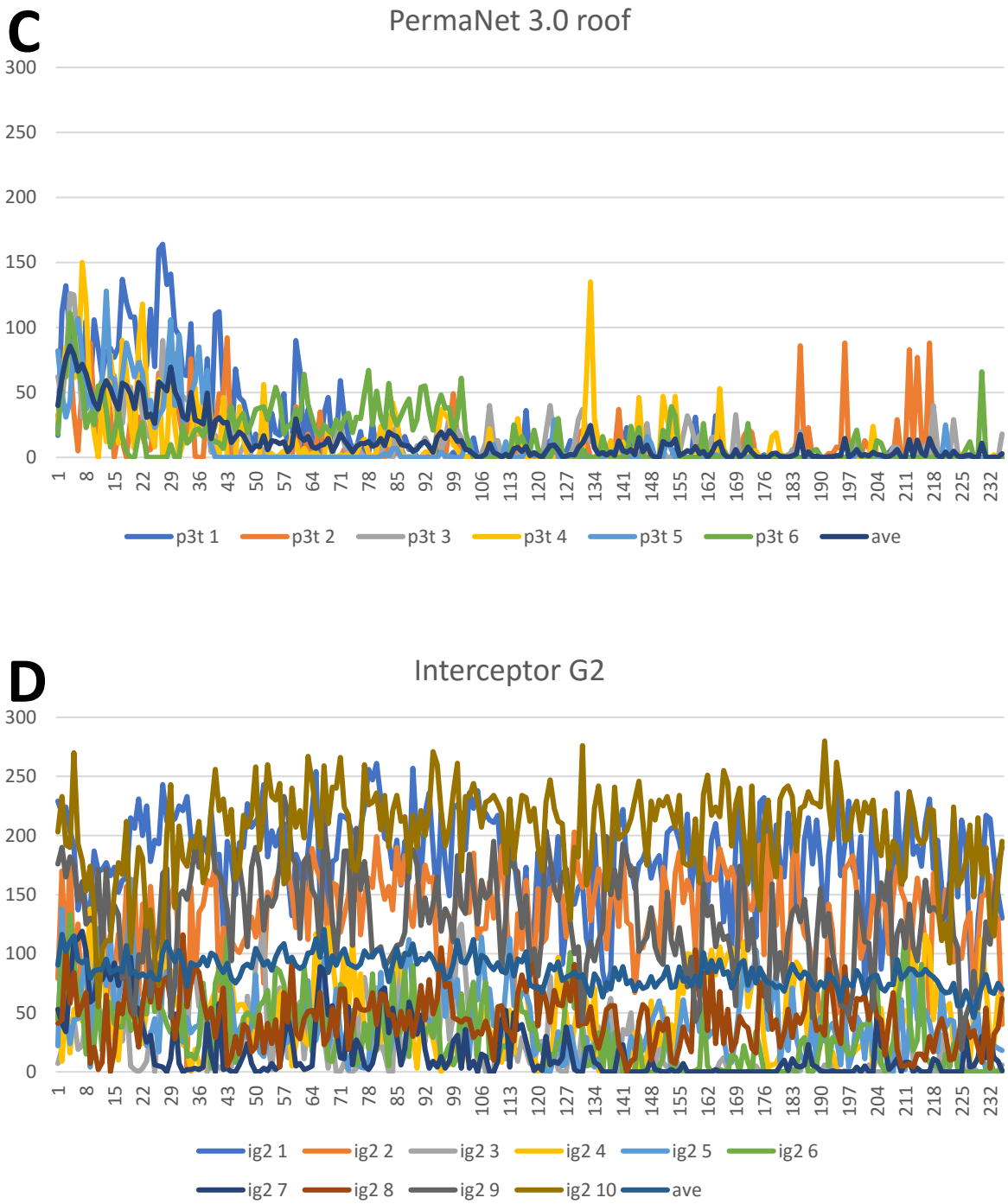


Figure A1.1. Unweighted activity over time detected in the entire box for each netting replicate during 20-minute exposure to (A) Untreated net, (B) PermaNet 3.0 side, (C) PermaNet 3.0 roof, and (D) Interceptor G2 in baited boxes.

Appendix 2

Publication: Murray, G.P.D., Lissenden, N., Jones, J. et al. (2020). Barrier bednets target malaria vectors and expand the range of usable insecticides. Nature Microbiology, 5, 40–47 (2020) doi:10.1038/s41564-019-0607-2.

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Barrier bednets target malaria vectors and expand the range of usable insecticides

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Transmission of *Plasmodium falciparum* malaria parasites occurs when nocturnal *Anopheles* mosquito vectors feed on human blood. In Africa, where malaria burden is highest, bednets treated with pyrethroid insecticide were highly effective in preventing mosquito bites and reducing transmission, and essential to achieving unprecedented reductions in malaria until 2015 (ref. [1]). Since then, progress has stalled[2], and with insecticidal bednets losing efficacy against pyrethroid-resistant *Anopheles* vectors[3,4], methods that restore performance are urgently needed to eliminate any risk of malaria returning to the levels seen before their widespread use throughout sub-Saharan Africa[5]. Here, we show that the primary malaria vector *Anopheles gambiae* is targeted and killed by small insecticidal net barriers positioned above a standard bednet in a spatial region of high mosquito activity but zero contact with sleepers, opening the way for deploying many more insecticides on bednets than is currently possible. Tested against wild pyrethroid-resistant *A. gambiae* in Burkina Faso, pyrethroid bednets with organophosphate barriers achieved significantly higher killing rates than bednets alone. Treated barriers on untreated bednets were equally effective, without significant loss of personal protection. Mathematical modelling of transmission dynamics predicted reductions in clinical malaria incidence with barrier bednets that matched those of 'next-generation' nets recommended by the World Health Organization against resistant vectors. Mathematical models of mosquito–barrier interactions identified alternative barrier designs to increase performance. Barrier bednets that overcome insecticide resistance are feasible using existing insecticides and production technology, and early implementation of affordable vector control tools is a realistic prospect.

Sleeping under a long-lasting insecticidal net (LLIN) is the most effective way of preventing malaria in Africa, where the widespread use of LLINs was the main contributor to 50% and 40% reductions in malaria prevalence and clinical disease incidence, respectively, between 2000 and 2015[1]. Those first-generation 'standard' LLINs used pyrethroids—fast-acting insecticides with minimal health risks for bednet users. By 2017, however, the annual reduction was replaced by an increase of 3.5 million malaria cases in the ten African countries[2] with the highest burden. Although its contribution to this alarming development is unclear, pyrethroid resistance is widespread in *Anopheles* spp. vector populations[4,5] and standard LLINs have lost efficacy against resistant vectors[3–6]. Thus, overcoming resistance is a global priority, demanding insecticides that do not share resistance mechanisms with pyrethroids or methods that reduce dependency on insecticides[7–9]. Recent trial results have identified insecticide combinations effective against pyrethroid-resistant vectors[3,10,11], but toxicity restrictions on risks to occupants, especially infants, and the higher cost of new insecticides limit bednet-treatment options.

Previous studies have shown that *A. gambiae* host-seeking activity predominates on a bednet roof, typically above the supine host's torso[12–15]. We also reported high numbers of flight paths traversing the space above the bednet roof, comprising flights with minimal ('visiting') or zero ('swooping') net contact[12,13]. To target these flights, we proposed intercepting mosquitoes with insecticidal net barriers projecting vertically from the bednet roof, where the insecticide would be beyond the reach of children, never touched by the bednet's occupants and rarely touched during routine human activity. If effective, then small net targets might control malaria vectors using a wider range of insecticides than possible with standard bednets[16].

As a proof of concept, we evaluated a single transverse barrier (0.5 m tall, 0.9 m wide) above a standard pyrethroid LLIN (Permanet 2.0 (P2)), positioned off-centre above the sleeper's torso (Fig. 1a,b). Barriers comprised P2 (P2B) or untreated netting dipped in fenitrothion (OPB, 0.02 g m^{-2}), an organophosphate widely used for indoor residual spraying (IRS) against pyrethroid-resistant mosquitoes[17–19], but never deployed on standard bednets. In initial laboratory bioassays (Fig. 1c), the unmodified P2 bednet killed 77% and 56% of insecticide-susceptible and resistant *A. gambiae* strains, respectively, within 48 h of exposure. Adding the P2B did not affect mortality rates with either strain, but the OPB was significantly better, killing 100% of resistant mosquitoes within 48 h (90% at 24 h; *t*-test, $P < 0.01$).

In a hut trial in a malaria-endemic setting in the Cascades region, Burkina Faso—where *A. gambiae* sensu lato (s. l.) vectors are highly resistant to deltamethrin but susceptible to fenitrothion (Extended Data Fig. 1)—we tested three different transverse barriers (Fig. 1d): P2B; fenitrothion-dipped netting (OPB; with 0.5 g m^2 fenitrothion, 20× higher than in the previous laboratory tests, equivalent to 25% of the target dose of IRS treatment); non-pyrethroid mixture (NPB; comprising indoxacarb and fenazaquin, each at 3–5%). The results show that all treatments significantly reduced mosquito entry rates

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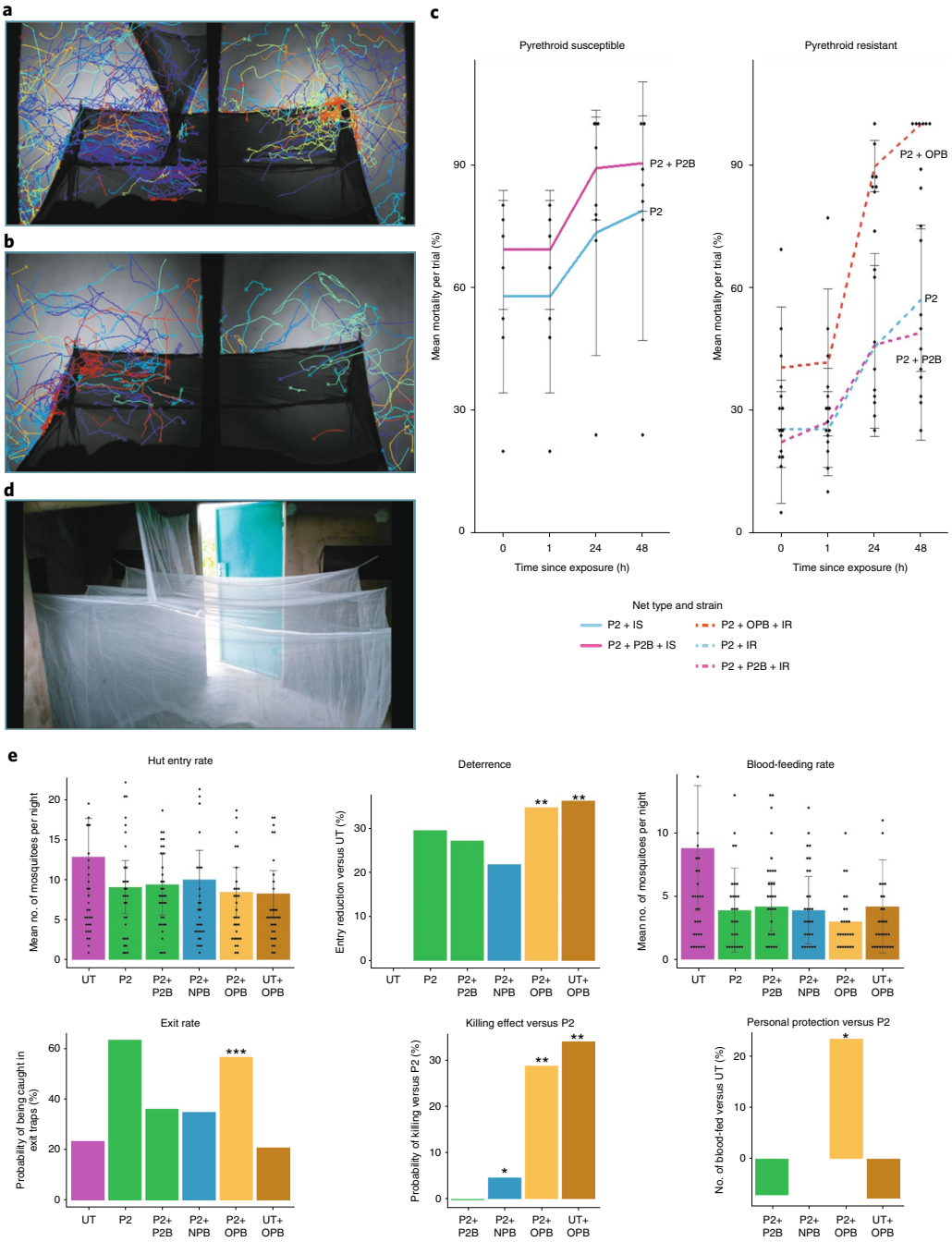

Fig. 1 | Performance of barrier bednets in laboratory and semi-field trials. a,**b**, Infrared tracks of mosquito flights at P2 bednets with a 50-cm-high transverse barrier (positioned off-centre, above the sleeper's torso) (**a**) and unmodified P2 (**b**). Tracks were recorded during bioassays; 25 mosquitoes, 60 min. **c**, Mean (±s.d., $n=6$ trials per treatment) mortality rates of *A. gambiae* strains susceptible (IS) or resistant (IR) to pyrethroids, following free-flight exposure to human-baited P2 nets, with or without barriers. P2 and P2 + P2B mortality rates were not significantly different for IS (*t*-test, $n=82$, d.f. = 5.3, $t=0.75$, $P=0.48$) and IR (*t*-test, $n=109$, d.f. = 8.7, $t=0.62$, $P=0.55$). P2 + OPB mortality at 24 h (90%) and 48 h (100%) significantly exceeded unmodified P2 mortality (IR 24 h, 45%; *t*-test, $n=91$, d.f. = 6.1, $t=5.21$, $P<0.01$; IR 48 h, 57%; *t*-test, $n=31$, d.f. = 5.1, $t=6.5$, $P<0.01$) and P2 + P2B (IR 24 h, 46%; *t*-test, $n=91$, d.f. = 5.8, $t=4.61$, $P<0.01$; IR 48 h, 49%; *t*-test, $n=41$, d.f. = 5.1, $t=4.74$, d.f. = 5.1, $P<0.01$). **d**, Barrier bednet in situ, Burkina Faso. **e**, Summary of key results from the hut trial; all comparisons versus UT, unless stated otherwise; asterisks denote significant differences ($0.05 \geq *P > 0.01$, $0.01 \geq **P > 0.001$ and $***P < 0.001$). Data are mean ± s.d. (Extended Data Fig. 2). Non-pyrethroid barriers (P2 + NPB, P2 + OPB and UT + OPB) killed significantly more than untreated controls (Poisson regression generalized linear model; $n=44$, d.f. = 5, $Z=2.12$, $P=0.03$; $n=133$, d.f. = 5, $Z=7.61$, $P<0.001$; and $n=152$, d.f. = 5, $Z=8.32$, $P<0.001$, respectively). Personal protection (number of blood-fed mosquitoes prevented relative to untreated nets) was significantly higher with P2 + OPB (66%; negative binomial GLM; $n=109$, d.f. = 5, $Z=-2.649$, $P<0.01$); the reduction with UT + OPB was not significant (negative binomial GLM; $n=153$, d.f. = 5, $P=0.954$). Killing effects of test net versus unmodified P2 were higher with P2 + NPB (Poisson regression GLM; $n=44$, d.f. = 5, $Z=1.82$, $P=0.043$), P2 + OPB ($n=133$, d.f. = 5, $Z=5.91$; $P=0.008$) and UT + OPB ($n=152$, d.f. = 5, $Z=7.53$, $P=0.044$) (Extended Data Fig. 2). Treatments: UT, untreated unmodified bednet; P2, unmodified Permanet 2.0 bednet with 55 mg m^{-2} deltamethrin; P2 + P2B (Permanet 2.0 and P2 barrier); P2 + OPB, P2 and fenitrothion barrier (0.02 g m^{-2} in laboratory, 0.5 g m^{-2} in field. Treatments P2 + NPB (P2 net and non-pyrethroid barrier (3–5% indoxacarb and fenazaquin)) and UT + OPB (untreated bednet and fenitrothion-dipped barrier) were tested in the field only.

and increased exit rates compared with untreated bednets (Fig. 1e and Extended Data Fig. 2; GLM, $P<0.001$). All three non-pyrethroid barriers increased killing, particularly OPB; OPB on P2 bednets killed 28.8% more mosquitoes than unmodified P2 and increased personal protection by 23% and 66% relative to unmodified P2 ($P<0.001$) and untreated bednets ($P=0.008$), respectively. Remarkably, OPB on untreated bednets increased killing by nearly 34% over unmodified P2 ($P=0.008$), without significant loss in personal protection ($P=0.954$).

We investigated these encouraging field results using a mathematical model of malaria transmission dynamics to estimate the expected public health impact in the Cascades region if existing nets were replaced with barrier bednets. By necessity, the model simplifies malaria transmission as a series of mechanistic processes on the basis of assumptions about the probability of transmission[20–22]. Replacement with barrier bednets was modelled to determine how this would: (1) reduce the numbers of mosquitoes entering the house to feed; (2) reduce the feeding success of mosquitoes that enter houses and; (3) increase mosquito mortality relative to a scenario without nets. LLINs reduce malaria infections in mosquitoes and humans by affecting vector survival and feeding rates, the strength and duration of which are specific to each LLIN type and parameterized from experimental hut data[4,23]. There are limitations to the model's capacity to predict LLIN impact (see Supplementary Information), particularly when considering net durability, though this can be simulated by washing nets[4,20,24].

Hut trial data (Extended Data Fig. 2) were converted to summary estimates of the probability of mosquitoes being killed, repeating host-searching behaviour or successfully feeding on each attempt, for each tested net and barrier type (Extended Data Fig. 3), with reductions in prevalence continuing until the active ingredient had waned. Over three years following replacement of P2 nets with P2 + OPB nets, the mathematical model predicted relative reductions in clinical malaria incidence of 10.4% (95% confidence interval (CI) 0–34.47%), 13.3% (95% CI 0–37.12%) and 16.4% (95% CI 1.15–39.76%), at net coverage rates of 60%, 80% and 95%, respectively. With OPBs on untreated (UT) nets (UT + OPB), predicted impacts were even greater, at 13.8% (95% CI 0–37.30%), 18.4% (95% CI 4.62–40.71%) and 21.4% (95% CI 11.66–43.67%) for the same coverage levels. We compared this result with next-generation pyrethroid LLINs that are co-treated with piperonyl butoxide (PBO) to disable resistance mechanisms, which are recommended by the World Health Organization (WHO) where pyrethroid resistance is confirmed[23,25]. From equivalent values calculated using the association between experimental hut mortality and bioassay mortality data[4], and similar vector resistance (99% survival in WHO bioassays), PBO nets were predicted to reduce clinical incidence by 13.0% (95% CI 0–36.09%), 16.2% (95% CI 0–39.14%) and 18.4% (95% CI 0–41.66%) at similar respective coverage levels (Fig. 2b). These results, and the 12% reduction reported with another new pyrethroid LLIN (Olyset duo, containing pyriproxyfen) also in the Cascades region, are similar to the predictions for barrier bednets.

We investigated how barriers target mosquitoes using infrared video tracking to map and quantify mosquito–netting contact (a proxy for insecticide exposure) using defined behavioural modes[12,13]. Contact predominated at the LLIN roof in all treatments (60–95% of total contact; Extended Data Fig. 4), demonstrating that barriers did not alter this characteristic behaviour at standard LLINs[12,13]. Adding P2Bs increased overall activity compared with unmodified LLINs ($P<0.001$) (Fig. 3a,b), but not contact; P2Bs increased flight activity in behaviour modes with zero or minimal contact ($P<0.001$) (Fig. 3c,d and Extended Data Fig. 6).

OPB killed resistant mosquitoes at contact durations of 12.5, 6.6 and 9 s per mosquito for P2 + OPB (laboratory), P2 + OPB (hut trial) and UT + OPB (hut trial), respectively. Although these times are too brief to kill immediately, they are similar to the minimum levels of contact accrued by susceptible *A. gambiae* during the critical first 10 min of activity at pyrethroid LLINs (range 11–57 s per mosquito), after which few survive[12]. A lethal dose of entomopathogenic fungus can be acquired from treated netting in only 5 s[26].

Fenitrothion surface residues can be strongly repellent[19], whereas P2 netting (deltamethrin) exerts a far weaker effect[12]. Thus without deltamethrin (P2 + OPB versus UT + OPB; Fig. 3c) contact increased with the untreated surface (Fig. 3c; $P=0.048$), but not with the treated barrier (Fig. 3e). All barrier treatments resulted in higher activity but less contact overall (that is, visiting or swooping: 60–95% of total activity; Supplementary Video) compared with unmodified P2 LLINs (12– 27%) (Fig. 3e). The exception was the low dosage P2 + OPB (0.02 g m^{-2} fenitrothion), where low-contact

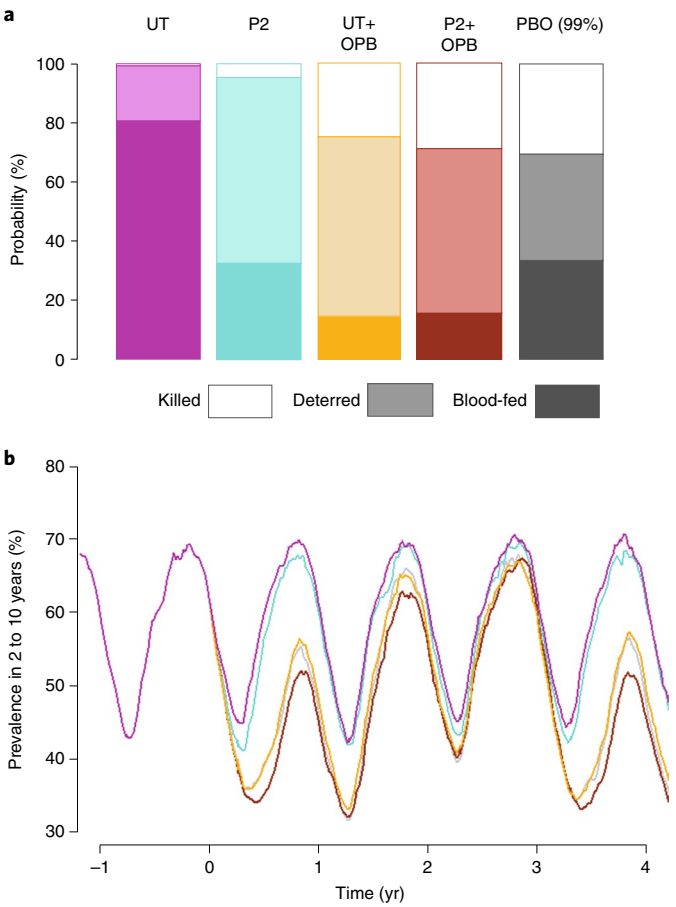

Fig. 2 | Summary of efficacy estimates of different bednet–barrier combinations and comparison with estimates for PBO bednets at high pyrethroid resistance. a, The probable outcome of a mosquito feeding attempt is determined for each net intervention: mosquitoes are either killed, deterred but return to feed again, or blood-feed successfully. Summary estimates were generated from hut trial data for UT and P2 with or without OPB (Extended Data Fig. 2). At a pyrethroid resistance level of 99%, the probability of an OPB bednet killing mosquitoes was comparable to that of the PBO nets, with fewer mosquitoes blood-feeding, regardless of whether the bednet was untreated (UT + OPB) or treated (P2 + OPB). **b**, The efficacy of these five bednet–barrier combinations drives the contrasting predicted reductions in prevalence among two- to ten-year-old children for the years following net-distribution campaigns at year zero and year three. Colour codes match the different bednet–barrier combinations in **a**. The model's parameters reflected the seasonality, entomology and endemicity of malaria in Cascades region, Burkina Faso.

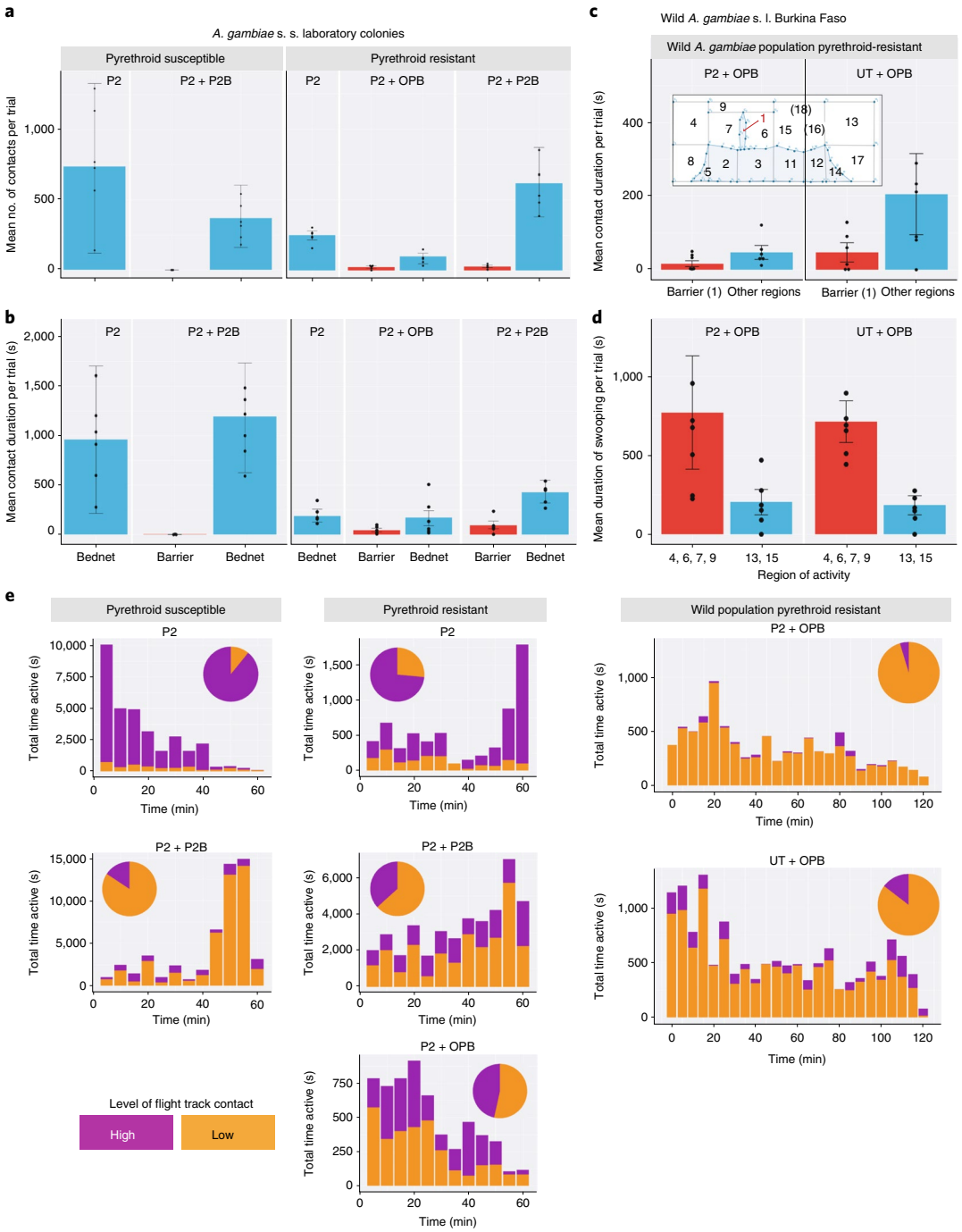

Fig. 3 | Behaviour at barrier bednets of *A. gambiae* s. l. laboratory colonies and wild population in Burkina Faso. a,**b**, Mean number (**a**) and duration (**b**) per test of flights contacting bednet or barrier for each treatment and mosquito laboratory strain. **c**,**d**, Mean duration of barrier or bednet contact in regions shown in the inset key (**c**) and mean total time spent in swooping mode (no net contact) (**d**) for wild mosquitoes. Inset: regions 16 and 18 correspond with 6 and 9, respectively, but activity in 15, 16 and 18 was pooled for analysis. Data are mean ± s.d.; number of independent samples is shown in Extended Data Figs. 4a,b, 5c and 6d. **e**, Activity at 5 min intervals during 60 min (laboratory) or 120 min (field) assays, showing mean durations of flight in high- (resting, bouncing) or low- (visiting, swooping) contact behaviour modes; pie charts show relative proportions of total duration per category. Treatment codes as in Fig. 1. Behaviour modes[12]: 'Swooping', tracks without net contact; 'Visiting', relatively lengthy flights with infrequent net contacts, trajectory turns of ≥ 80° and 0.4 s minimum interval between contacts; 'Bouncing', multiple rapid contact, intervals < 0.4 s or unbroken contact, never static; 'Resting', static > 0.75 seconds, velocity < 1.33 mm s^{-1}, unbroken net contact. Flight activity increased significantly with P2Bs (mean flight activity per trial; IS: 5,012 ± 1,975 s and 1,341.6 ± 741 s; Wilcoxon rank-sum test; $n = 25$, d.f. = 1, $W = 5422$, $P < 0.001$; IR, 577.2 ± 79 s and 464.4 ± 30 s; $n = 65$, d.f. = 1, $W = 23{,}017$, $P < 0.001$), but not OPBs (371.2 ± 45 s and 464.4 ± 30 s; $n = 65$, d.f. = 1, $W = 23{,}689.5$, $P = 0.155$, P2 and P2 + OPB respectively). Low contact activity increased with P2Bs in IR (*t*-test, $n = 65$, d.f. = 176, $t = 3.50$, $P < 0.001$) and IS (*t*-test, $n = 37$, d.f. = 73, $t = 2.519$, $P = 0.01$) mosquitoes, but not with OPBs ($P = 0.298$). Significantly more swooping activity occurred over the host's torso proximal to the barrier; *t*-test, $n = 5$, d.f. = 7.61, $t = 2.6976$, $P = 0.028$). Swooping (that is, zero contact) was significantly higher in both OPBs in the field (P2 + OPB, 79.5% of all flights; Pearson's χ^2 test; $n = 125$, d.f. = 3, $\chi^2 = 163.4$; UT + OPB, 64.2%; $n = 124$, d.f. = 3, $\chi^2 = 86.7$; $P < 0.001$). Netting contact duration (bednet plus barrier) was higher with OPBs on an untreated bednet than on a P2 bednet (*t*-test, $n = 5$, d.f. = 12, $t = -2.19$, $P = 0.048$).

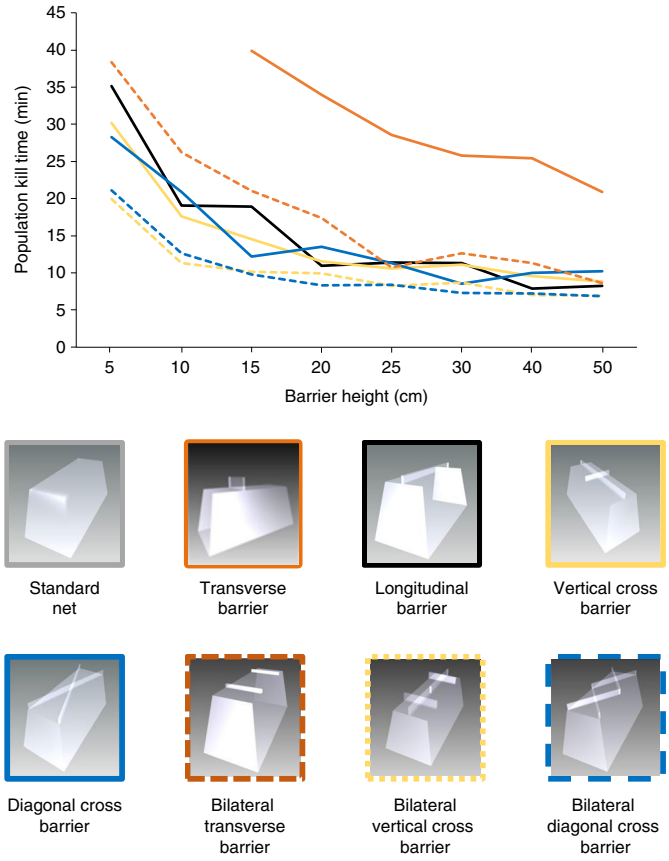

Fig. 4 | Comparing different barrier designs and heights by evaluating performance in silico. Population kill time (total time needed to achieve complete population death in minutes) for different barrier bednets when the bednet is untreated and insecticide is deployed only on the barrier. Values are weighted by surface area, using a transverse barrier of an equivalent height as reference. The eight designs are illustrated and include a standard (unmodified) bednet and the transverse barrier bednet tested in our experiments. Frame colour and pattern on the illustrations correspond with the lines on the graph, other than the standard net.

activity (53.3% total) was not significantly different from that with unmodified P2 ($P=0.298$), but markedly lower than with the higher dosages in the field trial (0.5 g m^{-2}; 85–95%; Fig. 3e). Increased flight without contact most probably combines a response to an insecticide's inherent repellent properties with the ability of *A. gambiae* to avoid net collisions[12] and may typify behaviour at barriers; thus careful selection of net and barrier treatments is required to maximize lethality.

Nevertheless, increased mosquito–netting contact directly increases insecticide exposure and we investigated whether alternative barrier designs and sizes could increase the frequency of contact. We used an agent-based, 3D spatiotemporal model of mosquitoes at an occupied LLIN in a virtual insectary to determine the effect of the 50 cm transverse barrier (Fig. 4). With untreated netting on bednet and barrier, transverse barriers showed only modestly increased contact duration over unmodified bednets (42.75 and 40.71 min, respectively; 25 mosquitoes, 1 h), whereas the complex bilateral diagonal cross barrier accrued 103.08 min of contact (Extended Data Fig. 7). When both bednet and barrier were treated with insecticide, contact and kill rates increased with greater barrier surface area and complexity (Extended Data Fig. 8). However, as larger complex barriers increase manufacturing costs, barrier area was weighted by cost per m^2, and the 30 cm longitudinal barrier performed almost as well as the 50 cm bilateral vertical cross (Extended Data Fig. 8). Encouraged by our semi-field trial result (Fig. 1e), we modelled performance where only barriers delivered insecticide, increasing the hypothetical dosage such that barrier-only contacts killed all mosquitoes within a 1 h simulation time window. Again, complex designs killed the population more rapidly, but performance levelled off at 20 cm barrier height. (Extended Data Fig. 7). Weighted by surface area however, and with the transverse barrier as reference, a simple 40 cm longitudinal barrier was nearly as effective as the more complex bilateral cross designs (Fig. 4) and was therefore a lead candidate for further development.

These results demonstrate that simple net barriers mounted on standard bednets can target *A. gambiae*. With appropriate insecticide, potentially including previously excluded classes, barriers significantly improved bednet performance, essentially restoring efficacy against pyrethroid-resistant mosquitoes. More effective barrier designs are possible, as are different combinations of net and barrier treatments, to maximize lethality and improve durability, with significant public health benefits[27].

We emphasize that we are not specifically proposing the use of organophosphate-treated barriers. We used fenitrothion primarily for its availability and efficacy against malaria vectors in west Africa[18,20], and expect comparable or better killing, repellency, net adherence and wash resistance from many insecticides or from non-insecticidal treatments[26,28]. Considerable industry and public sector investments in the past decade have delivered three new LLIN classes, all comprising a pyrethroid combined with a synergist[3], second insecticide[11] or insect-growth regulator[10]. If new or additional insecticides make LLINs more expensive, treating only barriers would reduce costs. The position of the barrier might enable relaxation of constraints on active ingredients for bednets (for example, knockdown rate or oral toxicity if ingested by infants), increasing the range of possible treatments. Furthermore, the potential to switch barrier treatments as resistance patterns shift would benefit resistance management and reduce insecticide waste. From manufacturing technology to correct nightly usage by communities in endemic settings, minimal change from existing LLIN processes and behaviours would be required to implement barrier bednets as an appropriate, safe and affordable method to extend LLIN lifespan in the fight against malaria.

Methods

Ethics review and research permission. All research methods were performed in accordance with approved guidelines for those procedures and written informed consent was obtained from all volunteers sleeping in experimental huts and laying under bednets during tracking experiments. The study was approved by the Research Ethics Committees at the Liverpool School of Tropical Medicine (LSTM) (Research Protocol 16–38, 11 October 2016, Liverpool) and Centre National de Recherche et de Formation sur le Paludisme (Deliberation no. 2016-9-097, 20 September 2016, Ouagadougou). No adverse effects of treatment or mosquito-borne infections were reported by volunteers during the course of the study.

Bednet and barrier materials. In all tests, rectangular bednets measuring 2 m × 0.9 m × 1.5 m tall were used as the standard bednet. To facilitate image capture, the net roof was tilted on its long axis when facing the cameras to ensure activity on the roof was visible (Fig. 1 and 2b,c). Hence, the net height was 0.93 m near the camera and 1.19 m at the rear. Pyrethroid-treated nets were Permanet 2.0 (75 denier polyester net impregnated with deltamethrin at 55 mg m^{-2} (Vestergaard)). New LLINs were hung for four weeks before use and tested for insecticidal activity using the standard WHO cone test and two laboratory strains ($n=4$ repeats per mosquito strain–LLIN combination; see Evaluation of barrier net performance in the laboratory).

The barrier comprised a vertical net panel positioned transversely on the net roof (Fig. 1a), one of the simplest barrier designs[16]. The barrier was 0.9 m wide (extending edge-to-edge across the LLIN) and was fitted above the tilted roof of the rectangular LLIN. It measured 0.8 m high (front) and 0.54 m (rear) to ensure the top edge was horizontal at a total height of 1.9 m from the floor. The lower edge was pinned to the roof of the net slightly off-centre, at 0.8 m from the head end (that is, 0.2 m from the midpoint) (Fig. 2b,c). To facilitate video tracking, creases, sagging and wrinkles were minimized by suspending the barrier from the ceiling using string and supporting the net and barrier edges with 5 mm carbon fibre rods.

Insecticidal barrier panels (0.6 m^2) were cut from new Permanet 2.0 LLINs or untreated polyester netting treated with the organophosphate fenitrothion (to make the OPB). We selected this low fenitrothion concentration (100 times less than that used in IRS) to minimize any potential repellent effects of organophosphate residues. OPBs (0.02 g m^{-2}) were prepared by immersing eight pre-cut untreated net barriers (plus 0.2 m^2 fragment to ensure all liquid was absorbed) into a 224 ml aqueous emulsion containing 0.1 g of fenitrothion (Greyhound Chromatography and Allied Chemicals). Unmodified Permanet 2.0 LLINs were used for comparison. Fresh barriers were used for each test repeat (six IR and five for IS).

Evaluation of barrier net performance in the laboratory. Initial tests were conducted on human-occupied bednets in a dedicated insectary in the UK (5.6 m × 3.6 m × 2.3 m high; climate controlled at 27 ± 2 °C, 70 ± 10% relative humidity), using *A. gambiae* s. l. strains from LSTM colonies of Kisumu (*A. gambiae* senso stricto (s. s.); IS, $n = 9$) or Tiassalé (*A. gambiae* s. s. and *Anopheles coluzzii* mix; resistant to pyrethroids and the majority of other insecticides used in public health, IR, $n = 17$ (ref. [25]). Three- to five-day-old unfed adult female mosquitoes (25 per experiment) were deprived of sugar and water for 4 h before transfer to the experimental room to acclimatize (1 h) before testing. All tests were conducted within 1–3 hours of the start of scotophase. Human volunteers lay uncovered on a fresh sheet over a 2 m × 0.9 m mattress (0.18 m thick; surface at 0.48 m above the floor). Mosquitoes were recorded using a video-tracking system of paired identical camera setups (one each for the upper or lower body of a supine human), each comprising a single infrared LED (850 mm wavelength, 1,000 mA minimum; M850L2, Thorlabs) aligned with a pair of Fresnel lenses (mounted either side of the bed, with a 43 cm gap between the lens and mattress on each side) and monochrome camera with 12.5 mm imaging lens (Baumer HXC40NIR, Camera Link, 4 megapixels; Lambda Photometrics). Video was recorded at 50 frames s^{-1} using StreamPix software (www.norpix.com), and data were saved as .seq files. Thirty minutes after the volunteer entered the bed, recording was started and mosquitoes were released from a paper cup at a height of 2 m, 1.4 m from the net. Activity was recorded for 60 min.

Bioassays of mosquito behaviour at human-occupied bednets. Eighteen human volunteers, 9 males and 9 females of different ethnicities, aged between 22 and 49, were recruited from staff and students at LSTM. Volunteers were clothed and barefoot and lay on their backs, as immobile as comfort permitted during the 1 h test. All were asked to eschew scented toiletries when testing. The majority were tested with both barrier-modified (P2B or OPB) and unmodified P2 nets on different days, with an average interval of 41 d between their tests. After each 1 h test, the number of live and dead mosquitoes in the room was recorded. Living mosquitoes were maintained with sugar and water and mortality was recorded at 1, 24 and 48 h.

Video tracking mosquitoes in the laboratory. Tracking individual mosquitoes or determining the number of responders among the 25 released was not possible as it was not possible to view the entire room. Each flight track, from entry to exit of the field of view, was analysed individually using segmentation and tracking algorithms through bespoke software in the Matlab framework (Mathworks). Data were extracted and interpreted to quantify the number and duration of contacts with different bednet regions and flight activity in spatial regions around the barrier. Mosquito flight tracks were categorized in four behaviour modes, using previously reported quantification algorithms[13,14]: 'Swooping', flight tracks without net contact; 'Visiting', extended flight tracks with infrequent net contacts; 'Bouncing', multiple rapid contacts with the bednet surface, including short flights between contacts, 'walking' and 'probing' behaviour; and 'Resting', static or slow movement. The field of view recorded by the cameras was divided into specific regions on the surface of barrier and bednet or in the airspace surrounding it. The limits of each region were delineated accurately to fit every barrier–bednet assembly, as shown in Figs. 2a and 3a. The number and duration of events in each behaviour mode were determined for every net and spatial region. When a single track included more than one behaviour mode, the time spent in each mode was recorded separately.

Quantifying mosquito contact at barriers and bednet regions. Bednet contact comprised all flight tracks in bouncing, visiting and resting behaviour modes. The number and duration of contacts were calculated for each test as total values and mean values per trial. Tracking individual mosquitoes throughout an entire assay is not possible with this system as it was not possible to view the entire room, and plausible estimates of minimum and maximum values of net contact per individual were calculated. The minimum value was total contact duration divided by the total number of released mosquitoes ($n = 25$); maximum net contact time per individual was calculated as the total contact duration divided by the maximum number of mosquitoes observed simultaneously ($n < 4$).

Evaluation of barrier bednets in the field. Between July and October 2017, barrier nets were tested against adult female mosquitoes morphologically identified as *A. gambiae* complex reared from wild larvae collected at Tengrela (10° 40′ N, 4° 50′ W) near Banfora, Burkina Faso. Species identification[29], conducted on a random selection of adult females tested, identified 87.41% ($n = 437$) of samples to be *A. coluzzii* Coetzee and Wilkerson, which have previously been found to be highly resistant to pyrethroids at this site[30].

Barrier bednets were assembled as described for the laboratory study, with the exception of OPB. These fenitrothion-dipped barriers were prepared by immersing pre-cut netting (0.65 m^2 or 0.8 m^2) in a solution of fenitrothion, prepared by adding 7.3 ml or 9 ml of fenitrothion stock solution (0.044 g ml^{-1} in acetone; AK Scientific) to 22 ml or 27 ml acetone, giving 29.3 ml or 36 ml of 0.01 g fenitrothion ml^{-1} acetone, respectively. At an absorbency rate of 45 ml m^{-2}, this deposited 0.5 g m^{-2} on the netting surface, equivalent to 25% of the target dose for IRS treatment. We selected this concentration, 25 times higher than in the initial laboratory experiment, on the basis of the absence of evidence for repellency in the initial laboratory experiments, and out of concern that durability of dipped nets at lower concentrations might be compromised in harsher field conditions.

Barriers (0.5 m high × 1.3 to 1.6 m) were placed across the full roof width of standard rectangular Permanet 2.0 (1.6 × 1.8 × 1.5 m) or untreated polyester nets (1.3 × 1.5 × 1.8 m), at an off-centre position, 0.7 m from the sleeper's head and 1.1 m from the foot of the net (Fig. 3a). Unlike the laboratory study, the bednet was not tilted to aid video tracking.

Hut trial design and protocol. The trial followed WHO guidelines[31] and was performed in six WHO standard cement huts of the West African design (3.5 × 2 × 2 m high) that had been used previously for evaluation of vector control tools including PBO nets[32]. The cement walls stand on concrete platforms with water-filled moats to minimize entry by ants and other scavengers. The roof is corrugated metal with a polythene sheet ceiling. Window and veranda traps were open during tests. To permit mosquito entry, holes were cut in all bednets as defined in the WHO Pesticide Evaluation Scheme guidelines: six 4 cm × 4 cm holes, two on the long sides and one on the short sides, were cut in each net. The experiment comprised six treatment arms:

1. Untreated control bednet (UT): untreated polyester netting of similar denier and mesh size as LLINs used in other treatments, no insecticidal properties and no barrier
2. Permanet 2.0 LLIN (P2): a WHO Pesticide Evaluation Scheme-recommended standard-size double LLIN (1.6 m × 1.8 m × 1.5 m) treated with deltamethrin at 55 mg m^{-2} with no barrier
3. Permanet 2.0 LLIN with Permanet 2.0 barrier (P2 + P2B): standard LLIN with a barrier element of identical Permanet 2.0 netting
4. Permanet 2.0 LLIN with non-pyrethroid insecticide barrier (P2 + NPB): standard P2 LLIN with an added barrier element treated with a combination of two non-pyrethroid insecticides: indoxacarb (3–5% oxadiazine) and fenazaquin (3–5% quinazoline)
5. Permanet 2.0 LLIN with fenitrothion barrier (P2 + OPB): standard LLIN with an added barrier element treated with the organophosphate fenitrothion, at a concentration of 0.5 g m^{-2}, equivalent to 25% of the level applied in IRS
6. Untreated net with OP barrier (UT + OPB): untreated polyester bednet with an added barrier element treated with 0.5 g m^{-2} of fenitrothion

To complete a full rotation for this comparison of six treatment arms, 36 experimental nights were required. Treatments were rotated between the huts weekly and the sleepers were allocated to different huts on each night (see Supplementary Information, Hut trial rotation plan). A new set of treated and untreated nets was prepared and used in each week of the trial. Before use, all manufactured LLINs and untreated control nets for use in any particular week were removed from their packaging and aired for seven days. OPB nets were dipped in fenitrothion as described above and aired for three days before use. To ensure the dipping process was successful, barrier samples were bioassayed before and after the trial (Supplementary Text). Human volunteers were recruited from the local community and each was used once with each treatment. After the clothed, barefoot volunteer had entered the bed, research staff checked the net to ensure it was secure. Sleepers remained under the net between 20:00 and 05:00. Seated at a distance of 10 m or more, a supervisor was on duty throughout the trial to ensure behaviour complied with the protocol and to assist the volunteers if required. At 05:00, volunteers collected mosquitoes inside their nets (using glass universal tubes with cotton wool plugs) before exiting the net and closing the veranda traps to prevent mosquito movement between the veranda and hut. Mosquitoes were then collected from the main hut and veranda before research staff entered huts to check for remaining mosquitoes.

Retrieved mosquitoes were sorted by treatment and hut, location (inside net, in hut or in veranda), alive or dead, sex and abdominal status (blood fed, semi-blood fed, unfed, gravid or semi-gravid). Live *A. gambiae* s. l. were sorted by hut and held in paper cups (5 mosquitoes per 250 ml cup), separated by feeding status and location, provided with 10% sugar solution on cotton wool pads and retained in a nearby hut until natural death. Mortality was assessed within 2 h of the test ending and at 24 h intervals thereafter until no mosquitoes remained alive. We quantified and compared a range of outcomes incorporating the standard parameters recommended by the WHO for evaluating LLINs[31]:

- Deterrence: the reduction in hut entry relative to control huts (untreated nets)
- Exophily or repellency: the proportion of mosquitoes found in the veranda traps

- Blood-feeding inhibition: the reduction in blood-feeding compared with the control huts (untreated nets)
- Immediate and delayed mortality: the proportions of mosquitoes entering the hut that are found dead in the morning (immediate mortality) or after being caught alive and held for 48 h with access to a sugar solution (delayed mortality)

Since deterrence and blood-feeding inhibition are indicators of personal protection, the personal protection effect of a treated net was calculated as:

$$\text{Personal protection (\%)} = \frac{100\times(B_u - B_t)}{B_u}$$

where B_u is the total number blood-fed mosquitoes in huts with untreated nets and B_t is the total number of blood-fed mosquitoes in huts with treated nets.

Mortality (immediate and delayed) is an indicator of the potential mass killing effect of LLIN use; that is, a reduction in the density and/or longevity of mosquitoes in areas with high net coverage, resulting in community-wide protection that also benefits non-users of LLINs. The potential killing effect of a treated net was estimated from:

$$\text{Mortality} = \frac{100\times(K_t - K_u)}{T_u}$$

where K_t is the number of mosquitoes killed in huts with treated nets, K_u is the number of mosquitoes killed in huts with untreated nets, and T_u is the total number of mosquitoes collected from huts with untreated nets.

Predicting barrier-bednet effectiveness for malaria control in a highly endemic context. An individual-based transmission dynamics model of malaria[20,22,33,34] was used to explore the public health impact of nets with organophosphate barrier panels fitted to the roof section. This model tracks *P. falciparum* infection in people and mosquitoes. Susceptible people are exposed to infectious mosquito bites at a rate dependent on local mosquito density and infectivity. Mosquito dynamics describe the effects of mosquito control and the resulting decline in egg laying[22].

The specific seasonal profiles[35] and historic scale-up of IRS and LLIN interventions from 2000 to 2015 were matched for the Cascades administration region in Burkina Faso (Malaria Atlas Project[1], as per ref. [36]). The mosquito density was adjusted to capture the underlying transmission intensity, which is high in the Cascades region. We used 60% prevalence in 2 to 10-year-old children at peak transmission as the baseline prevalence in this exercise. For all model simulations, the same baseline parameters were applied, but the parameters that determine net efficacy were estimated from the experimental hut data (Extended Data Figs. 2 and 3). Uncertainty in model predictions was generated by running the model 50 times with randomly drawn estimates from the posterior distribution of each model parameter, while fixing net-parameter estimates as recorded in the experimental hut trials. Next-generation nets are being developed to mitigate the potential lost impact of indoor interventions in the context of pyrethroid resistance. PBO synergist nets are the first next-generation nets to reach the market. PBO inhibits specific metabolic enzymes in mosquitoes that can detoxify pyrethroids, thereby extending the active life the insecticide in LLINs. We investigated how well barrier nets might perform relative to these PBO nets. Given that the average mortality in experimental huts for standard nets (unmodified Permanet 2.0) during the 8-week monitoring period was just 7.4%, and the relationship between discriminatory dose bioassay and experimental hut mortality determines that this corresponds to 99% resistance[4], we compared nets at this level of pyrethroid resistance. Extended Data Fig. 2 outlines the parameter changes made in the model to represent the predicted impact of organophosphate panels on prevalence in two- to ten-year-old children and all clinical cases in the Cascades administrative region in Burkina Faso. In the absence of wash data (used to simulate the natural wearing of the active ingredient of nets and to determine net durability)[4,23], we assumed a conservative estimate for the half-life of barrier nets based on maximum mortality estimates from the experimental hut data. This corresponds to approximately six months for the two barrier nets tested (P2 + OPB and UT + OPB). We compared the effect of PBO nets and barrier nets (P2 + OPB and UT + OPB) relative to P2 nets.

Video tracking mosquito flight in Burkina Faso. A dedicated experimental hut was constructed adjacent to the WHO huts at Tengrela to accommodate a video-tracking system based on a previously described system[37]. The room measured 6 m × 4 m in area and 3 m high, with a corrugated steel roof. Steel-shuttered windows and eaves were also present on two walls that were closed during recording to limit the movement of mosquitoes, airflow and external light sources. Conditions inside the hut were similar to ambient, with a mean (± s.d.) temperature and humidity during recording of 28 °C (± 3.1 °C) and mean (± s.d.) relative humidity of 75% (± 12.5%). Thirty minutes before tests, the volunteer entered the bednet, the mosquitoes were placed in a paper cup resting on the lip of the eave, 2 m above the ground, and the room was closed.
A section of eave screen was cut to enable a researcher to release the mosquitoes by uncovering and emptying the cup at the start of the trial before the eave screen and shutter were closed. Unfed females, insectary-reared from larvae collected at Tengrela and aged 4–7 days post-eclosion, were used in all tests. Mosquitoes were transferred to the experimental hut within 30 min of tests to acclimatize to the hut interior environment. All tests were run during the night, starting at or shortly after 19:30.

Five of each bednet–barrier combination (that is, P2 + OPB and UT + OPB) that had previously been used in the hut trial over six nights were used. Human volunteers lay on a 2 m × 0.88 m sleeping mat, with the bednet evenly tucked under by one of the researchers before filming. The recording period lasted 2 h from the time of mosquito release. Throughout, a researcher monitored the recording system from an adjacent control room. Before and after recording, mosquitoes in the room were collected with aspirators and the floor was swept to eliminate or recover any dead or knocked-down mosquitoes. The collected mosquitoes were maintained under ambient conditions in a separate hut nearby, were provided sucrose solution ad libitum and assessed (dead, knocked-down or alive) immediately at collection and 1, 24 and 48 h later. Video was recorded at 50 frames s^{-1} using StreamPix software (www.norpix.com) and saved as .seq files. Initial analysis was performed using segmentation and tracking algorithms through bespoke software in the Matlab framework (Mathworks) using these large files (>200 Gb video files). Following this, the video files were compressed with bespoke software using the .mp4 container and a dedicated video card (<5 Gb). This compression was designed to be compatible with the segmentation algorithms, allowing subsequent analysis to be performed on the compressed or re-rendered video files with negligible loss of information. All recorded video was then stored on multiple, redundant external drives.

Optimization of barrier size and shape. We developed an agent-based 3D spatiotemporal model of mosquito behaviour at a human-occupied LLIN in a virtual insectary to compare designs for optimizing barrier-net performance. Indoor vector control testing system (InVeCTS) is an attempt to create a virtual environment in which to assess mosquito populations' interactions with their host and their environment. This is a multi-agent approach using a fine-grained spatial representation in which a mosquito population can interact with a human host over time. Mosquito flight occurs in real time and all mosquito flight paths and interactions with the environment are recorded for subsequent analysis.
A population of mobile virtual mosquito insects are created. These individuals fly in a continuous 3D space representation inside a discretized spatial arena representing an insectary or hut containing a bednet and human host. For the experiments presented in this document an arena of size 5.6 × 3.6 × 2.3 m was used, corresponding to the experimental insectary at LSTM used previously[10,11]. Barrier bednets were designed from 3D triangular meshes, building on standard 'reference' simple unmodified bednet design (Fig. 4). The standard bednet design measured 2 m long x 0.9 m wide (at its widest point on the floor) and 0.8 m high. Barrier bednets of different designs and heights (5, 10, 15, 20, 25, 30, 40 and 50 cm) were assessed. The bednets were placed in the centre of a virtual insectary (5.6 m long × 3.6 m wide × 2.3 m high) and a population of 25 virtual mosquitoes were released from a wall-mounted position halfway along the longest axis (2.8 m) at a height of 2 m. A human-bait stimulus profile was centred in the bednet design with the head region furthest away from the release location. Each experiment was run for the equivalent of 1 h and results were recorded for further analysis. Five runs were performed at each barrier height. Experiments were performed under two treatment conditions. The untreated net condition was used to assess the contact time of the different net designs. The treated net condition was used to assess the effectiveness of the designs in reducing the activity of the virtual mosquito population.

Statistical analyses. Random effects generalized linear models were used for analyses of activity time, behavioural modes, region preferences, tortuosity, number of tracks, activity decay and effects of treatment type. Non-normality of data was tested for using Shapiro–Wilk tests. Welch's independent two-sample unequal variances *t*-tests were used. For all tests, an α-threshold of 0.05 was used. Statistical analyses were performed using R v.2.15.1 (R Development Core Team, 2012). In the hut trial, analysis was performed to assess the performance of the barrier bednet relative to the untreated control and standard PermaNet 2.0, with the extra arms allowing for a description of the relative benefits of the different insecticide treatments. The number of mosquitoes found inside the huts, blood-feeding rates and mortality were compared using Poisson regression generalized linear models or negative binomial generalized linear models to account for over-dispersion. In modelling barrier design and height, all statistical analyses were performed using R v.3.1.2 (http://www.R-project.org/). Comparisons of mortality and activity levels were performed on the basis of Welch's two-sample (unequal variances) *t*-test; when the assumption of normality was not met, they were based on a Shapiro–Wilk test, and then a one-sided Wilcoxon signed-rank test was used. Generalized linear models with Poisson distribution were used to compare hut trial outcomes, except in cases of over-dispersion, where negative binomial GLMs were used. For all tests, an α-threshold of 0.05 was used. Unless stated otherwise, data are reported as arithmetic means and associated standard deviation.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The hut trial dataset generated during the current study is available at Dryad Digital Repository (https://doi.org/10.5061/dryad.hqbzkh1b7). All data analysed during this study are available as described in the paper. All other data supporting the findings of this study are available within the article and its Supplementary Information files or are available from the authors on reasonable request.

Code availability

Data handling scripts and video segmentation and tracking software are available from the authors on reasonable request.

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Author contributions

P.J.M. conceived the barrier bednet and designed the study. G.P.D.M. collected most of the experimental data with assistance from N.L., K.H.T., S.N., W.M.G. and G.M.F. D.T. and C.E.T. designed the video-tracking capture and analysis systems, which V.V. and J.E.A.P. built. T.S.C. and E.S.-S. performed malaria impact predictions. J.J. performed barrier design simulations. G.P.D.M. performed statistical analyses with P.J.M. G.P.D.M. and P.J.M. interpreted results with H.R. and G.M.F. P.J.M. wrote the paper with input from G.P.D.M., G.M.F. and other authors. All authors approved the submitted version.

Competing interests

A patent application (WO2015063455A1) that names P.J.M. was filed by LSTM in respect of the barrier bednet, initially in the UK (7 May 2015), but has now entered the Patent Cooperation Treaty process. LSTM has a research agreement with Vestergaard, which provided LLIN materials but had no role in study design, data collection, analysis and interpretation, report writing or publishing. The authors declare no other competing interests.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41564-019-0607-2.

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Date	Insecticide	Knockdown at 1hr (%)	Mortality at 24hr (%)	No. mosquitoes tested
Aug 2016	Pyrethroid control	0	0	23
	Deltamethrin 0.05%	14.89	9.57	94
	Organophosphate control	0	5.26	19
	Fenitrothion 1%	0	94.44	90
Jun 2017	Pyrethroid control	--	0	57
	Deltamethrin 0.05%	--	35.67	157
Oct 2017	Organophosphate control	0	0	25
	Fenitrothion 1%	98.98	100	98
Mar 2018	Pyrethroid control	--	1.61	62
	Deltamethrin 0.05%	--	17.39	69
Sep 2018	Pyrethroid control	--	0	311
	Deltamethrin 0.05%	--	0	125

Extended Data Fig. 1 | Insecticide susceptibility status of the wild *Anopheles gambiae s. l.* population at Tengrela, Banfora in Cascades region of Burkina Faso. Adult female mosquitoes were tested using the WHO tube test. Mortality rates of less than 95% are indicative of resistance.

Outcome	UT	P2	P2 + P2B	P2 + NPB	P2 + OPB	UT + OPB
Total no. caught	522	368	381	408	341	334
Mean no. caught per night	14.5	10.2	10.6	11.3	9.5	9.3
% Deterrence	-	29.5	27.1	21.8	34.6**	36.1**
Total no. bloodfed	320	142	152	142	109	153
Mean no. bloodfed per night	8.8	3.9	4.2	3.9	3.0	4.2
Personal protection (%)	-	55.6	52.5	55.6	65.9**	52.1
Number dead on collection	8	27	26	44	133	152
Killing effect (%)	-	3.6	3.4	6.8*	23.9***	27.6***
Mean survival post collection (days)	12.0	11.6	11.3	11.1	11.4	10.6
% Exiting	23.4***	63.1	36.0	34.8	56.5	20.8
% collected inside net	31.6*	36.5	24.3	25.7	38.3	20.2
Killing effect (%) *vs.* P2	-	-	-0.27	4.61*	28.8**	33.96**
Personal protection (%) *vs.* P2	-	-	-7.04	0	23.23*	-7.74

Extended Data Fig. 2 | Complete results summary of the hut trial in Tengrela, Cascades Region, Burkina Faso. Treatment codes: UT (Unmodified untreated polyester bednet), P2 (unmodified Permanet 2.0), P2+P2B (Permanet 2.0 bednet and barrier of P2.0); P2+NPB (P2 net and non-pyrethroid mixture [indoxacarb/ fenazaquin, 3-5%]); P2+OPB (P2 and fenitrothion-dipped barrier, 0.5g/m^2); UT+OPB (untreated bednet and fenitrothion-dipped barrier). Outcomes are defined in Methods. Asterisks denote significant differences between treatments (P=0.05-0.01*; 0.01-0.001**;<0.001***). All comparisons *vs.* UT, unless stated otherwise. Percentage Deterrence: Poisson regression GLM; P2+OPB, n=6, df=5, Z=3.02 P=0.02; UNT+OPB, n=6, df=5, Z=2.21, P= 0.03. Personal protection: Negative Binomial GLM; P2+OPB, n=109, df=5, Z=−2.649, P=0.008. Killing effect: Poisson regression GLM; P2+NPB, n=44, df=5, Z= 2.127, P= 0.03; P2+OPB, n=133, df=5, Z= 7.612, P<0.001; UT+OPB, n=152, df=5, Z=8.320, P<0.001. Percentage exiting: Negative Binomial GLM; UT, n=121, df=5, Z= −5.805 P<0.001. Percentage collected inside net: Negative Binomial GLM; UT, n=163, df=5, Z=−2.047 P<0.0407. Killing effect vs. unmodified P2: Poisson regression GLM; P2+NPB, n=44, df=5, Z= 1.921, P= 0.04; P2+OPB, n=133, df=5, Z= 2.644, P=0.008; UT+OPB, n=152, df=5, Z=5.322, P=0.005. Personal protection vs. unmodified P2: Negative Binomial GLM; P2+OPB, n=109, df=5, Z=1.61, P=0.03.

Parameters	Parameter estimates for Tengrela, Cascades region simulation						
Baseline prevalence	60% (at peak transmission season)						
Assumed proportion *An. gambiae s.s.*	0.577						
Assumed proportion *An. funestus s.s.*	0.223						
Assumed proportion *An. arabiensis*	0.200						
Net coverage in 2015	95.7%						
Parameterization data from (*5*) *Estimated*				**Parameterization from experimental hut data** *Observed*			
	Permanet 2.0 (P2)		**PBO-net**	**Untreated net**	**P2 nets**	**P2+OPB**	**UT+OPB**
Assumed level of pyrethroid resistance	0%	99%	99%	-	-	-	-
Probability of repeating on encounter with net, r_{N0}	0.310	0.373	0.415	0.187	0.629	0.608	0.556
Probability of dying upon encounter with net, d_{N0}	0.510	0.140	0.203	0.007	0.047	0.247	0.288
Net half-life (years), γ_N	2.640	0.355	0.551	0.222	0.253	0.551	0.644

Extended Data Fig. 3 | Transmission model parameter estimates used to test the effect of organophosphate panels on bednets in the Cascades administration region of Burkina Faso. All other parameters match those previously reported *(21,29,30,33)*. Parameter estimates are noted for: i) standard nets (*for example*. Permanet 2.0) working optimally; ii) standard nets working as predicted for the resistance scenario where 99% of mosquitoes survive during a discriminatory dose WHO bioassay test in the presence of pyrethroid insecticides; iii) Permanet 2.0 with an organophosphate barrier, and; iv) an untreated net with an organophosphate barrier.

Net treatment	Net Region	Number of net contacts			Duration of contact (s)		
		Total	mean/test (SD)	% of all contact	Total	mean/test (SD)	% of all contact
P2+OPB	Barrier	40	10 (4.1)	10.9	78.8	19.7 (19.8)	26
	Net	329	82.3 (70.5)	89.1	224.1	56 (47.5)	74
UT+OPB	Barrier	174	43.5 (46.8)	6.3	220.7	55.2 (64.7)	17.7
	Net	2607	651.6 (915.6)	93.7	1024.9	256.3 (301.6)	82.3

Extended Data Fig. 4 | Frequency and duration of mosquito contact with bednets and barriers in the laboratory. The number, location and duration of mosquito contact at unmodified and barrier bednets; data from video recordings of the bioassays in Fig. 1b (25 female mosquitoes, 1hr). The bednet roof was the primary contact location in all treatments (*t*-test: IS, P=0.45; IR, P=0.19; IR/OPB, P=0.93). Contact with treated netting (bednet+barrier) was similar between treatments for IS (mean±SD contact/ trial: 959±1032s and 1099±1035s; *t*-test, P=0.839) and IR mosquitoes (185±144.8 vs. 519±455.7, *t*-test, P=0.478; Fig. 2g); and between P2 and P2+OPB (185.0±144.8 vs. 212.8±239.1, *t*-test, P=0.309) or number (249.4±7.2 and 123.5±13; *t*-test, P=0.056).

Net treatment	Net Region	Number of net contacts			Duration of contact (s)		
		Total	mean/test (SD)	% of all contact	Total	mean/test (SD)	% of all contact
P2+OPB	Barrier	40	10 (4.1)	10.9	78.8	19.7 (19.8)	26
	Net	329	82.3 (70.5)	89.1	224.1	56 (47.5)	74
UT+OPB	Barrier	174	43.5 (46.8)	6.3	220.7	55.2 (64.7)	17.7
	Net	2607	651.6 (915.6)	93.7	1024.9	256.3 (301.6)	82.3

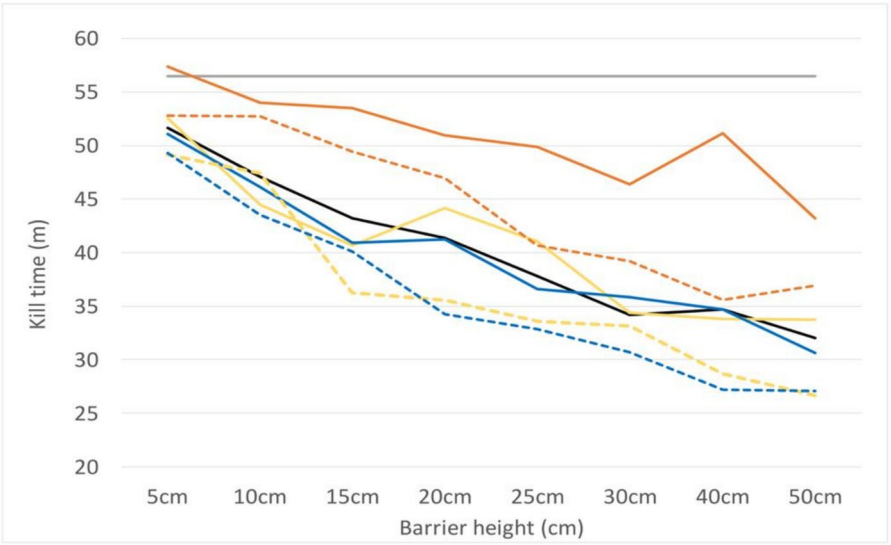
Extended Data Fig. 5 | Frequency and duration of contact at bednets with OP- treated barriers by wild *Anopheles coluzzii* in Banfora, Burkina Faso. The number, location and duration of mosquito contact on barrier bednets recorded during tests (Fig. 1b). Data refer to 2hr video recordings, with 25 female mosquitoes released. Comparisons of number or duration of contacts between treatments were not significant for the bednet or barrier, based on *t*-tests (normality tested using Shapiro–Wilk test). When bednet and barrier contacts were combined, duration was significantly higher in UT+OPB (*t*-test; n=5, df=12, $t = -2.19$, P=0.048).

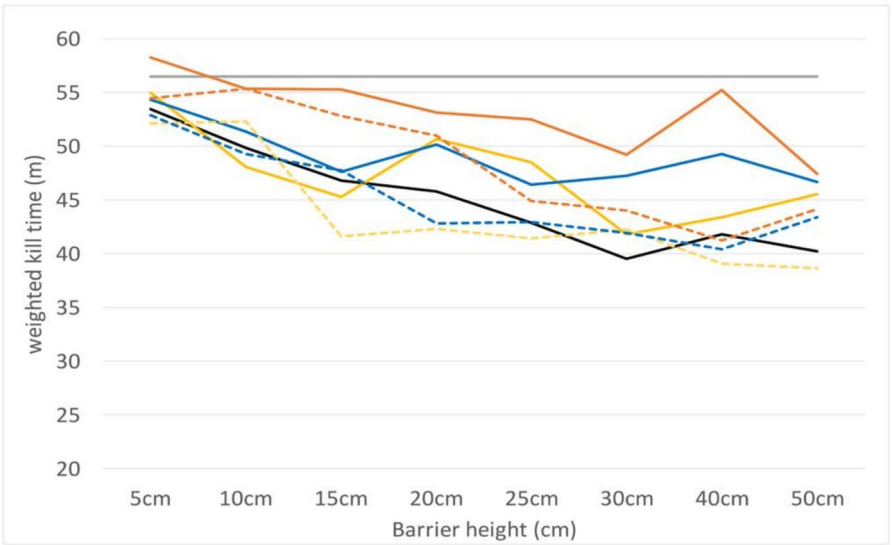
		Low contact		High contact	
Mosquito strain	**Treatment**	**Swooping**	**Visiting**	**Bouncing**	**Resting**
IS	P2	11.2 (0-36.7)	31.6 (0-117.8)	292.6 (0-1350.5)	11.2 (0-303.3)
IS	P2+P2B	1013.3 (0-3755.5)	194.8 (0-639.7)	45.0 (0-113.2)	1013.3 (0-1183.25)
IR	P2	13.9 (0-27.8)	22.2 (0-46.6)	78.6 (0-222.0)	13.9 (0-64.9)
IR	P2+P2B	20.8 (0-55.4)	45.7 (0-96.0)	77.7 (0-167.7)	20.8 (0-64.2)
IR	P2+OPB	16.5 (0-34.1)	25.7 (0-61.7)	25.5 (0-79.4)	16.5 (0-44.9)
	P2+OPB	62.1 (0-138.0)	12.6 (0-36.6)	2.6 (0-13.5)	62.1 (57.8-66.47)
Wild	UN+OPB	82.7 (0-173.1)	23.7 (0-64.7)	12.9 (0-39.1)	82.7 (68.2-96.6)

Extended Data Fig. 6 | Behaviour modes of Anopheles gambiae at bednets with or without barriers. Duration of activity in each behaviour mode; data from video recording of activity of 25 adult female *Anopheles gambiae s.l.* over 60min (pyrethroid susceptible [IS] or resistant [IR] strains; top) or 120min (wild Burkina Faso population, bottom). Total duration of all tracks classed in each behaviour mode (geometric mean ±SD, seconds). Since multiple mosquitoes were often active simultaneously in the field of view, the total activity times could exceed 60 minutes. Behaviour modes, defined previously[12], were as follows: Swooping - tracks that did not contact netting; Visiting - tracks of relatively long flight period interspersed with infrequent bednet contacts, characterized by sharp trajectory turns of ≥80° and 0.4s minimum interval between multiple contacts; Bouncing - tracks of multiple rapid netting contact, at intervals of less than 0.4s, including short flights between contacts, or unbroken contact without being static, *for example*. 'walking' and 'probing'; Resting - static for at least 0.75 seconds, or velocity less than 1.33mm/s, unbroken contact with net.

Barrier Height (cm)	Standard unmodified bednet	T Barrier	L Barrier	V Cross	D Cross	Bi T Barrier	Bi V Cross	Bi D Cross
A. Mean total mosquito population contact time (min)								
0	40.71	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5	N/A	39.98	44.77	44.36	49.60	43.46	44.36	51.58
10		41.06	49.86	51.31	53.23	47.69	51.31	58.15
15		40.79	56.13	56.83	58.04	51.97	56.83	66.98
20		41.45	60.33	62.00	62.89	55.64	62.00	73.09
25		41.63	64.65	66.49	65.74	57.87	66.49	80.23
30		42.23	68.52	69.45	67.41	61.86	69.45	84.06
40		42.50	72.61	73.94	73.09	66.35	73.94	94.29
50		42.75	77.01	78.11	75.73	69.96	78.11	103.08
B. Mean time to kill the entire mosquito population, when both bednet and barrier are insecticide-treated (min)								
0	56.50	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5	N/A	54.17	51.67	52.56	51.11	52.78	49.17	49.33
10		60.00	47.11	44.50	46.11	52.72	47.44	43.56
15		56.00	43.22	40.67	40.89	49.44	36.28	40.11
20		56.28	41.33	44.17	41.22	46.94	35.56	34.28
25		53.28	37.83	41.06	36.61	40.67	33.61	32.83
30		54.17	34.17	34.39	35.83	39.22	33.11	30.67
40		53.00	34.67	33.83	34.67	35.61	28.67	27.22
50		51.83	32.00	33.72	30.61	36.94	26.67	27.06
C. Mean population kill time when only the barrier is insecticide-treated (min)								
5		N/A	34.55	29.31	27.02	37.78	19.11	19.98
10		N/A	18.31	16.53	19.06	25.36	10.42	11.32
15		39.89	17.71	13.26	10.60	20.03	8.96	8.35
20		33.96	10.06	10.19	11.27	16.25	8.48	6.74
25		28.54	10.20	9.09	9.07	9.87	6.81	6.49
30		25.79	9.93	9.27	6.56	11.44	6.90	5.44
40		25.40	6.60	7.54	7.15	9.90	5.20	4.94
50		20.89	6.69	6.66	6.78	7.31	4.82	4.34

Extended Data Fig. 7 | Comparison of simulated performances of different barrier designs and heights. (**A**) Mean total mosquito population contact time (duration of all contact and resting events; minutes) per experiment for a standard untreated bednet and different untreated barrier designs at different heights. Note: with no negative impact from untreated net contact, virtual mosquitoes revisit the net *ad infinitum*, hence high contact rates within 1hr. (**B**) Mean time in minutes to kill the entire mosquito population, when both bednet and barrier are insecticide-treated, by each barrier design and barrier height on treated nets. All net contact areas deliver a dose of 0.05 units per contact. The insecticide treatment is identical on every surface treated, and equivalent to a Permanet 2.0 in terms of repellency. The agent response to contacting a *treated* net is to decrement health and to select a new random direction and fly away. Thus, the insecticide approximates contact irritancy and not spatial repellency. (**C**) Mean population kill time when only the barrier is insecticide- treated (dose=1 unit per contact). Note: 5 and 10cm T-barriers did not kill the entire mosquito population in all runs.





Extended Data Fig. 8 | Comparing different barrier designs and heights by evaluating performance *in silico*. (**A**) Population kill time (total time needed to achieve complete population death) when insecticide is delivered by both bednet and barrier, for different barrier designs at increasing barrier height. (**B**) Population kill time as in A, weighted by surface area with a standard unmodified bednet as reference. Plot colours correspond to barrier design borders in Fig. 4.

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☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
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☐	☒ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Video was recorded using StreamPix V8 software (www.norpix.com). Segmentation and analysis of video footage was performed using bespoke software under the Matlab framework (Mathworks) and C++ programming languages.
Data analysis	Video files were compressed using bespoke software and the .mp4 container. Statistical analyses were performed using R (R version 3.1.2) (R Development Core Team 2014).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
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The hut trial dataset generated during the study is available on Dryad Digital Repository under accession number: https://doi.org/10.5061/dryad.hqbzkh1b7. All data analysed during this study are available as detailed in the manusript. The authors declare that all other data supporting the findings of this study, are available within the article and its Supplementary Information files, or are available from the authors upon reasonable request.

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For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Hut trial sample size was based on WHO guidelines - 28) World Health Organization. "Guidelines for laboratory and field testing of long-lasting insecticidal nets" http://apps.who.int/iris/bitstream/handle/10665/80270/9789241505277_eng.pdf?sequence=1 (2013). Tracking and modelling studies were based on power calculations, with estimated effect sizes unless limited by the availability of biological samples.
Data exclusions	No data were excluded from the analysis.
Replication	Experimental findings were replicated across repeated trials, with general patterns conserved between laboratory and field trials. The key outcome, the hut trial, was based on standard WHO guidelines with reproducibility as a major consideration.
Randomization	Treatments and participants were assigned at random based on a blinded selection
Blinding	Investigators were not blinded to the groups during filming or hut trial work due to the ease with which bednet designs could be distinguished visually.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☐	☒ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	The study used two laboratory strains of mosquito: Anopheles gambiae s.s. Kisumu (insecticide susceptible) and a hybrid of Anopheles gambiae/ coluzzii Tiassale (pyrethroid resistant)
Wild animals	The study reared adult Anopheles gambiae s.l. from aquatic stages collected at the study site in Burkina Faso. Adult female mosquitoes were used in the experiments
Field-collected samples	No samples were collected in the field.
Ethics oversight	All research methods were performed in accordance with approved guidelines. The study was approved by the Research Ethics Committees at the Liverpool School of Tropical Medicine (LSTM Research Protocol 16-38, 11th October 2016, Liverpool) and Centre National de Recherche et de Formation sur le Paludisme (CNRFP Deliberation no. 2016-9-097, 20th September 2016, Ouagadougou).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics	For laboratory tests 18 human volunteers, 9 males and 9 females of different ethnicities, aged between 22 and 49, were

Population characteristics	recruited from institutional staff and students. For hut trial and field-based filming, 6 volunteers were recruited from the local community, aged between 21 and 35.
Recruitment	Participants were asked to volunteer to participate in the study, field-based research participants were recruited with the assistance of a local guide as some previous experience of collecting mosquitoes was required.
Ethics oversight	All research methods were performed in accordance with approved guidelines for those procedures and written informed consent was obtained from all volunteers sleeping in experimental huts and laying under bednets during tracking experiments. The study was approved by the Research Ethics Committees at the Liverpool School of Tropical Medicine (LSTM Research Protocol 16-38, 11th October 2016, Liverpool) and Centre National de Recherche et de Formation sur le Paludisme (CNRFP Deliberation no. 2016-9-097, 20th September 2016, Ouagadougou). No adverse effects of treatment or mosquito-borne infections were reported by volunteers during the course of the study.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Appendix 3

Publication: Hughes, A., Lissenden, N., Viana, M., Toé, K. H., and Ranson, H. (2020). Anopheles gambiae populations from Burkina Faso show minimal delayed mortality after exposure to insecticide-treated nets. Malaria Journal, 13(11):17.

Parasites & Vectors

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Anopheles gambiae populations from Burkina Faso show minimal delayed mortality after exposure to insecticide-treated nets

Angela Hughes[1†], Natalie Lissenden[1†], Mafalda Viana[2], Kobié Hyacinthe Toé[3] and Hilary Ranson[1*]

Abstract

Background: The efficacy of long-lasting insecticidal nets (LLINs) in preventing malaria in Africa is threatened by insecticide resistance. Bioassays assessing 24-hour mortality post-LLIN exposure have established that resistance to the concentration of pyrethroids used in LLINs is widespread. However, although mosquitoes may no longer be rapidly killed by LLIN exposure, a delayed mortality effect has been shown to reduce the transmission potential of mosquitoes exposed to nets. This has been postulated to partially explain the continued efficacy of LLINs against pyrethroid-resistant populations. Burkina Faso is one of a number of countries with very high malaria burdens and pyrethroid-resistant vectors, where progress in controlling this disease has stagnated. We measured the impact of LLIN exposure on mosquito longevity in an area of the country with intense pyrethroid resistance to establish whether pyrethroid exposure was still shortening mosquito lifespan in this setting.

Methods: We quantified the immediate and delayed mortality effects of LLIN exposure using standard laboratory WHO cone tests, tube bioassays and experimental hut trials on *Anopheles gambiae* populations originating from the Cascades region of Burkina Faso using survival analysis and a Bayesian state-space model.

Results: Following single and multiple exposures to a PermaNet 2.0 LLIN only one of the four mosquito populations tested showed evidence of delayed mortality. No delayed mortality was seen in experimental hut studies using LLINs. A delayed mortality effect was only observed in WHO tube bioassays when deltamethrin concentration was increased above the standard diagnostic dose.

Conclusions: As mosquito pyrethroid-resistance increases in intensity, delayed effects from LLIN exposure are substantially reduced or absent. Given the rapid increase in resistance occurring in malaria vectors across Africa it is important to determine whether the failure of LLINs to shorten mosquito lifespan is now a widespread phenomenon as this will have important implications for the future of this pivotal malaria control tool.

Keywords: Mosquito, *Anopheles*, Insecticide resistance, Delayed mortality, Longevity, Sub-lethal effects, Long-lasting insecticidal nets (LLINs), Burkina Faso

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Background

Long-lasting insecticidal nets (LLINs), which are the mainstay of many malaria control programmes in Africa, reduce contact between mosquitoes and humans by providing both a physical barrier and an insecticidal effect [1, 2]. In areas where LLINs are used on a large scale, they provide both personal and community-wide protection [3–5]. Across sub-Saharan Africa, ever-increasing numbers of people at risk of malaria are sleeping under an LLIN and this has been attributed to averting approximately two-thirds of potential malaria cases between 2000 and 2015 [6]. In Burkina Faso, malaria transmission remains high, and cases are increasing [7] despite high coverage of vector control tools, including three national LLIN distribution campaigns in 2010, 2013 and 2016. The majority of distributed LLINs were pyrethroid only, predominately deltamethrin; however, a small number of alphacypermethrin nets and nets containing piperonyl butoxide (PBO) were distributed in the 2010 and 2013 campaigns [8].

Insecticide resistance is defined as the ability of mosquitoes to survive exposure to a standard discriminating dose of insecticide [9]. Inevitably, after many years of prolonged use of pyrethroid insecticides to control agricultural pests and disease vectors, malaria vectors with increasing levels of pyrethroid resistance have emerged, and this has impacted on the ability of LLINs to control these mosquito populations [10, 11]. The impact of pyrethroid resistance on malaria transmission in Africa is contested [12–16]. The sometimes contradictory findings may be partially explained by the varying intensities of resistance in the study sites; a recent meta-analysis of bioassay studies and experimental hut trials data [17] shows that the community protection provided by nets reduces rapidly as resistance emerges whereas personal protection is only lost when resistance reaches much higher levels.

Although insecticide-resistant *An. gambiae* (*sensu stricto*), by definition, are not killed upon immediate contact with insecticides, fitness costs incurred from exposure may indirectly reduce their disease transmission potential [18]. Delayed mortality post-LLIN exposure has been demonstrated in a previous laboratory trial on pyrethroid-resistant colonies [19], and in a field study using *An. funestus* (*sensu lato*) and *An. gambiae* (*s.l.*) from Cameroon [20]. These studies found that the magnitude of the delayed mortality effects decreases in strains that have developed multiple resistance mechanisms and/or compensatory mutations [19, 20]. Given the rapid increase in resistance intensity observed in Burkina Faso and the emergence of additional potent resistance mechanisms [21, 22] we sought to quantify the presence of any delayed mortality following LLIN exposure in these highly resistant populations.

Methods

Study sites

Laboratory bioassays were performed in the insectaries at the Liverpool School of Tropical Medicine (LSTM), UK, and the Centre National de Recherche et de Formation sur le Paludisme (CNRFP) clinical research unit of Banfora, Burkina Faso (10°37′N, 04°46′W). Experimental hut studies were carried out at the CNRFP field station in Tengrela (10°40′N, 04°50′W). The huts are located on the outskirts of Tengrela village adjacent to rice growing fields. Tengrela is a rural town, mainly known as a rice and vegetable growing area, located in the Comoé Province approximately 440 km south-west of Ouagadougou, the country's capital, and 7 km from the province's capital, Banfora. Yendere (10°12′N, 04°58′W) is also a rural town with no specific agricultural practice. Cotton is grown in the areas surrounding the town. It is also in Comoé Province approximately 60 km from Banfora. Both sites are in the same health district of Banfora. The climate in this area of the country is characterised with a rainy season from June to October and a dry season from November to May. The average temperature is 27.5 °C and average annual rainfall is 1080 mm. Field experiments were conducted between 2016 and 2018 during the rainy season.

Mosquito strains

Two laboratory strains (VK7 2014, hereafter referred to as VK7, and Banfora) and two field populations, collected as larvae from Tengrela and Yendere, of insecticide-resistant *An. gambiae* (*s.l.*) from Burkina Faso were used. The insecticide-susceptible *An. gambiae* (*s.s.*) Kisumu reference strain [23] was used as a control in experiments conducted at LSTM, and to test the efficacy of netting used for tests in Burkina field studies. The Banfora laboratory strain was colonised from the Tengrela field site in 2015 and the VK7 strain from Valle du Kou, village no. 7 in 2014. Both are *An. coluzzii* colonies and have been maintained at LSTM under standard insectary conditions (27 ± 2 °C, 80 ± 10% relative humidity (RH) with a 12:12 h light:dark photoperiod). Field populations were collected as larvae from Tengrela and Yendere over several collection days. Mosquitoes were sampled from different types of breeding site (e.g. temporary pools, rice fields). Larvae were reared to adults in the insectaries (25 ± 3 °C and 75 ± 25% RH) at CNRFP; these mosquitoes were used for insecticide bioassays and in reared-release studies in experimental huts. In Tengrela, mosquitoes were largely collected from rice fields. In Yendere, rice is not a major crop, and mosquitoes were collected from more

temporary breeding sites, where typically *An. gambiae* (*s.s.*) predominate over *An. coluzzii* [24, 25]. Freely entering adults from Tengrela, of unknown age, were used in wild-entry experiments. Species identification of field strains was conducted using SINE PCR [26] at LSTM. *Anopheles coluzzii* predominates in Tengrela (87%, 437 mosquitoes tested) and *An. gambiae* (*s.s.*) in Yendere (90%, 203 mosquitoes tested).

Insecticide resistance status

The VK7 and Banfora laboratory strains are resistant to permethrin, deltamethrin and DDT [27]. Topical and tarsal permethrin dose-response assays suggest the Banfora strain to be more pyrethroid-resistant than VK7 although this difference is not significant. VK7 has a high frequency of the 1014F kdr mutation with the 1575Y sodium channel mutation present at a low level; several P450s (CYP6M2, CYP6P3 and CYP6P4) with known pyrethroid metabolism activity are upregulated in this strain. The Banfora strain is also heterozygous for the 1014F and 1575Y sodium channel mutations; metabolic resistance is less predominant in this strain and instead, topical assays suggest insecticide penetration barriers contribute to the resistance phenotype [27]. To establish the resistance status of larval-reared field populations, WHO susceptibility tube bioassays [9] were performed using control and deltamethrin papers at the diagnostic dose (0.05%), plus further assays using papers of increasing deltamethrin concentrations (0.05%, 0.25%, 0.50%, 0.75% and 1.0%); daily survival following exposure was assessed. Details of sample sizes are provided in Additional file 1: Figure S1.

Net treatments

PermaNet®2.0 (Vestergaard Frandsen, Switzerland, deltamethrin 1.4–1.8 g/kg) and untreated nets (purchased locally) were used for both LSTM laboratory tests and all field tests. Nets were aired for a minimum of one week prior to experiments (with the exception of the 2016 hut trials where nets were used on the same day, without airing) and acclimatised to the respective testing room before use. Details of sample sizes are provided in Additional file 1: Table S2.

WHO cone bioassay

Mosquitoes were exposed to randomly selected pieces of untreated or PermaNet 2.0 netting using a standard three-minute WHO cone bioassay [28]. For laboratory assays and 2017 field tests, one untreated net and one PermaNet 2.0 were used for all tests. For field assays, in 2017 two untreated nets and two PermaNet 2.0 nets were used. Netting pieces were randomly sampled from the roof and sides of the nets. Cohorts were exposed to nets once (Assay A) or several times (Assays B–E) using a variety of differing test regimes (Table 1). For laboratory assays, cohorts of 70 mosquitoes were exposed, and for field assays cohorts ranged from 25–125 mosquitoes depending on availably of mosquitoes (details of sample sizes are provided in Additional file 1: Table S2). The laboratory and field assays were carried out at different times and locations. The different exposure regimes approximate alternative types of exposure to LLINs that mosquitoes may experience during their lifespan [19]. Assay A (single exposure) provided a baseline level of net contact to compare untreated and treated netting. Assays B, C, and E (daily exposure for 2, 3 and 5 days, respectively) simulates the net contact a mosquito might encounter if it is repeatedly prevented from obtaining a blood meal. Assay D (exposure every 4 days for 4 exposures) simulates the level of net contact a mosquito might encounter every gonotrophic cycle. The exposure

Table 1 Summary of experimental factors in cone bioassays. Mosquitoes were exposed to PermaNet 2.0 and untreated nets

Cone assay ID	LLIN exposure (times exposed)	Exposure regime	Mosquito strain	Age (days)[a]
A	Single (×1)	Exposed once	VK7 (Lab)	4
			Banfora (Lab)	4
			Yendere (Field)	3–5
			Tengrela (Field)	5–8
B	Multiple (×2)	Daily exposure for 2 consecutive days	VK7 (Lab)	4
			Banfora (Lab)	4
C	Multiple (×3)	Daily exposure for 3 consecutive days	VK7 (Lab)	4
			Banfora (Lab)	4
D	Multiple (×4)	Exposure every 4 days, for a maximum of 4 exposures	Tengrela (Field)	4
E	Multiple (×5)	Daily exposure for 5 consecutive days	Tengrela (Field)	4

[a] Age at first exposure

Abbreviation: Lab, laboratory

regimes varied between the laboratory and field experiments for logistical reasons.

Age at first exposure to insecticides varied between 3 to 8 days post-eclosion and only non-blood-fed females were used. Mortality at 24 hours post-exposure was recorded. After the final exposure, all surviving mosquitoes were held with access to a sugar solution and daily mortality was recorded until all mosquitoes were dead.

Experimental hut trials

The semi-field experimental hut station contained six huts built to the West African design [28] and is situated adjacent to Tengrela's rice fields. Two trials (A and B) were conducted using either larval-reared mosquitoes or wild-entry mosquitoes, respectively over a two-year period (Table 2). Trials were replicated in 2016 and 2017. In Hut Trial B only mosquitoes without a visible bloodmeal were used to score longevity. Huts contained either untreated net (control) or unwashed PermaNet® 2.0. Nets were holed according to WHO guidelines [28]. Sleepers were randomly rotated within huts; however, small mosquito numbers for release meant this occurred on non-consecutive days, and between two and six huts were used for trials (full details Additional file 1: Table S1).

Volunteers entered the huts after ~20:00 h and remained under the nets until ~6:00 h. In the reared-release trial, window shutters, entries and door frames were closed or covered with untreated netting to prevent the exit of released mosquitoes. In the wild entry trial, window entries remained open. After acclimatisation (> 10 min) mosquitoes were either manually released into the hut (reared-release trial) or window traps opened to allow wild mosquitoes to enter (wild-entry trial).

The following morning, mosquitoes were collected individually using glass universal tubes and placed into labelled bags separated by location (i.e. under net, in the veranda, in the main hut). The remaining mosquitoes were collected using a Prokopack aspirator (The John W. Hock Company, Florida, USA). All mosquitoes were morphologically identified [29], sexed, recorded as dead or alive, and scored for abdominal status (unfed, partially-fed, blood-fed, semi-gravid/gravid). Dead female *Anopheles* mosquitoes were stored in silica, and male *Anopheles* and non-anopheline mosquitoes were recorded and discarded. Surviving female mosquitoes were transferred to paper cups and provided with 10% glucose solution. Mortality was recorded daily until all mosquitoes were dead, and dead mosquitoes were stored in silica.

Data analysis

Chi-square or Fisher's exact test was used for immediate mortality analysis. If a mosquito was censored (e.g. mosquito escaped) during the 24 hours following exposure, it was removed from immediate mortality analysis. In discrimination dose bioassays, immediate mortality following insecticide exposure was always less than 5 % so Abbot's correction [9] was not applied. In cone bioassays following single exposure control mortality was low across all treatments (< 5%). As control mortality during subsequent exposures in multiple exposure assays could be affected by mosquito age, cone bioassay mortality was not corrected in any exposures. For survival analysis, Kaplan-Meier curves were used to visualise the data, and Cox regression was used to compare post-exposure survival. Immediate mortality (24-h post-exposure, and/or dead on collection) was excluded, and censored data included. All analysis was conducted in IBM SPSS Statistics 24 (IBM Corp. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY, USA).

A Bayesian state-space survival model as developed by Viana et al. [19] was used to quantify the daily survival rate and the magnitude of any observed delayed mortality effect in each experiment. Briefly, the observed number of mosquitoes alive each day was modelled from a binomial distribution described by the total number of mosquitoes alive and the probability of daily survival which, in turn was described with a logit link to its nonlinear predictor parameterised as a function of the treatment previously published [19]. The results were generated using this model executed in JAGS. The model, structure and parameter priors have been previously published elsewhere [19]. The results were generated using a version of the model executed using Mathcad.

Table 2 Summary of experimental factors in experimental hut trials

Trial	Mosquito population (strain)	Year conducted	Date conducted	No nights	Age (days)	Blood-feeding status
A	Reared-release (larval-reared Tengrela)	2016	26 September–3 October	6	5–8	Unfed; blood-fed
		2017	10–22 September	10	5–8	Unfed; blood-fed
B	Wild-entry (Tengrela)	2016	10–21 October	10	Unknown	No visible blood meal
		2017	2–15 July	12	Unknown	No visible blood meal

Results

WHO cone bioassays

Immediate mortality

The Kisumu susceptible strain showed high immediate mortality against PermaNet 2.0 (LSTM strain, 100% mortality, $n=100$ mosquitoes; CNRFP strain, 98% mortality, $n=48$ mosquitoes). In laboratory strains, after single and repeated exposure to PermaNet 2.0, the immediate mortality of the Banfora and VK7 was < 15% (Fig. 1a; Additional file 1: Table S2). In the laboratory strains, a significant difference between PermaNet 2.0 mortality and untreated net mortality was only seen in the Banfora strain, following the single exposure (Assay A, Fig. 1a, $P=0.029$), and the second exposure of the two exposure assay (Assay B, Fig. 1a, $P=0.003$). In all other exposures, no significant difference in immediate mortality between laboratory mosquitoes exposed to treated or untreated net was seen (Fig. 1a; Additional file 1: Table S2).

In the field strains (Tengrela and Yendere) no difference in immediate mortality between PermaNet 2.0 and the untreated net was observed following single exposures (Assay A). However, significantly higher mortality was observed after the third exposure in Assay D (4 exposures every four days), and the 4th and 5th exposure in Assay E (5 exposures daily) (Fig. 1b; Additional file 1: Table S2).

Delayed effects

After a single exposure to LLINs, there was no significant reduction in survival compared to a single exposure to untreated netting in the laboratory VK7 strain (Cox regression, $P=0.57$), and field Tengrela (Cox regression, $P=0.27$) and Yendere (Cox regression, $P=0.52$) populations (Fig. 2a). Only the laboratory Banfora strain showed significantly reduced survival after a single exposure to LLIN compared to the control (Cox regression, $P=0.03$); Banfora mosquitoes exposed to PermaNet 2.0 had a 1.44-fold (95% CI: 1.13–1.84) increase in the risk of death compared to Banfora mosquitoes exposed to untreated netting.

After two exposures to LLIN (Fig. 3a), the Banfora strain showed no significant reduction in cumulative survival compared to two exposures to untreated netting (Cox regression, $P=0.26$), whilst the VK7 strain showed a small, but significant (Cox regression, $P=0.008$) increase in survival after two exposures to LLIN compared to the control;VK7 exposed to PermaNet 2.0 had a 0.72-fold (95% CI: 0.57–0.92) decrease in the risk of death compared to controls. After three exposures (Fig. 3b) neither laboratory strain showed a reduction in longevity compared to untreated netting (Banfora, $P=0.206$; VK7, $P=0.085$).

The Tengrela field population was exposed to LLINs either every fourth day, four times (Assay D) or daily for five days (Assay E). Neither exposure regime had any impact on long-term survival compared to untreated netting [Fig. 4a ($P=0.72$) and 4b ($P=0.97$)].

Experimental hut trials

Mosquito numbers, species identification and immediate mortality

Over the two-year study, a total of 1187 *Anopheles* and 602 non-*Anopheles* were collected during 22 nights by

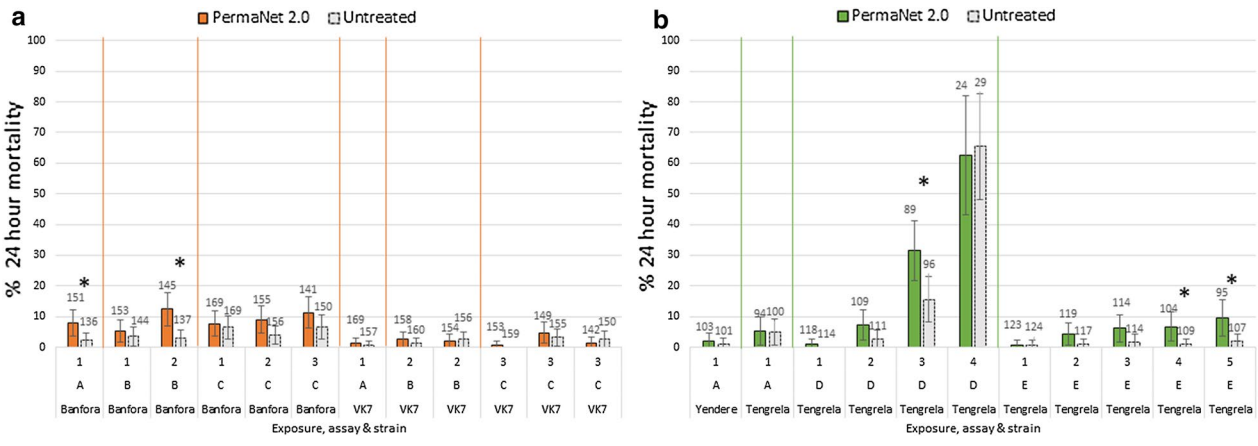

Fig. 1 24-hour mortality of laboratory Banfora and VK7 (**a**) and field Tengrela and Yendere (**b**) mosquitoes after WHO cone bioassay exposure. Mosquitoes were exposed to PermaNet 2.0 or untreated net single (Assay A), or multiple (Assays B-E) times and their mortality recorded. Error bars show 95% confidence intervals for the population proportion. Numbers above bars show the number of mosquitoes tested. Numbers below the graph show the number of exposures and letters refer to the experimental design (see Table 1). Asterisks show when untreated and PermaNet 2.0 mortality was significantly different ($P<0.05$). See Additional file 1: Table S2 for details of the mortality in each assay

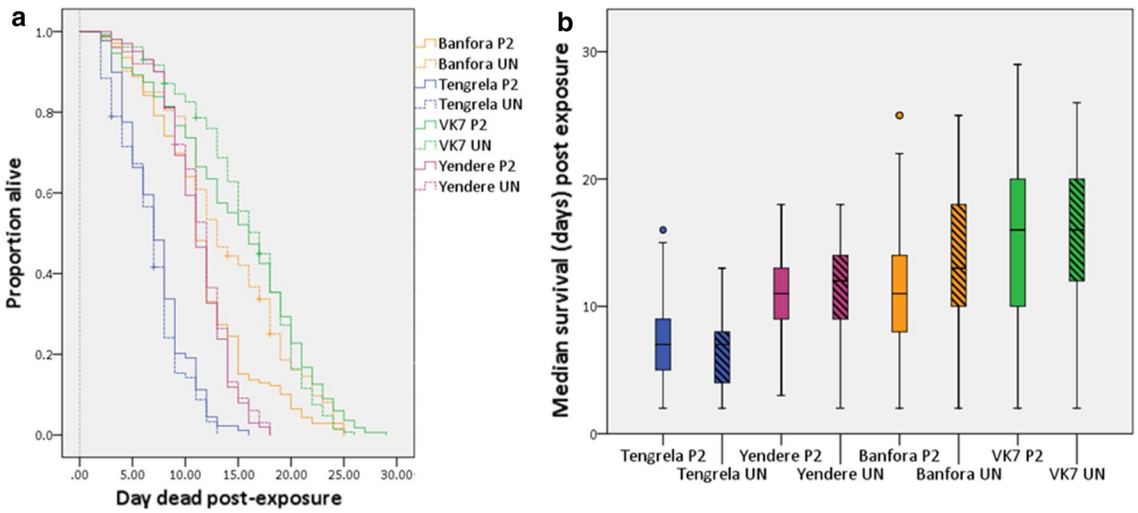

Fig. 2 The longevity of laboratory and field populations after single WHO cone bioassay exposures. **a** Kaplan Meier survival curves show the proportion alive each day post-exposure. The dashed grey line indicates the day mosquitoes were exposed. Crosses represent censored data at the point of censoring. **b** Box and whisker plots of median survival (days) dead post-exposure. Mosquitoes were 4 (VK7 and Banfora), 3–5 (Yendere), or 5–8 (Tengrela) days-old on exposure. Coloured dots show outliers in the data. In both **a** and **b** immediate (within 24 h) mortality is excluded. Banfora: 2 replicates (PN2, $n = 139$ mosquitoes; UN, $n = 133$ mosquitoes); VK7: 2 replicates (PN2, $n = 167$ mosquitoes; UN, $n = 156$ mosquitoes); Tengrela: 2 replicates (PN2, $n = 89$ mosquitoes; UN, $n = 95$ mosquitoes); Yendere: 2 replicates (PN2, $n = 101$ mosquitoes; UN, $n = 100$ mosquitoes)

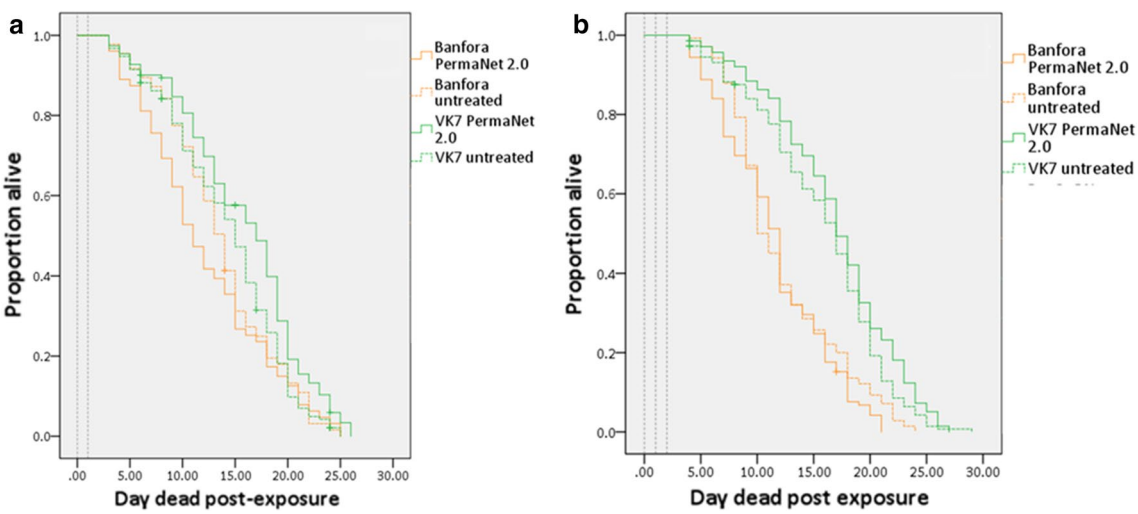

Fig. 3 The longevity of laboratory strains after multiple WHO cone bioassay exposures. Kaplan Meier survival curves show the proportion alive each day following two (**a**) or three (**b**) exposures. The dashed grey line indicates the day mosquitoes were exposed. Crosses represent censored data at the point of censoring. In both **a** and **b** immediate (within 24 h) mortality is excluded

volunteer sleepers in the wild-entry experimental hut trials in Tengrela (Additional file 1: Table S1, Table S6). The average number of female *Anopheles* caught per night/per hut were 16.9 in 2016 and 6.00 in 2017 for PermaNet 2.0 huts, and 20.6 in 2016 and 8.08 in 2017 in untreated hut (Additional file 1: Table S6). Lower mosquito numbers in 2017 may be due to the trial being conducted early in the rainy season (July), whereas mosquito numbers in 2016 (October) are comparable to other hut trials conducted at this site [30]. In the release-recapture hut trials, 782 *Anopheles* were released and 493 recaptured across all huts. A total of 92 non-target (non-*Anopheles* or male *Anopheles*) were collected. Recapture rates were greater in untreated compared to PermaNet 2.0 huts over the two years (Additional file 1: Table S6; Untreated: 76.21%; PermaNet 2.0: 49.87). Molecular ID confirmed

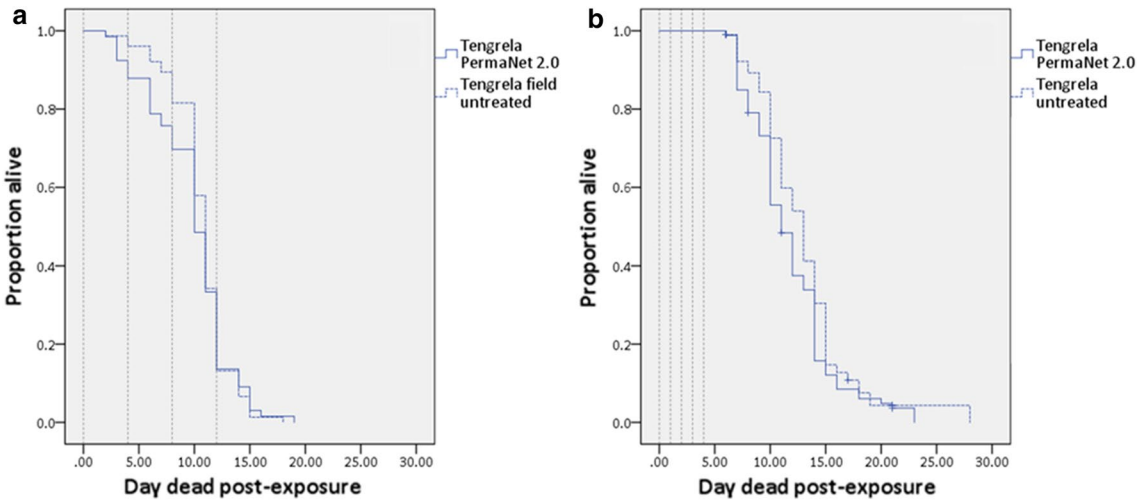

Fig. 4 The longevity of field populations after multiple WHO cone bioassay exposures. Kaplan Meier survival curves show the proportion alive each day following four exposures every four days (**a**); or five daily exposures (**b**). In both **a** and **b** the dashed grey line indicates the day mosquitoes were exposed. Crosses represent censored data at the point of censoring. Immediate (within 24 h) mortality is excluded

An. coluzzii to be the dominant species of mosquitoes collected from Tengrela (87.41% *An. coluzzii*; 2.97% *An. gambiae* (*s.s.*); 1.14% *An. coluzzii/gambiae* hybrids; 0.23% *An. arabiensis*; 8.24% unidentified; 437 mosquitoes tested in 2017), while *An. gambiae* (*s.s.*) were more abundant in mosquitoes collected from Yendere (90.15% *An. gambiae* (*s.s.*); 0.49% *An. coluzzii/gambiae* hybrids; 0.49% *An. arabiensis*; 6.40% unidentified; 203 mosquitoes tested in 2018).

In the reared-release trials, where adult mosquitoes aged 5 to 8 days, raised from larval collections were released into the huts, immediate mosquito mortality (dead on collection or within 24-h) in PermaNet 2.0 huts was 50% (95% CI: 38.61–61.39%) in 2016, and 45.50% (95% CI: 33.66–51.34%) in 2017 (untreated hut mortality: 2016, 11.01%, 95% CI: 5.13–16.89%; 2017, 16.22%, 95% CI: 10.90–21.53%). In the wild-entry trials, where mosquitoes were of unknown age, mortality in PermaNet 2.0 huts was 8.38% (95% CI: 4.18–12.59%) in 2016, and 13.57% (95% CI: 7.90–19.24%) in 2017 (untreated hut mortality: 2016, 4.93%, 95% CI: 1.95–7.90%; 2017, 5.29%, 95% CI: 2.10–8.48%). Mortality in the PermaNet 2.0 huts was always higher than in the huts with untreated nets but this difference was not significant in the wild entry trials. Further details of mosquito exophily and blood-feeding are provided in Additional file 1: Table S7.

Delayed mortality

The effect of date, feeding status, hut, net treatment, and collection locations (e.g. in net, in veranda) on mosquito survival post-collection was analysed. For the reared-released trials, in 2016, only blood-feeding status significantly affected mosquito longevity (Fig. 5, 92 blood-fed mosquitoes, 42 unfed mosquitoes, $P=0.001$). When non-significant variables were excluded from the regression analysis, blood-fed mosquitoes had a 0.561-fold (0.384–0.819) lower risk of death ($P=0.003$). In 2017, date of collection ($P=0.005$) and blood-feeding status ($P<0.0001$) both significantly affected mosquito longevity. When non-significant variables were removed from the model, and results were stratified by day, blood-fed mosquitoes had a 0.450-fold (0.327–0.618) reduction in the risk of death compared to unfed mosquitoes (Fig. 5b, 107 blood-fed mosquitoes, 113 unfed mosquitoes, $P<0.0001$). Data were hence stratified into unfed and blood-fed groups. In the reared-release trials, exposure to LLINs had no effect on longevity in either 2016 or 2017 (Fig. 5, Additional file 1: Table S4).

In the wild-entry trials, only unfed mosquitoes were retained for post-collection longevity analysis (as blood-fed mosquitoes were used in a separate experiment to investigate reproductive output not presented here). Again, in these trials, net treatment had no significant effect on mosquito longevity (Fig. 6) in either 2016 (untreated hut, $n=85$ mosquitoes; PermaNet 2.0 hut, $n=85$ mosquitoes, $P=0.405$) or 2017 (untreated hut, $n=55$ mosquitoes; PermaNet 2.0 hut, $n=53$ mosquitoes, $P=0.892$).

WHO intensity assays

In the discriminating dose assays, following exposure to the standard diagnostic dose of deltamethrin (0.05%), mortality was 2.01% for Tengrela (95% CI: -0.24–4.37%,

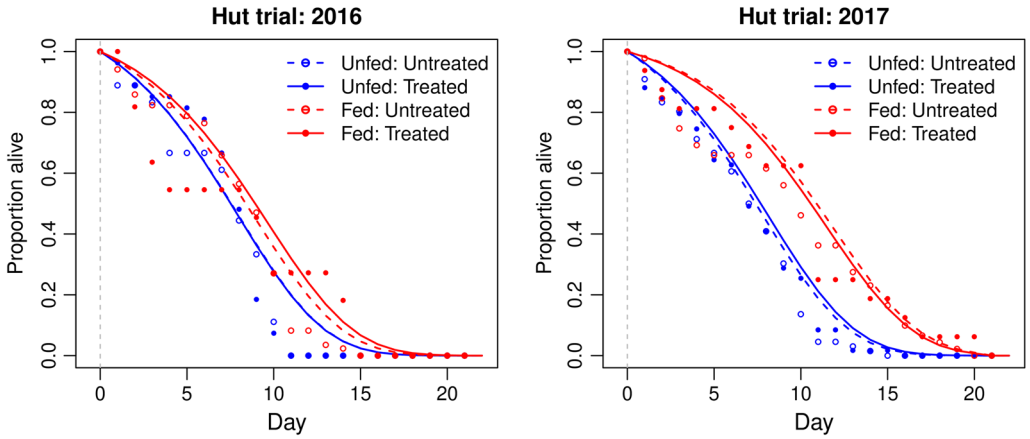

Fig. 5 The longevity of field populations after exposure in reared-release hut trial. Daily survival curves from the state-space model show the proportion alive each day following collections of blood-fed and unfed mosquitoes in 2016 and 2017. Dashed grey lines represent day of insecticide exposure in the hut trial

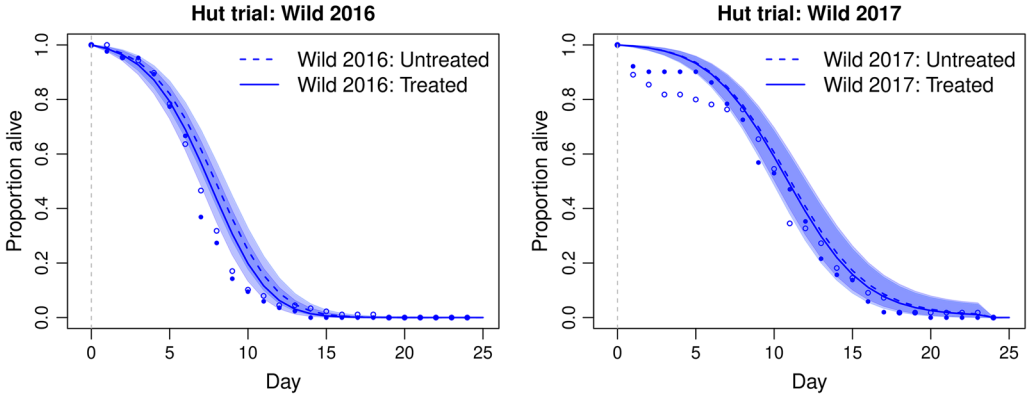

Fig. 6 The longevity of field populations after exposure in wild-entry hut trials. Daily survival curves from the state-space model show the proportion alive each day following collections of blood-fed and unfed mosquitoes in 2016 and 2017. Dashed grey lines represent day of insecticide exposure in the hut trial. Shading represents 95% confidence intervals

$n = 149$ mosquitoes). As the insecticide concentration was increased to 5× and 10× the diagnostic dose, mortality increased but it then plateaued or even decreased at 15× and 20× concentrations possibly indicating that the solubility limit of deltamethrin had been exceeded at these higher concentrations; a significant difference between treated and control mortality was seen following exposure to 0.25%, 0.5%, 0.75% and 1% deltamethrin papers (Additional file 1: Figure S1).

Excluding immediate mortality, there was no evidence of delayed mortality compared to untreated control at the standard dose of deltamethrin (0.05%, $P = 0.395$). However, as mosquitoes were exposed to increasing insecticide concentration, reduced longevity was observed in the treated versus the control tubes (Fig. 7; Additional file 1: Table S5).

Discussion

In our earlier publication [19], we showed that exposure to LLINs resulted in a delayed mortality effect that approximately halved the overall mosquito lifespan beyond the 24 hours post-exposure. The magnitude of this delayed mortality varied between strains, with LLIN exposure having a greater impact on median mortality in a moderately resistant Tororo laboratory strain than in the more highly resistant Tiassalé strain. However, the potential impact on malaria transmission of this delayed mortality was substantial for both strains, with exposure to LLINs estimated to reduce the malaria transmission by 3.3-fold and 7.8-fold in Tororo and Tiassalé, respectively. At the time of publication, we noted that although this delayed mortality effect may be mitigating the impact of pyrethroid resistance on LLIN efficacy in the field, this

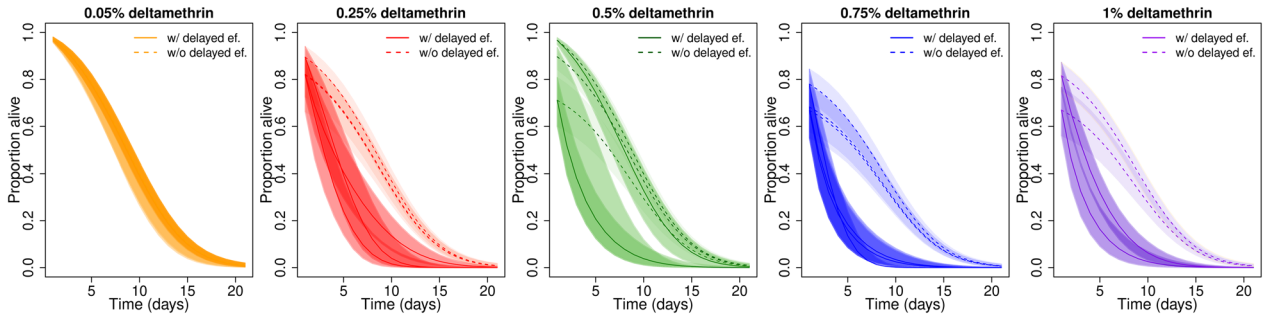

Fig. 7 The modeled daily survival curves of *An. gambiae* following a WHO longevity tube assay. Mosquitoes from Tengrela were exposed to increasing concentrations of deltamethrin or untreated control papers. Full lines represent the curve estimated from fitting the binomial model to the data, and the dotted lines represent the counterfactual curve predicted with no delayed effects. Lines correspond to the median prediction with shaded 95% credible intervals

effect may be eroded as resistance increases in intensity. We also recognised the importance of testing for delayed mortality in field populations, using more realistic methods of LLIN exposure. As a consequence, we have been routinely measuring daily survival post-insecticide exposure in our laboratory and field assessments of pyrethroid resistance. Here, we report data on the impact of LLIN exposure on lifelong survival in populations of *An. gambiae* (*s.l.*) from Burkina Faso.

Southwestern Burkina Faso is known as a hotspot for pyrethroid resistance [30]. We established two colonies of *An. coluzzii* from this region at LSTM in 2014 (VK7) and 2015 (Banfora), both of which have higher levels of pyrethroid resistance than our previous 'gold standard' resistant strain, Tiassalé [27]. Multiple exposures to LLINs in cone bioassays had very little impact on the 24-hour post-exposure with mortality levels less than 12% in all cases. Furthermore, there was no evidence of any delayed mortality in any of the exposure regimes for the VK7 strain. Delayed mortality was only observed in the Banfora strain although the magnitude of this effect was much smaller than observed in previous studies with Tiassalé and Tororo colonies (< 6% reduction in daily mortality in Banfora due to delayed mortality effects *vs* 46% for Tororo and 12% for Tiassalé).

When cone bioassays were performed directly on mosquitoes collected from the field, again there was very little immediate mortality following LLIN exposure and no evidence of any delayed mortality. The 3-minute exposure used in the cone bioassays is a simple means of evaluating the response in the laboratory but does not reflect the realities of mosquito exposure to LLINs in the field. Indeed, the duration of contact of mosquitoes with LLINs in response to a human baited bed net has been shown to be less than three minutes [31]. The use of experimental huts enabled us to mimic LLIN exposure in the field under controlled conditions. Again, we observed no difference between the longevity of mosquitoes exposed to LLINs or control nets.

In hut trials, feeding status had a significant effect on mosquito longevity with blood-fed mosquitoes surviving significantly longer post-collection than unfed mosquitoes. During blood meal digestion mosquitoes upregulate enzymes to detoxify harmful products from the blood meal. Subsequently, these enzymes could be providing an additional benefit following exposure to insecticides by assisting in insecticide detoxification [32]. In other laboratory trials acquiring a blood meal has been shown to improve survival following insecticide exposure [33] and increase longevity [34] and similar effects have documented in other field locations [35].

Reared released mosquitoes (Hut trial A, Fig. 6a, b), did not survive as long post-exposure as the wild entry mosquitoes in hut trial B (Fig. 7a, b). The experimental huts in Tengrela are situated between the rice fields and the village, and it is anticipated that a large proportion of mosquitoes in the wild entry experiments may be newly eclosed mosquitoes seeking their first blood meal. Females used in the reared release trials were five to eight days-old. The presumed difference in age structure between the wild mosquitoes entering the experimental huts and the reared and released, may explain the differences in observed longevity as it is well documented that mosquito susceptibility to insecticides increases as they age [36–38]. Additionally, by collecting and rearing mosquitoes in the insectary for release, we may be including mosquitoes of lower fitness which in the wild may have died before reaching the huts. Additionally, the extra handling and transportation of the larval-reared mosquitoes to the hut station in the reared-release trial may have led to increased mortality, although we note that only a slight increase is observed in the untreated arm of the reared-release trial, in

comparison to the wild-entry trial suggesting this may have a relatively minor impact on the differential mortality observed in the two tests.

Having observed almost no impact of LLIN exposure on mosquito longevity in any of the populations or exposure regimes, we sought to understand whether delayed mortality could be induced by increasing the amount of insecticide the mosquitoes were exposed to. Here we found that there was evidence of a delayed mortality effect at concentrations of >5× the discriminating dose in WHO tubes assay. These results indicate pyrethroids can induce sub-lethal effects even in the highly resistant populations, but under standard exposure conditions, these effects are rarely evident.

Conclusions

Mosquito longevity is the primary determinant of vectorial capacity. Our findings that standard pyrethroid nets are not impacting on the longevity of malaria vectors in southwestern Burkina Faso are of great concern. This study did not measure other potential sub-lethal effects of pyrethroid exposure in the resistant populations, such as reproductive output or re-feeding success, and these are now being investigated in follow-up studies. Further studies on the impact of exposure of pyrethroid-resistant mosquito populations on *Plasmodium* development are also needed to fully understand the impact of resistance on malaria transmission potential.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-019-3872-2.

Additional file 1: : Table S1. Summary of the number of nights volunteer and net treatment spent in each hut during trials. **Table S2.** Summary of 24-hour mortality from WHO cone bioassay exposures. **Table S3.** Estimated and counterfactual mean daily mosquito survival after WHO cone bioassay exposure. **Table S4.** Estimated and counterfactual mean daily mosquito survival after exposure in the reared-release trial. **Table S5.** Estimated and counterfactual mean daily mosquito survival after exposure in WHO tube assay. **Table S6.** Summary of mosquitoes in release-recapture and wild entry hut trials. In reared-release trials percentages show *Anopheles* recapture rate. **Table S7.** Summary of outcomes of *An. gambiae* s.l. in wild-entry hut trial in 2016 and 2017. **Figure S1.** The 24 hr mortality of An. gambiae s.l from Tengrela (2018) following exposure to deltamethrin diagnostic dose (0.05%) and intensity (0.10, 0.25, 0.50, 0.75, 1.00%) doses or an untreated control, in WHO tube bioassays. **Figure S2.** The longevity of laboratory populations after exposure in WHO cone assays.

Abbreviations

CNRFP: Centre National de Recherche et de Formation sur le Paludisme; LLIN: long-lasting insecticidal nets; LSTM: Liverpool School of Tropical Medicine; PBO: piperonyl butoxide; PPF: pyriproxyfen; WHO: World Health Organization.

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Authors' contributions

HR conceived the study. HR, AH and NL designed the study. AH and NL performed laboratory experiments. NL and KHT performed field experiments. MV performed model runs. AH and NL analysed the data. All authors interpreted the results and AH, NL and HR wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

Data supporting the conclusions of this article are included within the article and its additional file. The datasets used and/or analysed during the present study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Ethical approval for the for experimental hut trials was received from the Research Ethics Committees at the Liverpool School of Tropical Medicine (LSTM Research Protocol 16–38, Liverpool) and Centre National de Recherche et de Formation sur le Paludisme (CNRFP Deliberation no. 2016-9-097, Ouagadougou). Informed written consent was obtained from all volunteers, and no mosquito-borne infections, or adverse effects, were reported during the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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