The impact of insecticide resistance and parasite infection on vector behaviour

Thesis submitted in accordance with the requirements of the Liverpool School of Tropical Medicine for the degree of Doctor in Philosophy by Katherine Gleave.

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Abstract

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Katherine Gleave

Vector control remains one of the most important methods for reducing insect-borne diseases across the globe. However, heavy reliance on insecticides has led to the rapid spread of insecticide resistance, threatening the operational success of control programmes. In response to concerns over the sustained efficacy of our current tools, novel insecticides and products are now in development and various next-generation bednets are now in widespread use across Africa to combat resistance and restore ITN effectiveness. Understanding how resistance and new control methods may alter vector-specific disease transmission parameters is crucial. While insecticide resistance and parasite infection rates are documented in many populations, their effect on mosquito life-history traits and behaviour is less understood.

This thesis aimed to assess the impact of insecticide resistance and parasite infection on mosquito fitness and behaviour and how changes in either could impact the efficacy of new control tools and vectorial capacity.

This was achieved through work that 1) quantified and mapped insecticide resistance in Africa to document the spread of resistance in malaria vectors and the role of different resistance mechanisms, 2) evaluated next-generation ITNs for reducing malaria prevalence, 3) measured the impact of insecticide resistance on mosquito behavioural responses to ITNs and 4) studied the impact of exposure to insecticides and parasite infection on mosquito behaviour and longevity.

The results present data collated on the spatial distribution of insecticide resistance phenotypes and genotypes, which can be used to guide control programmes in resistance monitoring and consider changes in bed net distribution. The analysis generated by a systematic review showed that next-generation pyrethroid-PBO nets increase mosquito mortality, reduce blood-feeding success and lower clinical malaria incidence in areas with high insecticide resistance. Room-scale video tracking of mosquitoes around these next-generation nets has enabled us to investigate how these ITNs worked, capturing data showing the effects on mosquito behaviour of a number of different ITNs are remarkably consistent, with no significant differences in the responses between strains of different

pyrethroid susceptibility to different net treatments. Laboratory studies have explained how insecticide selection impacted mosquito fitness in male and female mosquitoes, demonstrating trade-offs in life-history traits that could limit or enhance disease transmission. It was also demonstrated that mosquitoes exposed to parasitic infection show a dynamic, stage-specific and density-dependent change in behaviour to host cues, decreased flight ability and reduced energy resources. Incorporating knowledge on the spread of insecticide resistance, the effects of next-generation nets on mosquito mortality and behaviour, and the impacts of resistance and infection on mosquito physiology will lead to a more holistic understanding of the impact of new vector control tools on mosquito-transmitted diseases.

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Contents

Table of figures

Table of tables

List of appendices

Abbreviations

%	Percent
<	Less than
>	More than
°	Degrees
g	Grams
µg/mL	Micrograms per millilitre
ACT	Artemisinin-based combination therapy
AI	Active ingredient
ATP	Adenosine triphosphate
ATSBs	Attractive toxic sugar baits
C	Centigrade
CDC	Centre for Disease Control
CENTRAL	Cochrane Central Register of Controlled Trials
CI	Confidence interval
cm	Centimetre
COEs	carboxylesterases
cRCTs	Cluster-randomised controlled trials
D	Denier
DALYs	Disability-adjusted life years
DPE	Days post-exposure
Dual AI	Dual active ingredient
EIP	Extrinsic incubation period
EIR	Entomological inoculation rate
ELISA	Enzyme-linked immunosorbent assay
ERG	Evidence Review Group
GAUL	Global Administrative Unit Layers
GPELF	Global Programme for the Elimination of Lymphatic Filariasis
GSTs	Glutathione S-transferases
IG2	Interceptor G2
IPTi	Intermittent preventative treatment of infants
IPTp	Intermittent preventative treatment of pregnant women
IR	Insecticide resistance
IRR	Incidence rate ratio
IRS	Indoor residual spraying
ITNs	Insecticide treated nets
kg	Kilogram
KD	Knock down
kdr	Knockdown resistance
km	Kilometres
L	Litre
L1	First instar larvae
L2	Second instar larvae
L3	Third instar larvae
LC	Lethal concentration
LF	Lymphatic filariasis
LLINs	Long-lasting insecticidal nets
LSTM	Liverpool School of Tropical Medicine
Ltd	Limited
LVP strain	Liverpool strain
m	Metres
MDA	Mass drug administration
m/s	Meters per second
mf	Microfilariae

mf/ml	Microfilariae per millilitre
mg	milligrams
mg/m²	Milligrams per metre square
mm	Millimetre
MoA	Mode of action
N	Number
NA	Not applicable
NR	Not recorded
NTD	Neglected tropical disease
OL	Olyset Net
OR	Odds Ratio
P450s	Cytochrome p450s
P3	PermaNet 3.0
PCR	Polymerase chain reaction
PBO	Piperonyl-butoxide
PE	Post-exposure
PQ	Prequalification
PQT-VC	Prequalification Team Vector Control Group
RDTs	Rapid diagnostic tests
REC-M	Malathion exposed mosquitoes
REC-P	Permethrin exposed mosquitoes
REC-R	Temephos exposed mosquitoes
REC-U	No insecticide selection mosquitoes
ROB	Risk of bias
RR	Risk Ratio
SMC	Seasonal malaria chemoprevention
VC	Vector control
VCAG	The Vector Control Advisory Group
Vgsc	Voltage-gated sodium channel
WHO	World Health Organisation
WHOPES	World Health Organisation Pesticide Evaluation Team

Introduction

1.1 Anopheles

Mosquitoes make up a large group of Diptera within the family Culicidae. They comprise two major subfamilies: the Anophelinae, which contains *Anopheles* mosquitoes, and the Culicidae, which contains *Aedes*, *Culex*, *Mansonia* and other genera.

Approximately 40 *Anopheles* species can transmit human malaria (Sinka *et al*., 2011), with the *An. gambiae s.l* complex and *An. funestus* group being the most important malaria vectors in Africa due to their susceptibility to *Plasmodium falciparum* and behavioural preferences, which contribute to their increased vectorial capacity (Battle *et al*., 2012; Wiebe *et al*., 2017). These species complexes and groups comprise morphologically indistinguishable sibling species that can possess different genetic and behavioural traits. The *An. gambiae* complex comprises eight sibling species; however, the most dominant in Africa are *An. gambiae* Giles (historically 'M-form'), *An. coluzzii* Coetzee & Wilkerson (historically 'S-form') (Coetzee *et al*., 2013) and *An. arabiensis* (Gillies, 1968; Gillies and Coetzee, 1987). The *An. funestus* group comprises eleven sibling species, with *An. funestus* Giles being the most competent disease vector (M. Coetzee & Fontenille, 2004) and one of the first species believed to have adapted to feeding on human hosts (Charlwood *et al*., 1995). *Anopheles* species display important differences in their geographical distribution, which can be influenced by factors such as temperature, humidity, vegetation type and proximity to humans (Wiebe *et al*., 2017) (Figure 1).

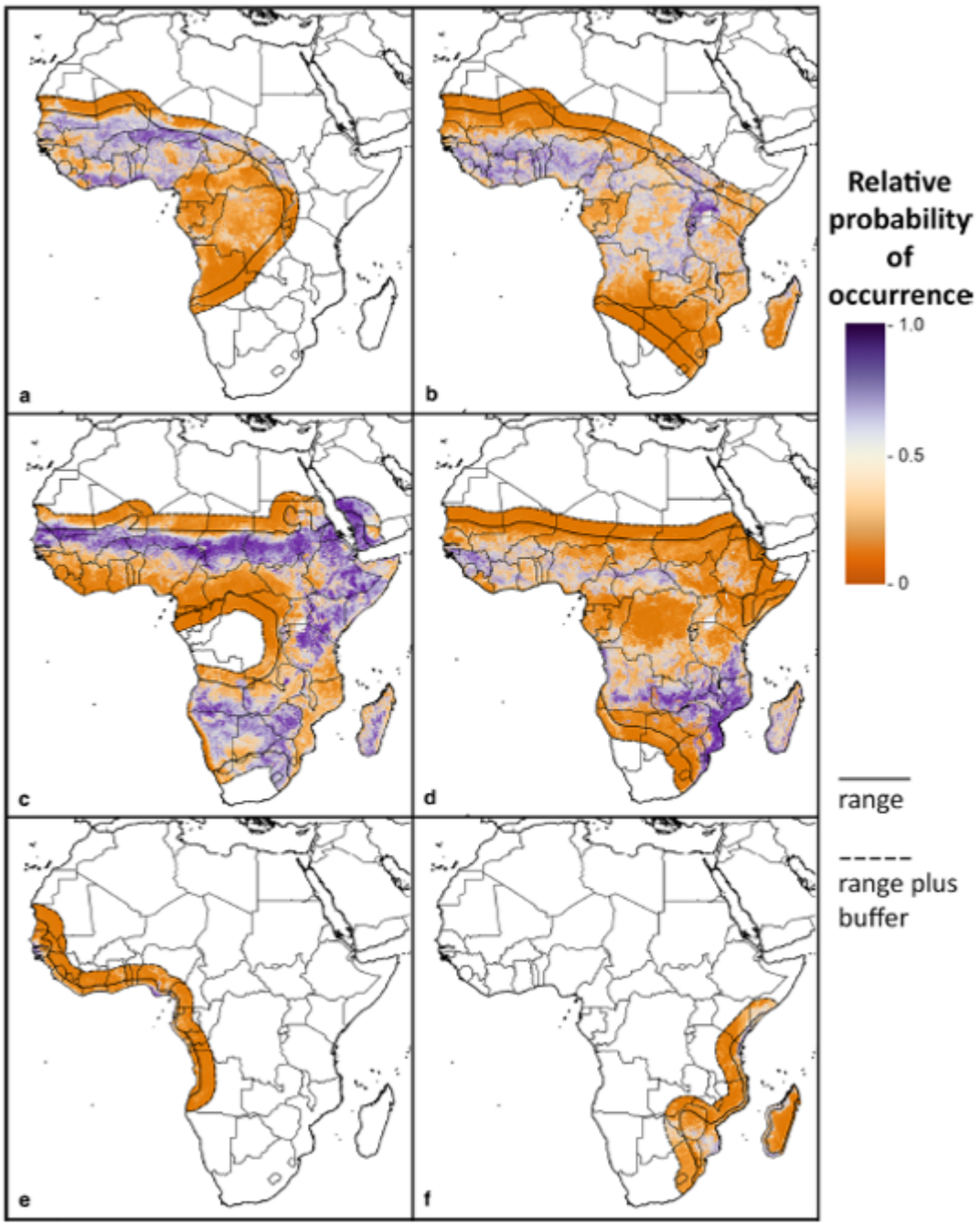


Figure 1. Predictive map for occurrence of sibling species. Relative probability of occurrence for each species is shown within its range plus a 300km buffer. (a) An. coluzzii, (b) An. gambiae, (c) An. arabiensis, (d) An. funestus, (e) An. melas, (f) An. merus (Wiebe et al., 2017).

Due to climate change, the global temperature is increasing, converting parts of the world that were never habitable to mosquitoes into more favourable climates. This could lead to an increase in population densities, shorter extrinsic incubation periods, longer disease transmission seasons and the distribution of vectors expanding to more temperate regions that are not equipped for disease control (Colón-González *et al*., 2021).

1.2 Mosquito behaviour

Mosquitoes have four distinct life cycle stages, developing from eggs to larvae and pupae and emerging as adults (Service, 2012). Mosquitoes mate soon after emergence, with females typically only mating once and storing enough sperm in the spermatheca to fertilise all eggs for a lifetime. Females are anautogenous, so they require a blood meal for vitellogenesis. Once egg development is complete, females will find a water source to lay their eggs in, where they will hatch within 1-2 days to first instar larvae (L1). Larvae feed in the water and undergo four moults before developing into the non-feeding pupal stage after around 7-8 days, depending on habitat conditions. Blood-feeding, resting post-blood meal, and subsequent oviposition is termed the gonotrophic cycle. Adult mosquitoes emerge and, depending on species, show distinct behaviours which influence their importance as vectors for disease. These include host or blood meal source preferences, whether they feed indoors or outdoors and whether they rest inside or outside to digest a meal. The entire process can take 10-23 days, depending on environmental conditions such as temperature, larval density, and nutrient availability.

1.2.1 Host-seeking behaviour

Female mosquitoes use a combination of thermal, olfactory, and visual cues to locate a host when searching for a blood meal (Bowen, 1991; McMeniman *et al.*, 2014; Takken, 1991; Zwiebel & Takken, 2004). These cues are utilised at different times during the host-seeking process (McMeniman *et al*., 2014). Over long-range host-seeking (55-70m), olfactory and visual cues play a significant role, while changes in behavioural responses at short-range utilise thermal, moisture and skin volatile cues (Cardé, 2015; J F Sutcliffe, 1994) (Figure 2).

Carbon dioxide activates mosquito flight and initiates the search for a host, with other olfactory cues coming from lactic acid, ammonia and carboxylic acids (Van Breugel, Riffell, Fairhall, & Dickinson, 2015). Females will navigate through an odour plume upwind, following a scent concentration through a flight process known as ‘casting’. While following an odour plume, females also use visual cues to aid flight towards a host (Rudolfs, 1922), assessing progress relative to cues below them (known as optomotor anemotaxis) (M. T. Gillies, 1980; Gibson & Torr, 1999). Mosquitoes have highly sensitive eyes even in low light, with diurnal species responding better to colour and brightness, while nocturnal species rely on visual contrast to assess flight progress (Allan, Day, & Edman, 1987; Bidlingmayer,

1994). Human hosts become visible when they are 5-15m away, showing that visual stimuli play an intermediate role between long-range plume tracking behaviour and short-range host cues (Van Breugel *et al*., 2015).

As a female approaches a host, short-range cues stimulate landing and probing, including heat detection and sensing volatiles from the skin, sweat and microbiota (De Jong & Knols, 1995; Gibson & Torr, 1999; Howlett, 1910; McMeniman *et al*., 2014). These short-range cues are crucial for distinguishing between anthropophilic and zoophagic preferences. In addition, the individual components of skin volatiles can result in variations in host attractiveness, resulting in the potential for different disease transmission dynamics (Smallegange, Verhulst, & Takken, 2011; Zwiebel & Takken, 2004). Some volatile organic compounds can either attract or repel mosquitoes. A study by Robinson *et al*., 2018, reported *Plasmodium*-induced increases in the attractiveness of skin odour and found that certain aldehydes produced in greater amounts by infected individuals were more favourable to host-seeking mosquitoes (Robinson *et al*., 2018). This is often termed 'deceptive signalling' where host cues favoured by host-seeking insects are exaggerated, thus increasing the host's attractiveness, even though the parasite-infected blood meal is unfavourable to the vector.

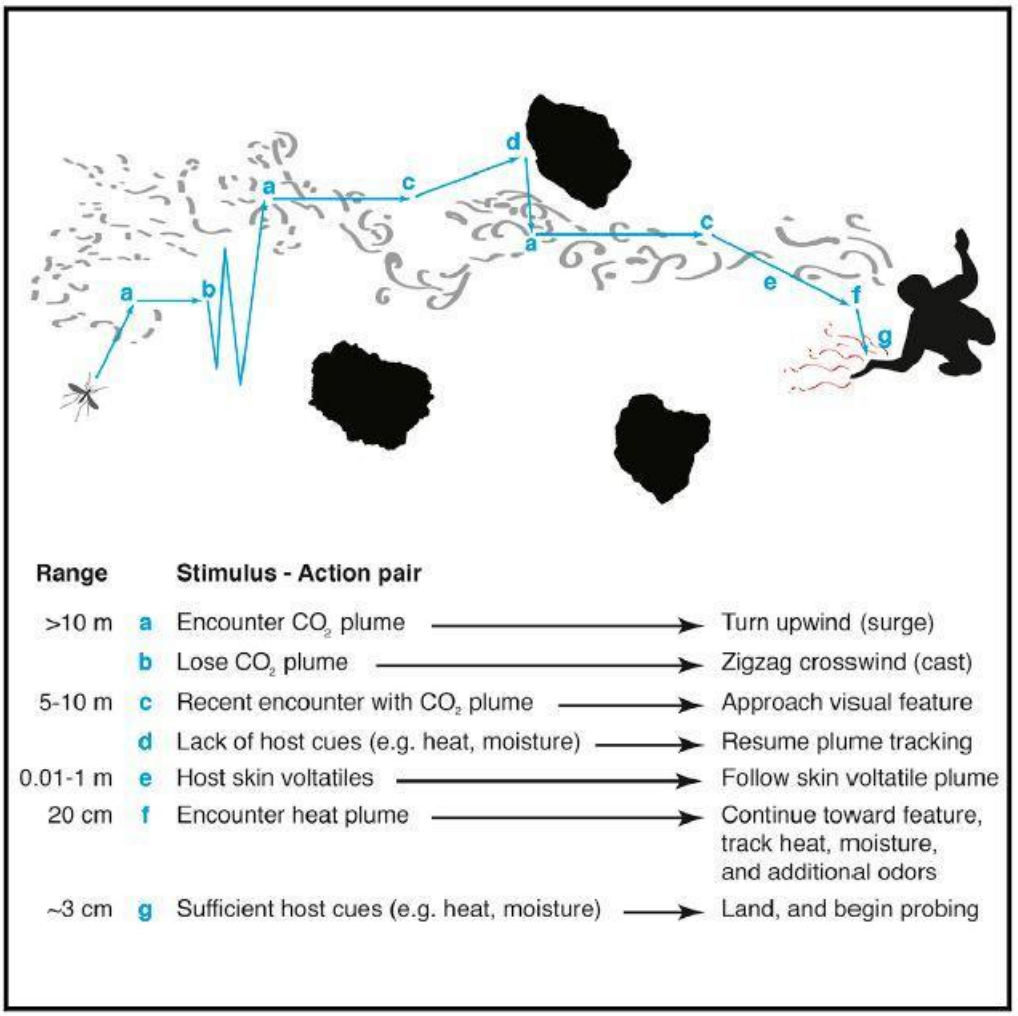


Figure 2. Sensory cues, range of detection and accompanying behaviour experienced by a female mosquito while host-seeking (Van Breugel et al., 2015).

1.2.2 Blood feeding behaviour

Female mosquitoes exhibit distinct feeding behaviours which differ between species, such as preferences for feeding on humans (anthropophagic), feeding on animals (zoophagic), feeding indoors or outdoors (endophagic/exophagic), time of day to take a meal, and resting inside or outside post blood meal (endophilic/exophilic). Most *Anopheles* species are crepuscular or nocturnal, with the most efficient vectors of disease belonging to the *gambiae* complex, which tend to bite humans indoors after 23.00. Conversely, *Aedes aegypti* are usually anthropophagic but prefer to feed by day and rest outdoors. Vector-host contact rate is a key parameter of parasite epidemiology and can vary with vector abundance (Smith *et al.,* 2007).

Females have elongated mouth parts, which have little difficulty penetrating through clothing that does not have a tight weave. When a female bites a host, her paired mandibles, maxillae, labrum and hypopharynx pierce the skin. Mosquito saliva contains antihaemostatic enzymes and anticoagulants, which both facilitate blood uptake. Once fully engorged, females will find a resting site. Blood meal digestion is temperature-dependent (Service, 2012); in the tropics usually taking between 2-3days, but in cooler climates taking between 7-14 days. Once the blood meal is digested, and eggs have fully developed, females are considered gravid and will search for a suitable oviposition site. After egg-laying, females will take another blood meal 2-3days later and start the process again. The gonotrophic cycle will be repeated several times during a female's life span.

1.2.3 Oviposition behaviour

Once gravid, females will seek out an oviposition site. As with host-seeking, mosquitoes use a range of olfactory cues to assess suitable breeding sites; sensing smells from nutrients and cues other mosquito larvae. In addition, oviposition cues are species-specific, with one site being attractive to a particular species but not to another (Afify & Galizia, 2015).

These sites vary depending on species and range from large permanent bodies of water such as marshes and rice fields to smaller, more temporary water sources such as pools, puddles, and ditches. Most water sources can provide a habitat for mosquito larvae unless they are also home to large numbers of predators, with natural containers (tree holes, bamboo stumps and split coconut shells) and artificial containers (discarded tyres, plant pots, water storage vessels) close to human dwellings being ideal breeding grounds. Again, depending on species, females will lay between 30-300 eggs in any one gonotrophic cycle, with *Anopheles* laying their eggs singularly and directly onto the water where they float.

1.3 Vectors of disease

The behaviour of mosquitoes plays a vital role in disease epidemiology, with certain specific behaviours influencing when females will encounter humans. For example, biting outdoors and late at night means endophagic vectors are more likely to bite adults and not children; however, during hot temperatures, people of all ages are more likely to sleep outside, so human contact increases. Disease transmission can occur between humans due to the

requirements of a parasite life cycle, where either development or replication needs to be completed within the mosquito.

The vast range of breeding sites that mosquitoes can oviposition in makes them an ideal vector for human-disease transmission. Large and small bodies of water around human dwellings, along with work environments such as rice fields, mean that as soon as females emerge and mate, they are near a blood meal source.

If female mosquitoes can avoid premature death from insecticide exposure or parasitic damage, then they are able to survive long enough to transmit a range of parasites. The time needed for parasites to develop within a vector is the extrinsic incubation period (EIP). Females need to take at least two blood meals to transmit disease, one to pick up an infection and one to pass it on. The risk of death before parasite transmission occurs increases if females host seek and attempt to feed before parasite development is complete. Females can lay an egg batch 2-3days after their first blood meal, and then host seek again 2-3days later for their next meal, so it would be detrimental to the parasite if the mosquito went searching for the second blood meal so soon. There have been multiple studies showing that mosquito host-seeking and blood-feeding behaviour is altered depending on the stage of parasite development (Wekesa, Copeland, & Mwangi, 1992; Anderson, Koella, & Hurd, 1999; Vézilier *et al.*, 2012).

1.3.1 Malaria

Anopheles species are vectors for malaria, lymphatic filariasis and several arboviruses. 2020 saw an estimated 241 million malaria cases across 85 disease-endemic countries, with the World Health Organisation (WHO) African Region accounting for 95% of all cases (WHO, 2021). Despite previous global reductions in malaria burden, this increased by around 14 million cases from the year before, with malaria deaths increasing by 12% in the same period. These increases are associated with disruptions in health care access and disease intervention measures caused by the COVID-19 pandemic.

Malaria is an acute illness caused by five *Plasmodium* species: *Plasmodium falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi* (WHO, 2021), with disease severity depending on species and host immunity. Parasites infect human liver cells and red blood cells to mature and replicate, where they are responsible for causing anaemia, fever, chills, headaches and muscle aches. Malaria parasites are picked up by female *Anopheles* mosquitoes when they ingest a blood meal from an infected vertebrate host containing *Plasmodium* gametocytes,

where it then takes between 9-16 days for parasites to develop within the mosquito (the extrinsic incubation period) before they become infective (Ohm *et al*., 2018; Paaijmans *et al*., 2010; Vaughan, 2007; Venugopal, Hentzschel, Valkiūnas, & Marti, 2020).

1.3.2 Lymphatic filariasis

Lymphatic filariasis (LF) is a neglected tropical disease (NTD), causing 863 million people in 50 countries to require preventative chemotherapy to stop the spread of infection (WHO, 2022). LF is the second largest cause of permanent and long-term disability worldwide, with an estimated loss of 2.8 million disability-adjusted life-years (DALYs). Symptoms can be incredibly painful and disfiguring and include lymphoedema, elephantiasis and the enlargement of multiple body parts, all of which can lead to not only physical disability but impact mental health and a person's ability to work, resulting in monetary losses. Decreasing the burden of the disease will improve quality of life and help reduce poverty. While infection can lead to acute or chronic disease, many people remain asymptomatic and fuel community transmission of the parasite.

Three filarial nematode species are responsible for causing LF: *Wuchereria bancrofti* (responsible for 90% of all cases), *Brugia malayi* and *Brugia timori.* Adult worms will live in the lymphatic vessels of humans for up to eight years, during which time they produce and release millions of microfilariae (mf) into the blood. Disease transmission is indirect, with parasites developing within a mosquito vector before being transmitted to the definitive vertebrate host. This requires a mosquito to ingest a blood meal from a human with circulating mf in their peripheral blood. Mf then escape out of the midgut by penetrating the midgut wall, upon which they migrate to the thoracic muscles and undergo two larval moults to become the infective L3 stage. Finally, the infective stage moves to the head, where they will burst out of the mouthparts when the mosquito takes its next blood meal, actively penetrating the bite site to enter the host bloodstream. Unlike malaria parasites, there is no parasite reproduction within the mosquito, so infective L3 numbers are limited on initial mf uptake (Figure 3).

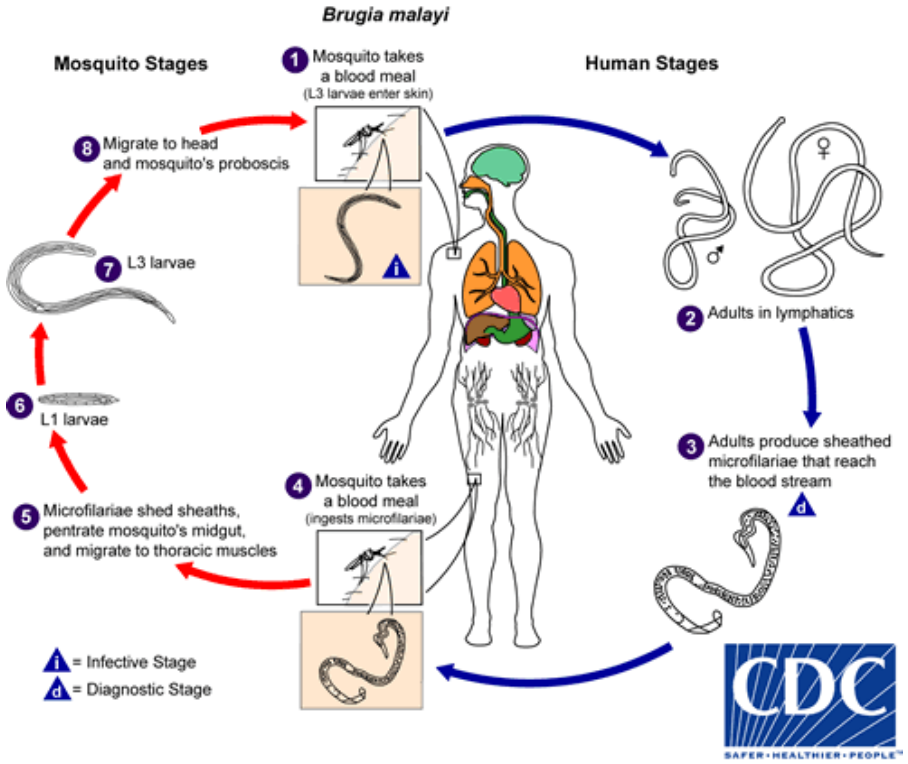


Figure 3. The life cycle of Brugia malayi. (https://www.cdc.gov/parasites/lymphaticfilariasis/biology_b_malayi.html)

Different mosquitoes from the four major genera *Culex*, *Anopheles*, *Aedes* and *Mansonia* can transmit LF, but susceptibility differs between species due to multiple barriers to parasite development, such as the cibarial armature found in *Anopheles* species. In addition, filarial worm development is also highly damaging to the mosquito, with midgut penetration, movement through the flight muscles and consumption of energy reserves for moulting through life stages all taking a toll on mosquito fitness and health.

1.4 Conventional vector control

With parasite development occurring within the vector, interventions that shorten the lifespan of mosquitoes will reduce vectorial capacity and disease transmission. Vectorial capacity is a measure of transmission potential of vector-pathogen systems and describes the total number of potentially infectious bites that would arise from all the mosquitoes biting a single infectious human on a single day (Garrett-Jones ', 1964; Macdonald & Director, 1956) (Figure 4). An infectious person will be subject to the attention of '*m*'

mosquitoes (if we assume that everyone is equally attractive to mosquitoes) and will receive '*ma*' bites each day. For mosquitoes to become infectious they must survive the extrinsic incubation period (EIP, the time it takes for a vector to become infectious with probability 'P^n'). Adult mosquitoes on average live for '*1/(-lm(p)*' days of biting, and potentially infecting, humans at a rate of '*a*' per day. Changes in vector fitness and transmission dynamics can change depending on vector competence, with not all mosquitoes that are exposed being able to transmit pathogens effectively. To be effective, control measures need to be in line with mosquito behaviour, for example, targeting endophagic and endophilic species using interventions in the home, which are different to those that can be used outside to target exophilic and exophagic vectors. Reducing the vector population has proven to be a very effective measure for disease prevention, with the main method being the use of insecticide-treated nets (ITNs) (Bhatt *et al*., 2016; Pryce, Richardson, & Lengeler, 2018). By 2020, 65% of households in sub-Saharan Africa had at least one insecticide-treated net (ITN) (WHO, 2021) (Figure 4). ITNs provide a physical barrier to reduce biting and contain an insecticide to kill mosquitoes. By inducing mosquito mortality, ITNs will reduce vector density and reduce the age structure of a population, which will contribute to 'community protection' where people without an ITN still benefit from others using them (Hawley *et al*., 2003).

$$\text{Vectorial capacity} = \frac{ma^2 \times VC \times P^n}{-\log_e P}$$

m = vector density in relation to the host
a = probability of host being fed upon
VC = vector competence
n = extrinsic incubation period
P = probability of daily survival

Figure 4. Vectorial capacity equation

Pyrethroids are the most commonly used insecticides to treat ITNs, and prior to 2017, they were the only class approved for use due to their low mammalian toxicity but rapid

insecticidal activity. Pyrethroids are neurotoxins that target an insect's peripheral and central nervous system, altering the voltage-gated sodium channel (*Vgsc*), which causes the repeated, uncontrollable firing of neurons, leading to paralysis and ultimately death (Bloomquist, 1996; Davies *et al*., 2007; Soderlund *et al*., 2002).

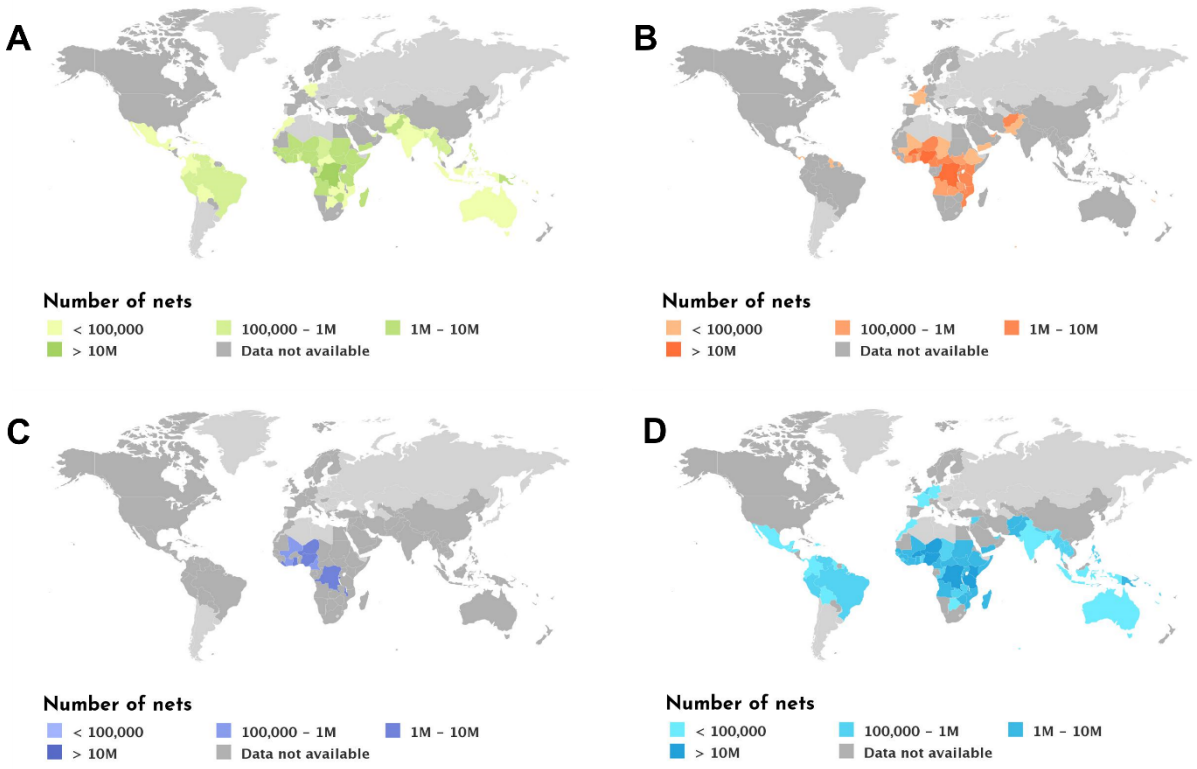


Figure 5. The cumulative number of insecticide-treated nets shipped worldwide by 2021. (A) standard pyrethroid only nets, (B) pyrethroid-PBO nets, (C) new active-ingredient nets, and (D) all net types. (sourced from The Alliance for Malaria Prevention, netmappingproject.allianceformalariaprevention.com/).

1.5 Resistance to standard insecticides

Despite substantial improvement in reducing clinical incidences of mosquito-borne diseases, progress has stalled in recent years (WHO, 2019). When mosquitoes can survive exposure to a previously determined standard dose of insecticide, due to physiological or behavioural mechanisms, they are deemed to be insecticide-resistant (WHO, 2016). We can now see an association between the increase in the mass distribution of ITNs across Africa and a rise in mosquito insecticide resistance during the same period (H Ranson & Lissenden, 2016; WHO, 2018).

Unfortunately, resistance to pyrethroids is now widespread in African malaria vectors and threatens vector control interventions' operational success. Resistance to pyrethroids was first recorded in *An. gambiae s.l* (Elissa *et al.*, 1993) and *An. funestus* (Hargreaves *et al*., 2000) 30 years ago and in the past ten years, 78 malaria-endemic countries have reported resistance to at least one insecticide class in at least one malaria vector (Figure 5). Nineteen of these have recorded resistance to all four classes of insecticide approved for use (pyrethroids, organochlorines, carbamates and organophosphates) (WHO, 2021). A multi-country trial (Kleinschmidt *et al*., 2018) recently showed that the personal protective qualities of ITNs were not impacted by insecticide resistance; however, it is accepted that resistance in major disease vectors will eventually weaken the efficacy of pyrethroid-only ITNs and threatens the operational success of many vector control programmes (Figure 6). This has led to innovative new bed nets becoming essential to overcome the threat of resistance and continue to protect millions of people.

The mechanisms leading to insecticide resistance in mosquitoes can be split into four main groups; target site, metabolic, cuticular and behavioural resistance.

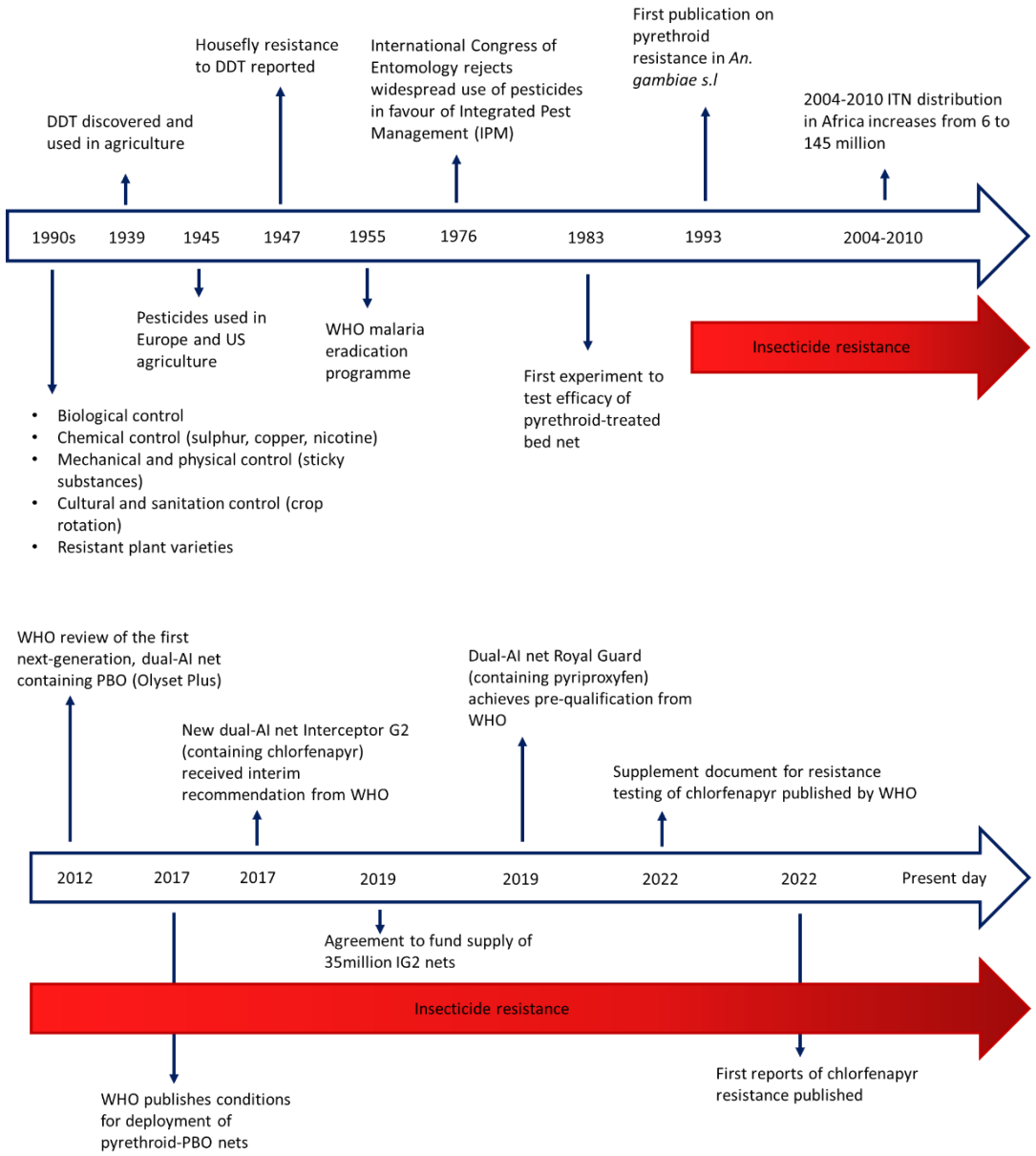


Figure 6. Timeline of insecticide use for agricultural and public health purposes.

1.5.1 Target-site resistance

Target-site resistance involves genetic point mutations that alter the site that insecticides bind to, with the commonly reported target site mutations in *Anopheles* being in acetylcholinesterase (resistance to organophosphates and carbamates) and the voltage-gated sodium channel (*Vgsc*) (resistance to pyrethroids and DDT). In *An. gambiae* and *An. coluzzii* there are three mutations (L99SF, L99SS, N1570Y) in the *Vgsc* that are frequently documented to cause resistance (Jones *et al*., 2012; Martinez-Torres *et al*., 1998; H Ranson *et al*., 2000; Silva, Santos, & Martins, 2014).

1.5.2 Metabolic resistance

Metabolic resistance acts by increasing the sequestration rate of insecticides before they can reach their target site within an insect through alterations or increases in detoxification and/or metabolism enzymes. The three enzyme groups that have been highly studied in relation to this form of resistance are the carboxylesterases (COEs), glutathione S-transferases (GSTs) and cytochrome p450s (p450s), with the latter well categorised in *An. gambiae* (Adolfi et al., 2019; Müller et al., 2008; Stevenson et al., 2011).

1.5.3 Cuticular resistance

Insecticides used on ITNs or treated surfaces, as with indoor residual spraying (IRS), must first penetrate through the cuticle of mosquitoes before they can reach their target site. Therefore, alterations in cuticle thickness or composition can affect how well insecticides can enter inside the mosquito and is termed cuticular resistance (Balabanidou et al., 2016; Wood *et al.,* 2010). Cuticular resistance is measured using insecticide penetration assays or by comparing cuticle thickness between different mosquitoes species (Yahouédo *et al*., 2017).

1.5.4 Behavioural resistance

Any alterations to standard mosquito behaviour, such as a change in biting time, biting location or host preference, can reduce the chance of females encountering insecticides and limit the success of control measures (Gatton *et al*., 2013; Killeen, 2014; Killeen *et al*., 2006; Pates & Curtis, 2005). Following mass ITN distribution campaigns, multiple studies have shown a shift in biting time to earlier in the evening or morning when people are less likely to be under the protection of their bed nets (Moiroux *et al*., 2012; Thomsen *et al*., 2017), as well as changes in host preferences (Charlwood & Graves, 1987). Behavioural resistance can be challenging to quantify as it requires studies conducted over a long time frame that describe and quantify mosquito behaviour before and after a vector control intervention, and collect regular data on species-specific identification as sibling species will impacted by interventions in different ways.

Studies looking at the effects of insecticide resistance on behavioural alterations are limited and the topic requires further investigation.

1.5.5 Insecticide resistance monitoring

Insecticide resistance of a population is a constantly evolving state, but resources to monitor vast numbers of mosquito populations simultaneously are not readily available. Entomological data is necessary to determine when and where to employ new ITNs. Standard resistance surveillance methods include the use of WHO tube assays, CDC bottle bioassays and WHO cone assays (ITNs). These methods can be straightforward, but complications can arise when we consider the large numbers of mosquitoes required, the various insecticides to test (including synergist testing), the need for resistance mechanism testing and the importance of standardised protocols and reporting across multiple sites.

1.6 Next-generation bed nets

In response to insecticide resistance, bed net manufacturers have developed 'next generation' ITNs, which contain a pyrethroid insecticide plus an additional chemistry. These next generation nets include dual-active ingredient (AI) ITNs which contain an additional insecticide that has a different mode of action (MoA) to pyrethroids, ITNs that have a synergist incorporated into the net to target enzymes in the mosquito responsible for resistance, or nets that contain chemicals to sterilise adult female mosquitoes. There are currently 23 LLINs with WHO Prequalification (PQ) listing, eight of which are defined as next-generation nets (WHO, 2019a) (Table 1). Of these eight, six contain the insecticide synergist piperonyl butoxide (PBO), one contains the growth hormone regulator pyriproxyfen, and one is impregnated with the non-pyrethroid insecticide chlorfenapyr.

Table 1. List of WHO Prequalified Insecticide Treated Nets.

Product name	Manufacturer	Active ingredient	Date of prequalification
DuraNet LN ®	Shobikaa Impex Private Ltd	Incorporated net, Alpha-cypermethrin (150D, 5.8g/kg)	Dec 2017
DuraNet Plus ®	Shobikaa Impex Private Ltd	Incorporated net, Alpha-cypermethrin (150D, 6.0g/kg), Piperonyl Butoxide (2.2g/kg)	Aug 2020

Interceptor®	BASF AGRO B.V Arnhem (NL) Freienbach Branch	Coated net, Alpha-cypermethrin (75D, 6.7g/kg) (100D, 5.0g/kg)	Dec 2017
Interceptor G2 ®	BASF AGRO B.V Arnhem (NL) Freienbach Branch	Coated net, Alpha-cypermethrin (75D, 3.2g/kg; 100D, 2.4g/kg), Chlorfenapyr (75D, 6.4g/kg; 100D, 4.8g/kg)	Jan 2018
MAGNet LN ®	V.K.A Polymers Pvt. Ltd	Incorporated net, Alpha-cypermethrin (150D, 5.8g/kg)	Feb 2018
MiraNet®	A to Z Textile Mill Ltd	Incorporated net, Alpha-cypermethrin (130D, 4.5g/kg)	Feb 2018
OLYSET Net ®	Sumitomo Chemical Co., Ltd	Incorporated net, Permethrin (150D, 20g/kg)	Dec 2017
OLYSET PLUS ®	Sumitomo Chemical Co., Ltd	Incorporated net, Permethrin (150D, 20g/kg), Piperonyl Butoxide (10g/kg)	Jan 2018
Panda Net ®	Life Ideas Biotechnology Co., Ltd	Incorporated net, Deltamethrin (120D, 1.8g/kg)	May 2018
PermaNet 2.0 ®	Vestergaard Sarl	Coated net, Deltamethrin (75D, 1.8g/kg; 100D, 1.4g/kg; 150D, 1.4g/kg)	Dec 2017
PermaNet 3.0 ®	Vestergaard Sarl	Coated net, Deltamethrin (roof: 100D, 4.0g/kg; sides: 75D, 2.8g/kg; 100D, 2.1g/kg; 150D, 2.1g/kg), Piperonyl Butoxide (roof: 25.0g/kg)	Jan 2018
Reliefnet Reverte™	Real Relief Health ApS	Incorporated net, Deltamethrin (120D, 1,8g/kg)	Jan 2021
Royal Guard®	Disease Control Technology LLC	Incorporated net, Alpha-cypermethrin (120D, 5.5g/kg; 150D, 5.0g/kg), Pyriproxyfen (120D, 5.5g/kg; 5.0g/kg)	Mar 2019
Royal Sentry®	Disease Control Technology LLC	Incorporated net, Alpha-cypermethrin (150D, 5.8g/kg)	Dec 2017
Royal Sentry 2.0 ®	Disease Control Technology LLC	Incorporated net, Alpha-cypermethrin (120D, 5.8g/kg)	Feb 2019
SafeNet®	Mainpol GmbH	Coated net, Alpha-cypermethrin (75D, 6.7g/kg)	Feb 2018

Tsara®	Moon Netting FZCO	Incorporated net, Deltamethrin (120D, 2.2g/kg)	Aug 2020
Tsara Boost ®	Moon Netting FZCO	Incorporated net, Deltamethrin (130D, 3.0g/kg), Piperonyl Butoxide (11.0g/kg)	Jan 2018
Tsara Plus ®	Moon Netting FZCO	Incorporated net, Deltamethrin (roof: 130D, 3.0g/kg; sides: 100D, 2.5g/kg), Piperonyl Butoxide (11.0g/kg)	Jan 2018
Tsara Soft ®	Moon Netting FZCO	Incorporated net, Deltamethrin (75D, 2.7g/kg; 100D, 2.0g/kg; 150D, 2.0g/kg)	Oct 2020
VEERALIN ®	V.K.A Polymers Pvt. Ltd	Incorporated net, Alpha-cypermethrin (130D, 6.0g/kg), Piperonyl Butoxide (2.2g/kg)	Jan 2018
Yahe LN ®	Fujian Yamei Industry & Trade Co. Ltd	Coated net, Deltamethrin (50D, 2.3g/kg; 75D, 1.85g/kg; 100D, 1.4g/kg)	Feb 2018
Yorkool LN ®	Tianjin Yorkool International Trading Co., Ltd	Coated net, Deltamethrin (75D, 1.8g/kg; 100D, 1.4g/kg; 150D, 1.4g/kg)	Feb 2018

(Note: D = denier)

1.6.1. Pyrethroid-PBO nets

Pyrethroid-PBO nets are one example of a dual AI ITN as they contain a pyrethroid and the synergist PBO. Synergists are generally non-lethal themselves, but PBO improves the efficacy of these ITNs as it specifically targets the metabolic enzymes within the mosquito that are responsible for resistance to pyrethroids. By inhibiting the action of these enzymes, the lethal action of the pyrethroid on the bed net can be restored. PBO targets the metabolic enzymes cytochrome p450s (p450s), which in resistant mosquito populations typically sequester or detoxify pyrethroids and inhibit their neurotoxic action.

Pyrethroid-PBO nets vary in their design and AI concentration depending on the manufacturer. Some have PBO throughout all parts of the net (Olyset Plus), while others only contain PBO on the roof of the net (PermaNet 3.0), working on the results from studies that have shown the majority of mosquito contact with a bed net occurs on the roof (Lynd & Mccall, 2013). Differences between manufactured nets make it challenging to directly compare the efficacy of these ITNs. However, the results from recent cluster-randomised

controlled trials in Tanzania (Protopopoff *et al*., 2018) and Uganda (Staedke *et al*., 2020), where mosquito populations have high levels of resistance to pyrethroids, have shown that pyrethroid-PBO nets reduced malaria parasite prevalence by 60% in Tanzania and 17% in Uganda two-years post-deployment. Using the results from these trials, the WHO has now released new guidelines stating that pyrethroid-PBO nets are recommended in places that meet the following criteria: pyrethroid resistance that results in 10-80% mortality in susceptibility tests, which is conferred at least in part by monooxygenase-based resistance mechanisms (WHO, 2017). There is a need for more data on comparisons of different net types, with standardised testing being used throughout studies, alongside results collected on both epidemiological and entomological outcomes to help feed into malaria transmission models (Churcher, Lissenden, Griffin, Worrall, & Ranson, 2016) and better equip control programmes.

Mosha *et al*., (2022) have been undertaking a cluster-randomised trial, again in Tanzania, to investigate the effectiveness and cost-effectiveness of three types of dual active-ingredient bed nets in comparison with pyrethroid-only ITNs. In this study, the pyrethroid-PBO nets showed increased effectiveness compared to standard nets, but this was sustained for a shorter time than in the previous Protopopoff *et al*., (2018) trial. One explanation for this is that net use declined more quickly in the latest study, which the authors state may have been a consequence of a high proportion of the nets being more torn than the other LLINs, and they recommend that manufacturers need to improve the physical integrity of these nets.

1.6.2 Interceptor G2

The ITN currently on the market containing an additional, non-pyrethroid insecticide is Interceptor G2 (IG2) (BASF). IG2 combines the pyrethroid alpha-cypermethrin (100mg/m^2) alongside chlorfenapyr (200mg/m^2). Chlorfenapyr is from the pyrrole class of insecticides and is a broad-spectrum pro-insecticide that shows stomach and contact toxicity in insects by acting at the cellular level to disrupt respiratory pathways and proton gradients. This pro-insecticide requires initial activation by mixed-function oxidases to produce the active compound. Oxidative removal of the N-ethoxymethyl group of chlorfenapyr leads to the toxic form identified as CL 303268, which functions to uncouple oxidative phosphorylation in the mitochondria, resulting in disruption of adenosine triphosphate (ATP) production and loss of energy leading to cell dysfunction and subsequent death of the mosquito (Black *et al*., 1994; Treacy *et al*., 1994).

The above-mentioned study by Mosha *et al*., (2022) examined chlorfenapyr nets and those containing either PBO or pyriproxyfen and reported that chlorfenapyr nets are a safe, effective and cost-effective alternative to standard pyrethroid-only ITNs. In a highly pyrethroid-resistant setting, chlorfenapyr nets provided significantly better protection over two years than a standard pyrethroid net, with children aged six months to ten years having a 44% lower malaria incidence. The entomological inoculation rate (EIR) was 85% lower, arising from a reduced vector population density and reduced longevity.

Unfortunately, it has not proven easy to replicate these results within a laboratory setting. There are challenges to finding a laboratory assay predictive of the mortality levels observed in experimental hut studies, with studies reporting a failure to reach 100% mortality in pyrethroid susceptible An. gambiae Kisumu strain (Camara *et al*., 2018; N'Guessan *et al.,* 2016). This has proven confusing, as the alpha-cypermethrin content within these nets should still be effective at killing susceptible mosquitoes, despite being lower than the pyrethroid only counterpart Interceptor G1. There is currently no easily accessible WHO recommended protocol for testing chlorfenapyr on ITNs, but the overnight tunnel test using an animal bait has shown promising results when other method such as cone and tube tests do not perform well. However, Oxborough *et al*., (2015) performed work under different conditions and stated that temperature, length of insecticide exposure, time of day and exposure method could all have different effects on the mortality results (Oxborough *et al*., 2015). There is currently limited knowledge on the bioefficacy of pyrethroids and chlorfenapyr when used in combination on a net or their impact on mosquito life-history traits and behaviour.

To date, there is no published evidence of any cross-resistance with chlorfenapyr; however, activation to the lethal form (tralopyril) requires mixed-function oxidases that are upregulated in some resistant populations giving the potential for negative cross-resistance to arise. As these nets are being used across Africa, insecticide resistance needs to be closely monitored and planned into control programmes, with susceptibly to chlorfenapyr currently being confirmed using CDC bottle bioassays.

1.6.3 Royal Guard

Pyriproxyfen (PPF) is an insect juvenile hormone analogue which interferes with reproduction and the development of mosquitoes by inhibiting embryogenesis and metamorphosis (Dhadialla, Carlson, & Le, 1998). PPF has been shown to inhibit oogenesis

and sterilise adult female mosquitoes (Harris *et al*., 2013; Ohashi *et al*., 2012), meaning that adults can no longer contribute to the next generation of potential disease-transmitting vectors. Previous hut trials in Benin and Cote d'Ivoire using one type of pyrethroid-PPF net, Olyset Duo, have shown that nets provided personal protection through the excito-repellent property of the included pyrethroid and sterilisation of female mosquitoes which survive exposure to pyrethroids due to resistance (Ngufor *et al*., 2014), and also protected against clinical malaria compared to pyrethroid only ITNs in areas of high *Plasmodium falciparum* transmission (Tiono *et al.,* 2018). Royal Guard is a newer pyrethroid-PPF net created by Disease Control Technologies (DCT) and has the pyrethroid alphacypermethrin and PPF incorporated into the net fibres. Laboratory studies have shown to induce >80% mortality and sterilised mosquitoes that survived after exposure. In experimental hut trials in Benin, Royal Guard nets gave an 83% reduction in mosquito oviposition rate and a 95% reduction in viable offspring when unwashed (Ngufor *et al*., 2020). Current work is ongoing to establish the regeneration time of the Royal Guard after the nets have been washed three times to ensure that PPF returns to the nets surface in amounts capable of inducing oviposition inhibition (Lees *et al*, in progress).

1.7 The challenges of evaluating new control tools

Understanding insecticide mode of action (MoA) is critical for assays to measure ITN efficacy and durability accurately. The WHO is responsible for evaluating VC products for global use, and in January 2017, the WHO Pesticide Evaluation Team (WHOPES) changed to the WHO Prequalification Team Vector Control Group (PQT-VC). All ITN manufacturers must submit documents on the efficacy, safety and content of their products for this panel to review. If a product does not fall within a current class of net type that is already predetermined, then it requires epidemiological evidence of its efficacy. For the evaluation of ITNs, products undergo laboratory testing, small-scale trials and large-scale trials and must pass through a set of criteria before moving on to the next testing phase. For example, in Phase I laboratory studies, mortality must exceed 80% in a standard WHO cone bioassay after washing 20times > in Phase II nets must perform as well, or better than, those currently on the marker > and for Phase III nets that are collected after three years of use must retain an efficacy of more than 80% mortality on WHO cone tests. Laboratory trials are performed under strict, standardised conditions and include the WHO cone bioassay and tunnel test (WHO, 2013). These bioassays measure the efficacy, wash resistance and

insecticide regeneration time of ITNs by measuring knock-down at 60minutes and mortality at 24hours. The tunnel test also measures blood-feeding inhibition, as mosquitoes have access to a live animal to feed on if they pass through holes in the net. Similarly, small-scale studies using experimental huts collect data on wash resistance and efficacy by examining 24hour mortality and blood-feeding inhibition, but they also record mosquito deterrence and exophily. The last phase of prequalification testing is large-scale village trials which assess ITN efficacy over multiple time points (bed nets should have a field life of three years), loss or attrition of nets, durability and community perceptions of bed nets.

Pyrethroid only ITNs are well categorised, and the bioassays to study their efficacy are well understood and used globally. However, new active-ingredients with different MoAs used on next-generation nets may require different testing methods. For example, the synergist PBO used to improve the efficacy of pyrethroids is generally itself not insecticidal, so a susceptible strain must be tested alongside a resistant strain to ensure that we can detect the effect of PBO separate to the pyrethroid as the measured outcome for both is mortality. For other MoAs, we need to consider measuring endpoints that are not considered in previous protocols, such as delayed mortality (mortality after 24hours) and impact on fecundity (egg laying, larval hatching).

Recent work by Lissenden *et al*., (2022) has produced consensus standard operating procedures (SOPs), through collating and interrogating several different assay methods and working with multiple partners, to evaluate the biological durability of new ITNs (Lissenden *et al*., 2022). These SOPs explain how a large number of factors need to be considered and include but are not limited to: temperature and humidity, time of day (light/dark cycle for mosquitoes), acclimatisation of nets and mosquitoes, recorded details of larval collection sites, details of the location of the sample taken from net (to account for some ITNs containing different concentration of AIs on different areas, like PermaNet 3.0), the number of samples tested and the number of replicates performed. The processes used must allow data to be generated which is directly linked to how the MoA functions, and to encourage novel technologies, we need to keep an open mind to new testing methods.

As resistance status, resistance mechanisms, and intensity vary between mosquitoes over time, ITN evaluation methods must consider this when deciding which populations to utilise (Lees *et al*., 2022). The Vector Control Advisory Group (VCAG) proposed criteria on suitable mosquitoes to test; at three strains, of which two must have different metabolic resistance, showing a range of mechanisms and have a resistance level greater than ten-fold that of the

susceptible colony (VCAG, 2015). However, as the list of different resistance mechanisms is already long and continues to increase with the discovery of more, covering all of these different requirements in only three strains will be a challenge, and adding more strains would increase the workload for each new net tested.

1.8 Impacts of insecticide resistance and infection on behaviour

While insecticide resistance reduces the impact that control methods have on vector populations, it is essential to consider what effects resistance can have on the mosquito. Removing insecticide pressures from an area results in lower resistant allele frequencies in populations, suggesting that it is costly for mosquitoes to maintain resistance in the absence of insecticides (David *et al.,* 2018). Resistance mechanisms can cause multiple alterations in key physiological functions, such as depleting energy reserves, affecting larval development time (Rahim, Ahmad, & Maimusa, 2017; Ramos *et al*., 2018) and impacting vector immune responses (Vontas *et al*., 2005).

Lipids and glycogen are necessary energy resources, being used for short and long-range flight, vitellogenesis, oogenesis, larval moulting and while undergoing an immune response (Beenakkers, Horst, & Marrewijk, 1981; Steele, 1981). However, the elevated enzyme activity involved in metabolic resistance can be energetically costly, diverting these resources to be used for the metabolism and detoxification of insecticides (Saingamsook *et al,*, 2019), with lipids being used for amino-acid synthesis. Multiple studies (Diniz *et al*., 2015; Martins *et al*., 2012; Viana-Medeiros, Bellinato, Martins, & Valle, 2017) have reported that temephos resistant females produce smaller egg batches than susceptible controls. Considering the impact on male fecundity, Belinato *et al*., (2012) found that temephos resistant *Aedes aegypti* had a significantly reduced frequency of female insemination compared to their susceptible counterpart, and this effect was more pronounced with a higher resistance ratio (Belinato, Martins, & Valle, 2012).

The effects of insecticide resistance may not always have negative physiological impacts. For example, Chan & Zairi, (2013) found that permethrin resistant Aedes albopictus survived for longer under harsh rearing conditions with reduced food, and produced larger females when larvae were reared at crowded densities. Increasing longevity in resistant mosquitoes increases the likelihood of surviving through a parasite extrinsic incubation period, improving vectorial capacity.

When studying parasites and vectors within the environment, there are multiple factors to consider: how different parasites interact with various vector species and hosts, what impact changes in environmental conditions between different geographical areas have, and how larval crowding and competition for nutrients affect vectorial capacity? (Figure 7).

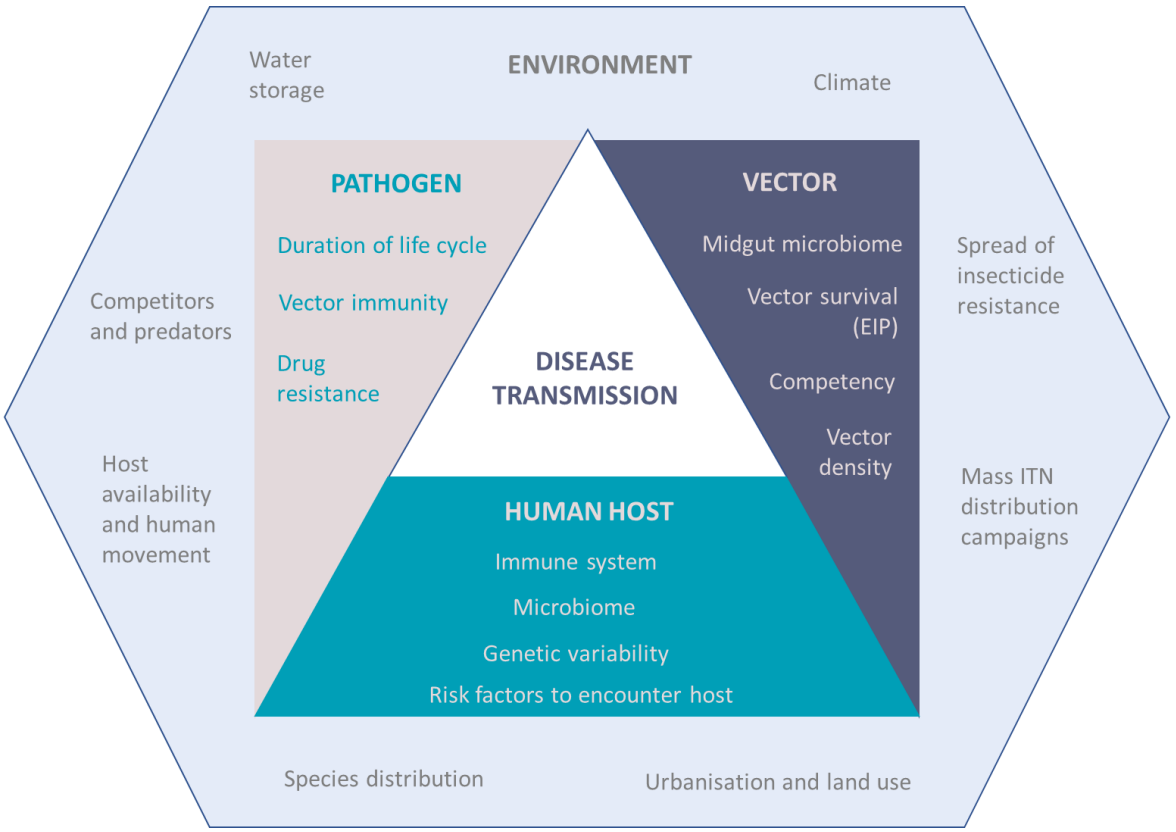


Figure 7. Interactions to consider for vector-parasite disease transmission.

Parasite infection can cause mechanical damage to mosquitoes through tissue destruction and can also lead to an increased susceptibility to other infections, a reduction in energy content (lipid and glycogen reserves) and changes in the immune response. Melanisation is one example of an innate immune response whereby filarial worms are encased and melanised, stopping their development within the mosquito; however, this is specific to certain species, and little is known on why it occurs in some and not others.

The relationship between mosquitoes and parasites can result in different transmission outcomes, which is particularly relevant for LF transmission. For example, anopheline mosquitoes are generally categorized as poor LF vectors due to their inability to support filarial worm development at low densities because of the damage caused by cibarial

armature. However, work performed by Erickson *et al*., (2013) investigated mosquito survivorship post-exposure to filarial parasites and saw more significant mortality in *An. farauti* at high mf densities compared to low mf densities, with mosquito survival being no different to that of the uninfected controls and reaching 14DPE (when infective L3 are present). This suggests that despite not being the 'ideal' vectors for transmission, those parasites that do avoid damage do not cause increased mortality to the vector, so transmission can be sustained at low parasite levels.

Long-lived mosquitoes that survive that extrinsic incubation period of parasites maximise the chance of disease transmission, which is an essential consideration for control programmes. While some studies show that infection leads to a shorter lifespan, others have demonstrated that fecundity is reduced instead, implying an adaptive strategy to divert resources to longevity. Vézilier *et al*., (2012) demonstrated that the number of eggs laid was strongly dependent on whether females were infected with *Plasmodium* parasites or not, with those harbouring an infection producing a significantly smaller egg batch. We have discussed how behavioural resistance can cause shifts in biting times, but we also need to consider how parasite infection could affect mosquito feeding and how this would alter disease dynamics. A study looking at biting behaviour (Anderson *et al*., 1999) showed that in *An. stephensi*, feeding persistence decreased in the presence of malaria oocysts (non-transmissible stage) but increased when parasites had developed in the transmissible sporozoite stage.

Similarly, Wekesa *et al*., (1992) observed that malaria-infected females probed for nearly twice as long as uninfected females. One hypothesis for this is that salivary gland apyrase functions as an anticoagulant that minimises the time needed to take a blood meal, but this apyrase function is reduced in mosquitoes when they harbour a *Plasmodium* infection, thus increasing probing and feeding time. Again, these behavioural modifications could increase the chance of multiple contacts with a host, or contact with multiple hosts, when parasites are in the salivary glands increasing the risk of transmission.

Cator *et al*., (2013) demonstrated that mosquitoes with an early-stage *Plasmodium* infection showed reduced attraction to a human host, whereas those with a late-stage infection showed increased attraction towards the host compared to controls. However, further studies found that the observed behavioural changes could also be generated by an immune challenge and were hence not explicitly linked to the presence of *Plasmodium* parasites. Heat killed *E.coli* displayed the same behavioural alterations as those observed with malaria parasites suggesting that altered behavioural phenotypes could arise from

host resource allocation amongst immunity, blood-feeding and reproduction that is not specific to *Plasmodium* infection (Cator *et al*., 2013).

The variety of potential alterations and differing results between studies highlights the importance of taking multiple endpoint measures and considering a range of factors when planning experiments to assess mosquito behaviour and fitness.

Investigating the impact of insecticide resistance and parasite infection is becoming increasingly important as we move towards elimination. Transmission dynamics such as vector biting rate and parasite density depend on the vector, parasite and interactions between them. Many models assume that uninfected mosquitoes share the same fitness costs as infected counterparts and do not take into consideration the possible negative or positive effects of an infection or insecticide selection phenotypes.

Aims and objectives

This project investigated the effects of insecticide resistance and parasite infection on the behaviour of mosquitoes. The specific aims were:

1. To document the spread of insecticide resistance in the main malaria vectors in Africa by quantifying and mapping the spatial distribution of resistance phenotypes and genotypes.

2. To evaluate the epidemiological and entomological efficacy of next-generation ITNs through a meta-analysis of experimental hut studies and cluster-randomised controlled trials with data on pyrethroid-PBO nets.

3. To measure the efficacy of next-generation ITNs compared to standard pyrethroid-only nets and assess the effect of insecticide resistance on mosquito behaviour using a room-scale tracking system.

4. To study the impact of insecticide and parasite exposure on mosquito physiology and behaviour using laboratory bioassays.

Summary of studies

To achieve the aim of the thesis, this body of work was completed between 2014 – 2022 (Table 2) with publication dates ranging from 2016 – 2022. A full list of author's publications can be found in Appendix 1.

Table 2. Characteristics of the publications submitted as part of this thesis

Chapter	Number	Title	Journal and date	Author list
1	1	Developing global maps of insecticide resistance risk to improve vector control	Malaria Journal February 2017	M. Coleman J. Hemingway **K. Gleave** A. Wiebe P. W. Gething C. L. Moyes
	2	Analysis-ready datasets for insecticide resistance phenotype and genotype frequency in African malaria vectors	Nature Scientific Data July 2019	C. L. Moyes A. Wiebe **K. Gleave** A. Trett P. A. Hancock G. G Padonou M. S. Chouaibou A. Sovi S. A. Abuelmaali E. Ochomo C. Antonio-Nkondjio D. Dengela H. Kawada R. K. Dabire M. J. Donnelly C. Mbogo C. Fornadel M. Coleman
2	3	Piperonyl butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa	Cochrane Database of Systematic Reviews May 2021	**K. Gleave** N. Lissenden M. Chaplin L. Choi

				H. Ranson
3	4	Behaviour of pyrethroid resistant *Anopheles gambiae* at two dual active-ingredient bed nets, assessed by infrared room-scale video tracking	Malaria Journal April 2022	**K. Gleave** A. Guy F. Mechan A. Matope M. Emery A. Murphy V. Voloshin C. E. Towers D. Towers G. Foster H. Ranson P. J. McCall
4	5	The effects of temephos, permethrin and malathion selection on the fitness and fecundity of *Aedes aegypti*	Medical and Veterinary Entomology November 2021	**K. Gleave** F. Mechan L. J. Reimer
	6	Filarial infection influences mosquito behaviour and fecundity	Nature Scientific Reports October 2016	**K. Gleave** D. Cook M. J. Taylor L. J. Reimer
	7	The consequences of *Brugia malayi* infection on the flight and energy resources of *Aedes aegypti* mosquitoes	Nature Scientific Reports December 2019	A. G. T. Somerville **K. Gleave** C. M. Jones L. J. Reimer

These studies show a combination of data collation activities, a systematic review and extensive laboratory work within the Liverpool School of Tropical Medicine.

Chapter 1. Quantifying and mapping insecticide resistance in Africa

Paper 1 – Developing global maps of insecticide resistance risk to improve vector control.

Rationale

A 40% reduction in the clinical incidence of malaria-causing *Plasmodium falciparum* is predominantly attributed to long-lasting insecticidal nets (Bhatt *et al*., 2016). However, increasing insecticide resistance threatens these gains as we are now observing a decrease in mosquito mortality to the most used class of insecticides, the pyrethroids. This reduction in mortality poses a threat to vector control programmes as the number of alternative insecticide classes approved for use in public health is limited. As resistance continues to spread, control programmes must have the capacity to monitor mosquito populations and share information quickly to provide accurate vector surveillance. Previous open-access databases have contained differing amounts of resistance data, all of which are displaying data as single points (Dialynas *et al.*, 2009; Knox *et al*., 2014; Mnzava *et al*., 2015), but they do not consider potential confounding factors within the data, which are important for robust and comprehensive estimates of resistance. This global mapping project (IR-MAPPER) collated and assessed all available field data on insecticide resistance, developing a modelling framework to analyse spatiotemporal patterns of resistance, which can be combined with species and disease prevalence information.

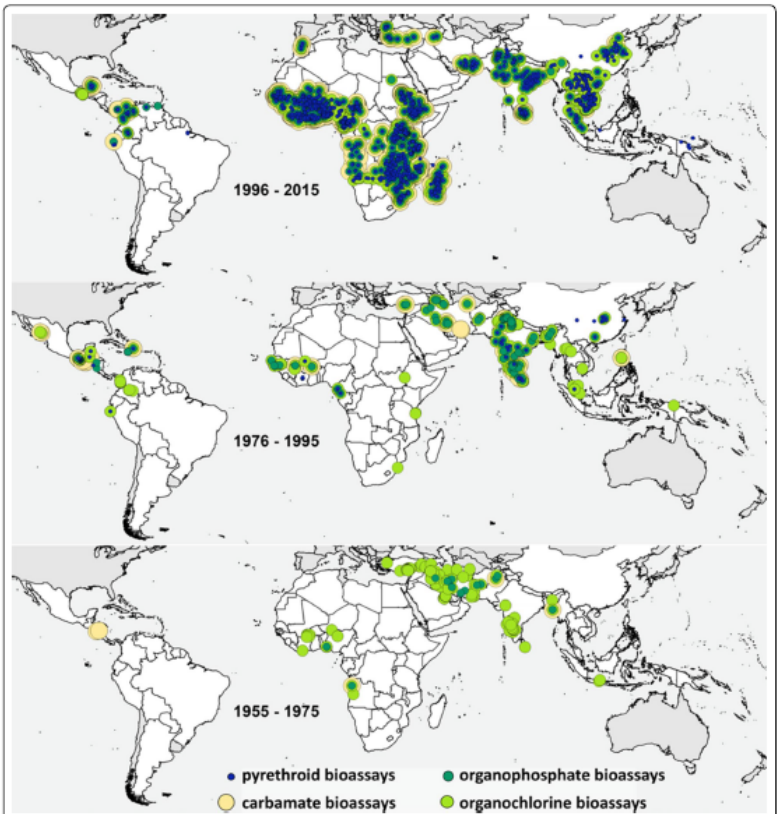
Methods

Resistance data were collated from three sources: published articles, contacting authors; and contacting custodians of unpublished data sets. The data was disaggregated to single sites and collection periods to give a fine spatial resolution. We extracted data on mosquito collection methods, identification methods, bioassay conditions including protocol followed, insecticide concentration and exposure time, generation tested, tested synergists, information about the collection site and information on the data source. Sites less than 25km^2 were assigned coordinates either from those provided or using an online gazetteer. We defined areas of more than 25 km^2 by either their borders using GIS software or defined them from the FAOs Global Administrative Unit Layers (GAUL). To best visualise any trends in the data, we filtered all results to those that used a pyrethroid and then these results were split over three time periods to correspond to data availability and the introduction of pyrethroids in global health and agriculture. Mortality data was plotted onto

maps, and when resistance mechanism data were also available, it was linked to the relevant bioassay.

Results

The data set as of October 2016 includes data from 1955, spans 71 malaria-endemic countries and 74 anopheline species or species complexes. This data includes 1018 locations reporting carbamate resistance, 1655 reporting organochlorine resistance, 1056 reporting organophosphate resistance and 3127 reporting pyrethroid resistance. Analysis of the data (Figure 8) shows that information for each insecticide class is highly clustered, indicating that interpretation needs to consider any bias in the location sampled.



Summary interpretation and conclusion

The establishment of IR-MAPPER has aided in developing tools that can use the data available to provide the best estimates of the spatial distribution of insecticide resistance, in turn helping to prolong the life of current insecticides, reduce control programme costs and aid in reducing malaria prevalence. As up to date data on insecticide resistance is crucial for deploying all insecticide- based control strategies , IR Mapper provides a free, user-friendly tool for policymakers, control programme managers and researchers to visualise current information on insecticide resistance across multiple vectors. Reported data can be continually added to the global database. While volumes are increasing, there is still a need to increase reporting on resistance mechanisms and species identification, mainly because anopheline species will differ in the rise and spread of different mechanisms.

There are multiple sibling species within the Gambiae complex, and they can differ in biting and resting locations, geographical distribution and insecticide resistance status. With this in mind, we built upon this work and used the new IR Mapper resource to look into the geographical distributions of African malaria vector sibling species and evidence for insecticide resistance (Wiebe *et al*., 2017) (Appendix 1). The work from this can be coupled with the publicly available data on insecticide resistance and vector distribution to improve policy decisions and provide a more focused vector control. Similarly, we analysed the relationships in resistance across the insecticides most commonly used in malaria vector control (Hancock *et al*., 2018) (Appendix 1), examining resistance prevalence, focusing on resistance phenotype and frequency of resistance genes. We looked for associations within insecticide class, between insecticide class, and between the prevalence of resistance phenotype and allele frequency. By building on previous work and analysing patterns of variation in insecticide resistance, we were able to find relationships across different types of insecticides used across Africa, allowing predictions of resistance to be improved.

Author contribution

KG compiled insecticide resistance data from published articles, contacted authors for unpublished or missing data and by contacted custodians of unpublished data sets. KG geopositioned all collated data and contributed to the interpretation and write up of results.

Paper 2 – Analysis-ready datasets for insecticide resistance phenotype and genotype frequency in African malaria vectors.

Rationale

Vector control (VC) activities to combat malaria rely heavily on insecticides; however, the rise and spread of insecticide resistance mechanisms disrupt current control efforts (Hemingway *et al*., 2016). Previous work carried out in multiple countries has been undertaken to investigate the impact of insecticide resistance (Kleinschmidt *et al*., 2018). To obtain fully understand the situation we need to have access to more quantitative resistance information on species distributions and malaria infection prevalence. There are currently several databases containing this information; however, one platform that combines all these factors and information on insecticide resistance that users can download as analysis-ready datasets is vital (Eisen *et al*., 2011; Moyes *et al.*, 2013). To predict the impact of insecticide resistance, diverse datasets over time and space are needed (Coleman *et al*., 2017).

This work aimed to collect data from studies that characterise resistance phenotype and genotype across multiple species, locations, and time points. Our main objective was to generate standardised datasets to address questions that would aid control programmes by using geospatial analysis. In addition, this work will provide measures of insecticide resistance for a representative sample of a mosquito population at a specific time and place rather than at the level of an individual mosquito.

Methods

We obtained the data used in this work was obtained from published journal articles, reports and unpublished data sets. Our data search ran from 1956 up to December 2017 and yielded 3,685 articles, of which 342 provided data on field samples of mosquitoes in malaria-endemic countries in Africa for either insecticide resistance phenotype and/or genotype. Once all data were collected, replicates from the same collection site and period were combined. Datasets were constructed based on mosquito samples representing either a single species or a species complex or subgroup. To use the data collected in a geospatial model with a resolution of ~5km, each collection location was classified as either a point (a site located within a 2.5 arc-minute grid cell, giving an area of ~ 5km x 5km) or a polygon (a site with an area great than that of a point). We assessed all data for quality and internal consistency.

Results

The database was used to generate eight individual data files to address specific questions (Table 3).

Table 3. Summary of each of the eight data files released (Moyes et al., 2019)

Number	Title	No. data points
1	Standard WHO susceptibility test results for the *Anopheles gambiae* complex and *Anopheles funestus* subgroup.	13,618
2	Standard WHO susceptibility test results for individual species.	3,525
3	Standard CDC bottle bioassay results for the *An. gambiae* complex and *An. funestus* subgroup.	1,061
4	Paired WHO susceptibility test or CDC bottle bioassay results with and without a synergist (*An. gambiae* complex and *An. funestus* subgroup).	1,013
5	WHO and CDC intensity bioassay results (*An. gambiae* complex and *An. funestus* subgroup).	1,816
6	*Vgsc* allele frequencies for the *An. gambiae* complex and *An. funestus* subgroup.	1,068
7	*Vgsc* allele frequencies for individual species	1,890
8	Paired *Vgsc* allele frequencies from dead and alive subsamples after an insecticide susceptibility test.	296

All data are available to download from the Dyrad Digital Repository (https://datadryad.org/stash/dataset/doi:10.5061/dryad.dn4676s). Data File 1 is the largest dataset, but all eight have similar spatial distributions with clustered sampling in east and west Africa. These datasets also show similar temporal distributions, with phenotypic data volumes increasing over time, particularly from 2008. In addition to extracting data on voltage-gated sodium channel (*Vgsc*) allele frequencies, we also examined data for *Ace-1* allele frequencies and metabolic resistance mechanisms such as cytochrome P450s, esterases and glutathione-S-transferases; however, the amount of data available at the time did not meet our requirements for providing standardised data for many locations. The spatial and temporal distributions of standard WHO susceptibility test results for the *Anopheles gambiae* complex and *Anopheles funestus* subgroup are shown in Figure 9 (Date File 1).

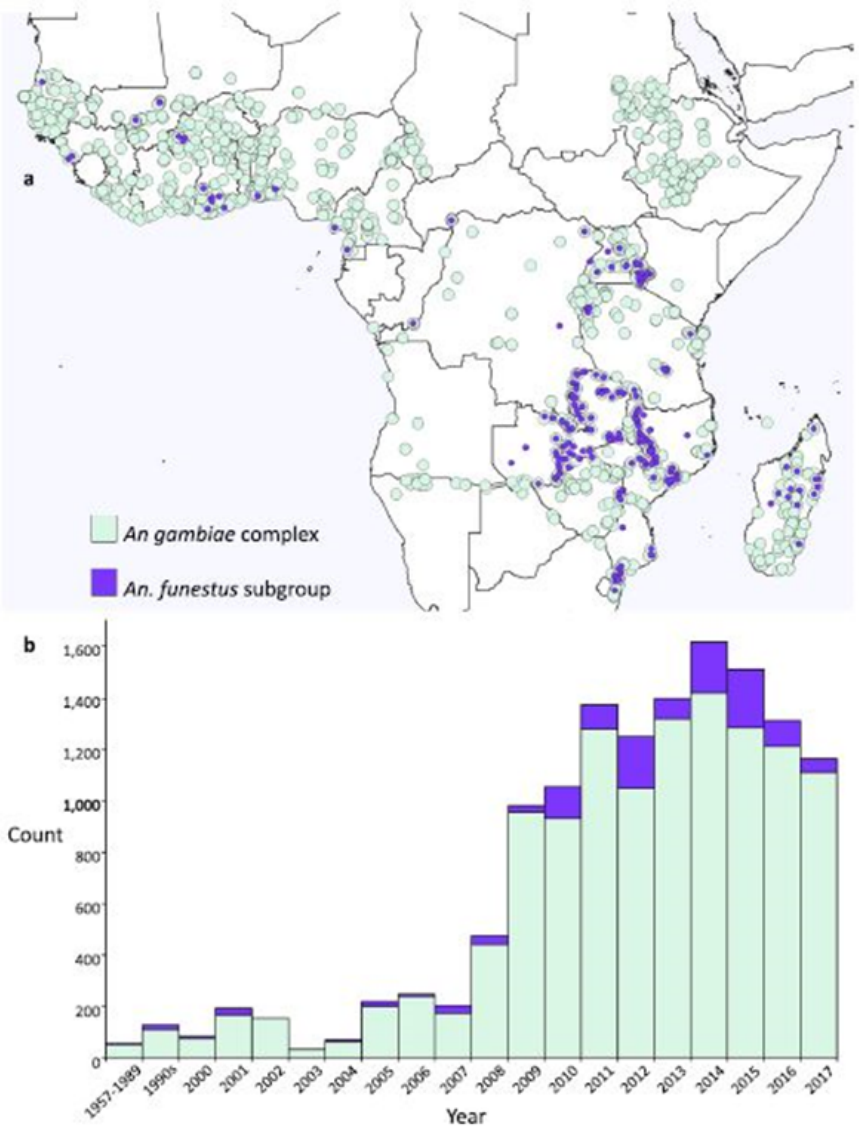


Figure 9. Spatial and temporal distributions of Data File 1. (a) The locations of mosquito collections of the An. gambiae complex and the An. funestus subgroup that were used in standard WHO susceptibility tests. (b) The number of data points available for each year for the An. gambiae complex and An. funestus subgroup (Moyes et al., 2019).

Summary interpretation and conclusion

The data files provided here show results for a representative sample of a species complex or subgroup, with the files having been designed for use in geospatial analyses, giving precise location and date information. This allows results to be matched with environmental information and previous vector control interventions. The information released provides sufficient volumes of standardised values to support a range of analyses of insecticide resistance in malaria vectors in Africa and is freely available to all. However, due to the difficulties of rearing *An.funestus* within an insectary setting, there is still a

shortage of data points compared to the volume that we have for *An.gambiae.* After this paper was published, predicted values for the prevalence of resistance (i.e. mortality in a standard WHO susceptibility test) at every location in a ~5km resolution grid for 2005 – 2017 were modelled and released.

Moyes *et al*., 2020 used the data on pyrethroid resistance in all African malaria vectors from this paper to produce maps that show the probability that the mean prevalence of pyrethroid resistance in an area meets a set of thresholds linked to specific malaria control programme recommendations. This allowed them to provide data for areas with gaps in their resistance results over space and time. The work mentioned above will allow for more focused and resource-efficient vector control and aid in future resistance modelling.

Author contribution

KG extracted, processed and geopositioned collected data. KG extracted recommended sample sizes, doses and exposure durations from all WHO and CDC protocols. KG contributed to final manuscript.

Chapter 2. Evaluating next-generation ITNs

Paper 3 – Piperonyl-butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa.

Rationale

An estimated 663 million cases of malaria have been prevented in Africa between 2000 and 2015 (Bhatt *et al*., 2016; WHO, 2017), attributed mainly to the use of insecticide-treated nets (ITNs) (Bhatt *et al*., 2016), which are used to target the malaria vector. All ITNs currently in use contain a pyrethroid insecticide due to their dual properties of low mammalian toxicity yet rapid insecticidal activity (Zaim, Aitio, & Nakashima, 2000). However, widespread pyrethroid insecticide resistance within mosquito populations now threatens ITN effectiveness (Churcher *et al.,* 2016; Ranson & Lissenden, 2016), increasing the urgency for novel bed net chemistries and insecticide innovation to maintain the efficacy of this vector control method. One way to overcome resistance is to add the insecticide synergist – piperonyl butoxide (PBO) – to the net. PBO inhibits mosquitoes' specific metabolic enzymes (cytochrome P450s) and results in a new combination net (pyrethroid-PBO nets) with efficacy in insecticide-resistant mosquito populations.
This Cochrane systematic review aimed to assess evidence of the effectiveness of pyrethroid-PBO nets against African malaria vectors in areas of different resistance levels to their standard pyrethroid-only counterpart. Accordingly, we conducted a meta-analysis of all relevant trials and examined epidemiological and entomological endpoints.

Methods

Included studies in this review were either randomised trials that measured epidemiological outcomes, entomological outcomes or both, and experimental hut trials. Participants in trials were adults and children living in malaria-endemic areas in Africa and mosquitoes from the *Anopheles gambiae* complex or *Anopheles funestus* group. Studied nets must have been treated with both a pyrethroid and PBO and have received a minimum of interim-WHO approval. Control nets had to contain a pyrethroid only but could be treated with a different dose from the intervention to allow for the critical appraisal of all pyrethroid-PBO nets on the market. Primary epidemiological outcomes were malaria parasite prevalence or incidence of clinical malaria, and entomological outcomes were mosquito mortality, mosquito knock-down (KD), blood-feeding success and sporozoite rate. We assessed risk of bias (ROB) for each trial using a set of predetermined criteria used to

judge certainty of evidence using the Cochrane GRADE approach (Schünemann, 2013), to low-, moderate- or high-certainty of evidence.

When possible, analyses were stratified by trial design and mosquito resistance level (Table 4). We also performed analyses for the primary outcomes stratified by follow-up time (4-6months, 9-12months, 16-18months and 21-25months).

Table 4. Stratification of resistance level

Outcome	Low	Moderate	High	Unclassified
Mosquito mortality %	61-90%	31-60%	<30%	Unknown

Results

We identified 389 records, removed duplicates, and screened all articles for possible inclusion. After abstract and title screening, we excluded ineligible trials and were left with 25 full-text articles to assess eligibility (Figure 10). Overall, 16 trials conducted between 2010 and 2020 compared standard pyrethroid nets to pyrethroid-PBO nets that m*et a*ll the inclusion criteria. These consisted of ten experimental hut trials, four village trials, and two cluster-randomised controlled trials (cRCTs). The two cRCTs measured the impact of pyrethroid-PBO nets on malaria infection in humans; all other studies recorded their impact on mosquito populations (mortality and blood-feeding inhibition).

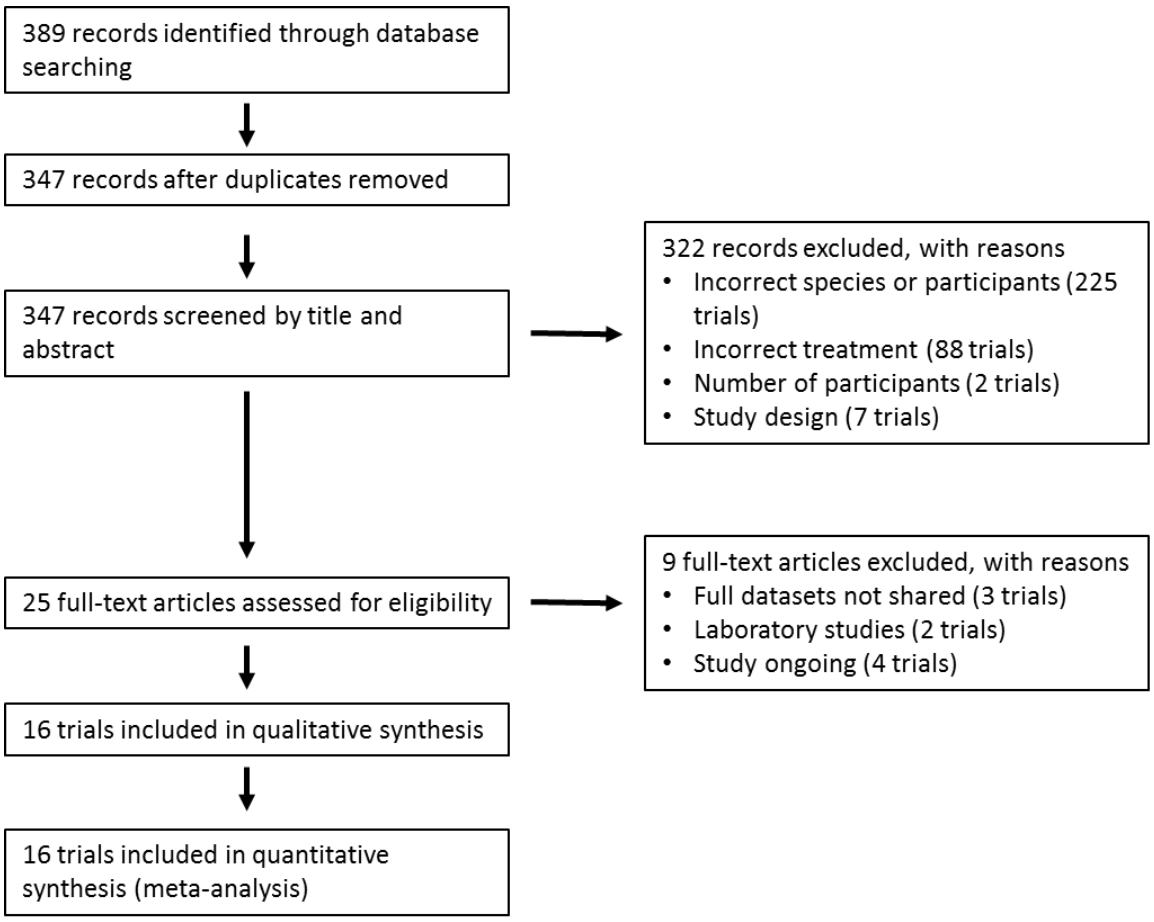


Figure 10. Study flow diagram.

The first cRCT, conducted in Tanzania (N Protopopoff *et al*., 2018), compared parasite prevalence in children using Olyset Plus (pyrethroid-PBO net) with that of children using Olyset Net (standard pyrethroid ITN) where mosquito populations are highly resistant to pyrethroids and found that at the final sampling time-point (21 months), pyrethroid-PBO nets reduced parasite prevalence by 60% (Table 5) (Table 6). The second cRCT compared parasite prevalence in children using Olyset Plus or PermaNet 3.0 nets with children using Olyset Net or PermaNet 2.0 nets across East and West Uganda (Staedke *et al*., 2020), where mosquito vectors are also highly resistant to pyrethroids and found that pyrethroid-PBO nets reduced parasite prevalence by 17% at the latest time point (25months). Examining results from both studies showed that at 21-25 months post-deployment of nets, parasite prevalence was lower in the intervention arm (Odds Ratio [OR] 0.79, 96% Confidence Interval [CI] 0.67 to 0.95; 2 trials, 2 comparisons; moderate-certainty evidence). Stratifying data from experimental hut studies by resistance levels showed that in areas where mosquitoes are highly resistant to pyrethroids, new and unwashed pyrethroid-PBO nets will cause significantly higher mosquito mortality (Risk Ratio [RR] 1.84, 95% CI 1.60 to 2.11; 14,620 mosquitoes, 5 trials, 9 comparisons; high-certainty evidence), and will reduce

blood-feeding rates (RR 0.60, 95% CI 0.50 to 0.71; 14,000 mosquitoes, 4 trials, 8 comparisons; high-certainty evidence), compared to their non-PBO counterpart. These effects were not sustained once nets had been washed. We found no evidence for any difference in the performance of pyrethroid-PBO nets from different manufacturers against highly resistant mosquitoes.

Table 5. Adapted summary of findings (Gleave, at al., 2021). Pyrethroid-PBO nets compared to long-lasting insecticidal nets (LLINs) for malaria control when insecticide resistance is high.

Outcomes	Anticipated absolute effects* (95% CI)		Relative effect (95% CI)	Number of participants (trials)	Certainty of the evidence (GRADE)
	Risk with LLIN	Risk with pyrethroid-PBO nets			
Parasite prevalence (4–6-month follow-up)	254 per 1000	201 per 1000 (174 to 233)	OR 0.74 (0.62 to 0.89)	11,582 people (2 trials, 2 comparisons, 61 PBO clusters, 64 non-PBO clusters)	**HIGH**
Parasite prevalence (9–12-month follow-up)	180 per 1000	136 per 1000 (118 to 159)	OR 0.72 (0.61 to 0.86)	11,370 people (2 trials, 2 comparisons, 61 PBO clusters, 64 non-PBO clusters)	**MODERATE** *Due to inconsistency*
Parasite prevalence (16–18-month follow-up)	248 per 1000	228 per 1000 (196 to 255)	OR 0.88 (0.74 to 1.04)	10,603 people (2 trials, 2 comparisons, 61 PBO clusters, 64 non-PBO clusters)	**MODERATE** *Due to inconsistency*
Parasite prevalence (21–25-month follow-up)	350 per 1000	298 per 1000 (265 to 338)	OR 0.79 (0.67 to 0.95)	10,603 people (2 trials, 2 comparisons, 54 PBO clusters, 60 non-PBO clusters)	**MODERATE** *Due to inconsistency*
Mosquito mortality (un-washed nets)	238 per 1000	438 per 1000 (381 to 503)	RR 1.84 (1.60 to 2.11)	14,620 mosquitoes (5 trials, 9 comparisons)	**HIGH**
Mosquito mortality (washed nets)	201 per 1000	242 per 1000 (177 to 328)	RR 1.20 (0.88 to 1.63)	10,268 mosquitoes (4 trials, 5 comparisons)	**VERY LOW** *Due to imprecision and inconsistency*
Blood-feeding success (un-washed nets)	428 per 1000	263 per 1000 (241 to 311)	RR 0.60 (0.50 to 0.71)	14,000 mosquitoes (4 trials, 8 comparisons)	**HIGH**
Blood-feeding success (washed nets)	494 per 1000	400 per 1000 (356 to 454)	RR 0.81 (0.71 to 0.92)	9674 mosquitoes (3 trials, 4 comparisons)	**HIGH**

*The risk in the intervention group (and 95% CI) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI).

CI: confidence interval; LLINs: long-lasting insecticidal nets; OR: odds ratio; PBO: piperonyl butoxide; RR: risk ratio

Table 6. GRADE Working Group grades of evidence.

High certainty	We are very confident that the true effect lies close to that of the estimate of the effect.
Moderate certainty	We are moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
Low certainty	Our confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect.
Very low certainty	We have very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of the effect.

Summary interpretation and conclusion

Data from the studies included in this review show that pyrethroid-PBO nets were more effective than standard pyrethroid-only nets in reducing the number of malaria infections, killing mosquitoes, and preventing blood-feeding in areas where mosquito populations are highly resistant to pyrethroid insecticides. We presented results from this review to the WHO Evidence Review Group (ERG) for consideration. Results support a recent WHO policy recommendation that pyrethroid-PBO nets should be considered for use in areas were insecticide resistance to pyrethroids has been confirmed in the main malaria vectors (WHO, 2017). There is no evidence suggesting that pyrethroid-PBO nets are less effective than standard LLINs for inducing mosquito mortality in any setting, so if pyrethroid-PBO nets perform as well or better than standard LLINs, then the choice to switch to these next-generation nets relies on economics.

The durability of pyrethroid-PBO nets requires further investigation and protocols need to be adjusted to utilize pyrethroid-resistant colonies so that the impact of PBO, separate from a pyrethroid, can be measured over the intended lifespan of a net. A recent paper involving the authors of this Cochrane review proposed a pipeline for monitoring the residual efficacy of pyrethroid-PBO nets (Lissenden *et al*., 2022) (Appendix 1).

Author contribution

KG conceived and designed the protocol along with co-author and corresponding author. KG conducted trial screening, data extraction and analysis along with co-author. KG prepared the first drafts of the manuscript with co-author, all authors contributed to the final manuscript.

Chapter 3. The impact of insecticide resistance on mosquito behavioural response to insecticides

Paper 4 – Behaviour of pyrethroid resistant *Anopheles gambiae* at the interface of two dual active-ingredient bed nets assessed by room-scale infrared room-scale video tracking

Rationale

Our current main defence line against malaria-transmitting vectors is insecticide-treated bet nets (ITNs) containing a pyrethroid. However, with resistance to pyrethroids now well documented across Africa in multiple mosquito species (Penelope A. Hancock *et al*., 2020), there is a growing need for new compounds and combinations to help restore the efficacy of ITNs (Pryce *et al*., 2018). The success of ITNs relies mainly on the well characterised daily behaviour of the main malaria vectors, with *Anopheles* species being anthropophagic (feed on humans), endophagic (bite indoors), endophilic (rest indoors) and feed during the evening when most people are more likely to be underneath bed nets (Killeen *et al*., 2006; Pates & Curtis, 2005). There have been reports of mosquito behavioural alterations contributed to widespread ITN use (Gatton *et al*., 2013). Moiroux *et al*., (2012) reported a shift in *An. funestus* biting times in Benin, from a peak late at night to early morning, following a mass ITN distribution campaign in two villages. With the introduction of next-generation nets, it is essential to understand how mosquitoes will behave around new chemistries. Room-scale video tracking allows recording of active mosquito behaviour around an entire net, capturing visits towards the human host, contact number and contact duration (Parker *et al*., 2017). This study reports the first room-scale behavioural tracking of insecticide-resistant *Anopheles* mosquitoes around an untreated net, a standard pyrethroid only net and two next-generation nets, Interceptor G2 and Permanet 3.0.

Methods

Two insecticide-susceptible (Kisumu and N'gousso) and two insecticide-resistant (VK7 and Banfora) strains of *Anopheles gambiae* were reared at the Liverpool School of Tropical Medicine (LSTM) under standard insectary conditions (27 °C and 80% relative humidity, 12:12 light/dark cycle). The ITNs used in this study are shown in Table 7, and all experiments required a human volunteer to act as 'bait' underneath a bed net.

Table 7. Insecticide treated nets used in room scale tracking assays. (Gleave et al., 2022)

Net type	Specification	Manufacturer
Polyester control	Untreated	Bayer AG, Leverkusen, Germany
Olyset Net	150 denier polyethylene net with permethrin at 800 mg/m^2	Sumitomo Chemical Company, Tokyo, Japan
PermaNet 3.0	roof is 100 denier polyethylene net with deltamethrin at 120mg/m^2 and PBO at 750mg/m^2, sides are 75 denier polyethylene net with deltamethrin at 84mg/m^2	Vestergaard Sarl, South Africa
Interceptor G2	75 denier polyester net with alphacypermethrin at 100mg/m^2 and chlorfenapyr at 200 mg/m^2	BASF AGRO B.V Arnhem (NL), Germany

Experiments were performed in a climate-controlled custom-built free-flight testing room (7m x 4.8m x 2.5m), with all assays performed during the 'night' phase of a mosquito's circadian rhythm.
We release 25 mosquitoes into the room and recorded behaviour over 2hours using paired identical recording systems. Figure 11 shows a schematic diagram of the filming apparatus. ITN treatments were rotated approximately every three weeks, and the testing room was deep cleaned.

Data extracted during video analysis included trajectory duration, distance travelled, the number, duration and location of contact with the bed net, and track velocity, all of which have been previously described by Parker *et al.,* (2015). Since we released multiple mosquitoes into the room it was impossible to track them individually, and so analyses were performed on individual flight tracks, which could be categorised into different behavioural modes; swooping, visiting, bouncing or resting (Table 8). After each tracking assay, mosquitoes were collected and underwent sub-lethal pipeline monitoring to assess 60minute knock-down (KD), 24hour mortality, willingness to feed at 60minutes or 24hours, overall longevity and wing length. Table 9 summarises all outcomes and measured endpoints of this study.

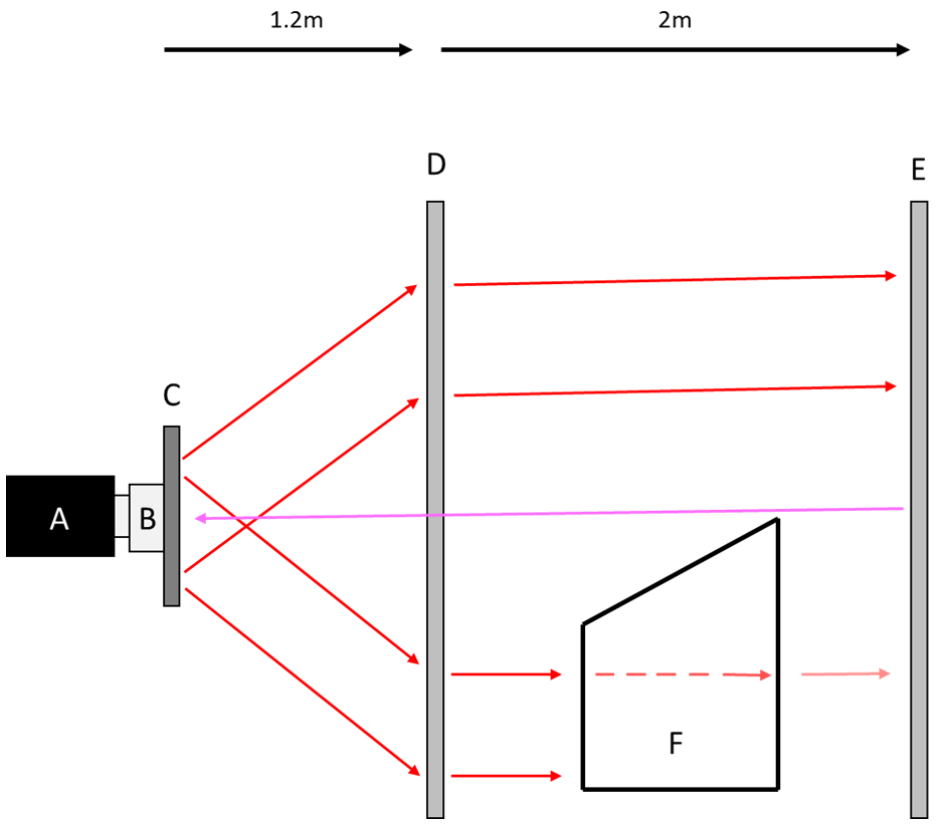


Figure 11. Set-up of room scale tracking recording system. Adapted from (Voloshin et al., 2020). (A) camera, (B) lens, (C) LED ring light, (D) Fresnel lens, (E) Retroreflective screen, (F) bednet and volunteer.

Table 8. Definition of mosquito behavioural modes.

Behavioural mode	**Definition**
Swooping	Flight tracks without net contact.
Visiting	Tracks where extended periods of flight were interspersed with infrequent contacts with the bed net. Contacts were characterized as sharp 80° turns or more in the trajectory, and when multiple contacts occurred with the net, the minimum interval between each contact was 0.4 seconds (*i.e.,* an interval of at least 20- frames, at 50 frames per second).
Bouncing	Tracks where the mosquito made multiple contacts at intervals of less than 0.4 seconds with the bed net surface; including tracks with short flights between the contacts, or tracks maintaining contact with the bed net surface without being static. This includes 'walking' or 'probing' the net with gaps in movement lasting less than 0.75 seconds

Resting	Tracks where the mosquitoes were static for at least 0.75 seconds on the net surface, or where the velocity of mosquito movement was less than 1.33 mm/s. Dead mosquitoes were excluded by limiting resting periods to a maximum of 300 seconds, however, no dead mosquitoes were found on nets at the end of each test

Table 9. Outcome and measured endpoints.

Outcome	Measured endpoint
Bioefficacy of nets	Mosquito mortality at 24hours post-exposure
Mosquito longevity	Overall longevity, monitored until natural day of death
Mosquito activity	Total activity, calculated as the sum of all mosquito activity, regardless of behavioural mode
Mosquito activity per behavioural mode	Total activity time split between the four behavioural modes
Total net contact number	The sum of the number of all contacts occurring with the bed net where contacts are obtained from visits, bounces or resting tracks
Total net contact duration	The sum of the total duration of all contacts occurring with the bed net where contacts are obtained from visits, bounces or resting tracks
Contact location	Filming field of view was divided into 16 regions, 10 of which are on the bed net and so contact number and duration in each region could be assessed
Flight speed	Speed (m/s) using whole swooping tracks around the net
Activity decay	Total activity in first 5minutes of recording subtracted from total activity in final 5minutes of recording
Willingness to feed	Mosquitoes offered blood meal at 60minutes and 24hours post-exposure
Mosquito size	Wing length

Results

One thousand six hundred ninety mosquitoes were tested across 73 assays, using 18 different volunteers as human 'bait'. Olyset (OL), PermaNet 3.0 (P3) and Interceptor G2 (IG2) killed more than 90% of susceptible strains at 24hours, however, mortality rates were significantly lower for resistant strains on all ITNs (OL: VK7 20.4%, Banfora 45.4%; P3: VK7 71.4%, Banfora 72.4%; IG2: VK7 15.9%). Mortality at 72hours for VK7 after exposure to IG2 was 25.3%.

Across all treatments, flight track length ranged from 2.51mm to 20249.09mm (20.25metres), and track duration ranged from 0.077 seconds to 1010.03 seconds (16,83minutes). For all four strains, total activity around an untreated net was significantly longer than that around any ITNs. There was no difference in total activity observed between susceptible and resistant strains around any of the ITNs tested (Figure 12). The number of contacts and contact duration was also similar for all ITNs and strains.

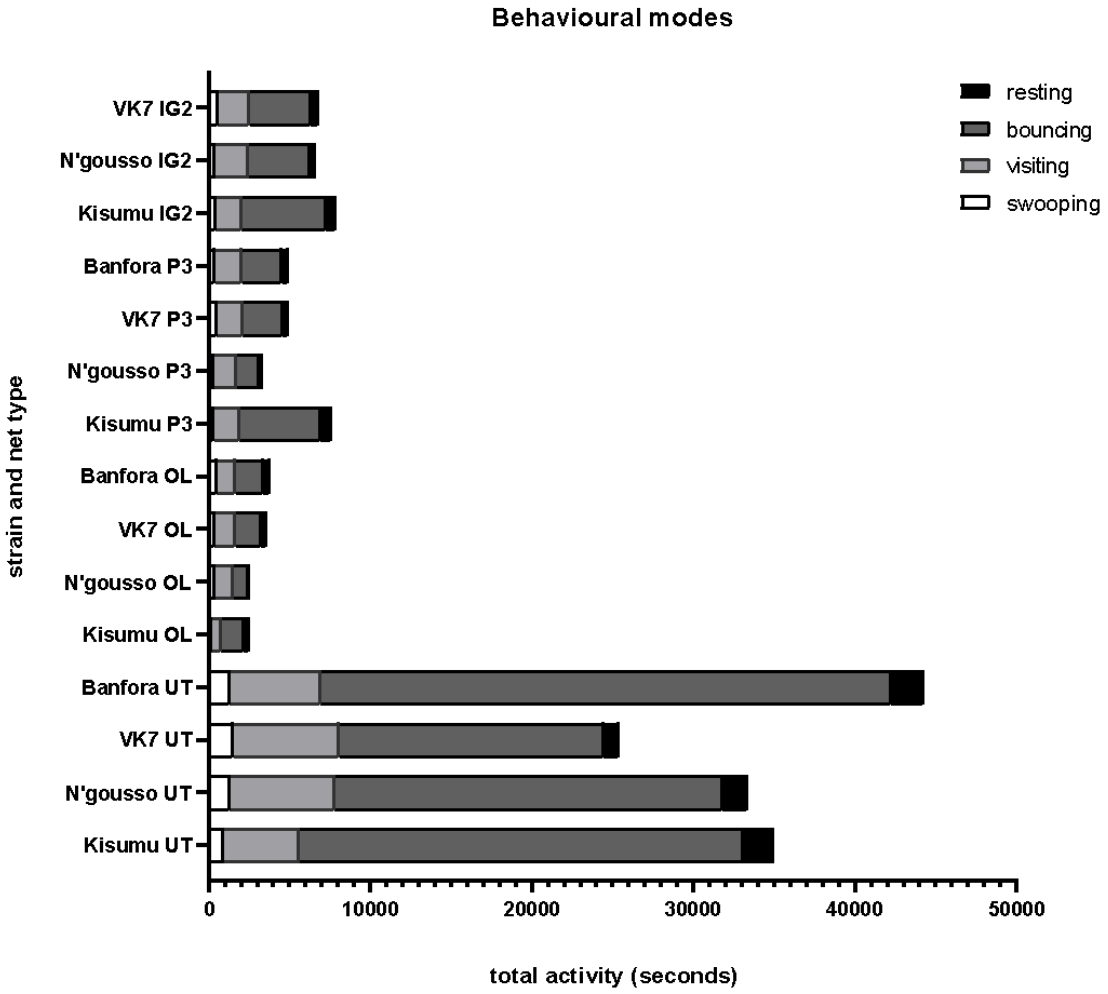


Figure 12. Behaviour of Anopheles gambiae at human baited bed nets. Mean total activity time of Anopheles gambiae recorded for each behavioural mode over two-hour recording period. As multiple mosquitoes were active simultaneously in the field of view, the total activity time could exceed the total recording time of 2 hours (7,200 seconds) (Gleave et al., 2022).

The distribution of total activity for all strains was heavily focused on the roof of all nets (>90% on UT, >85% OL, >72% P3 and >87% IG2) and did not change throughout the assay. We observed a steep decay in activity for both susceptible strains in the presence of OL and P3, but only a decrease in activity over time for Kisumu with IG2. Resistant strains showed a

less dramatic decay in activity when OL and P3 were present, but decay was still more pronounced than with an untreated net (Figure 13).

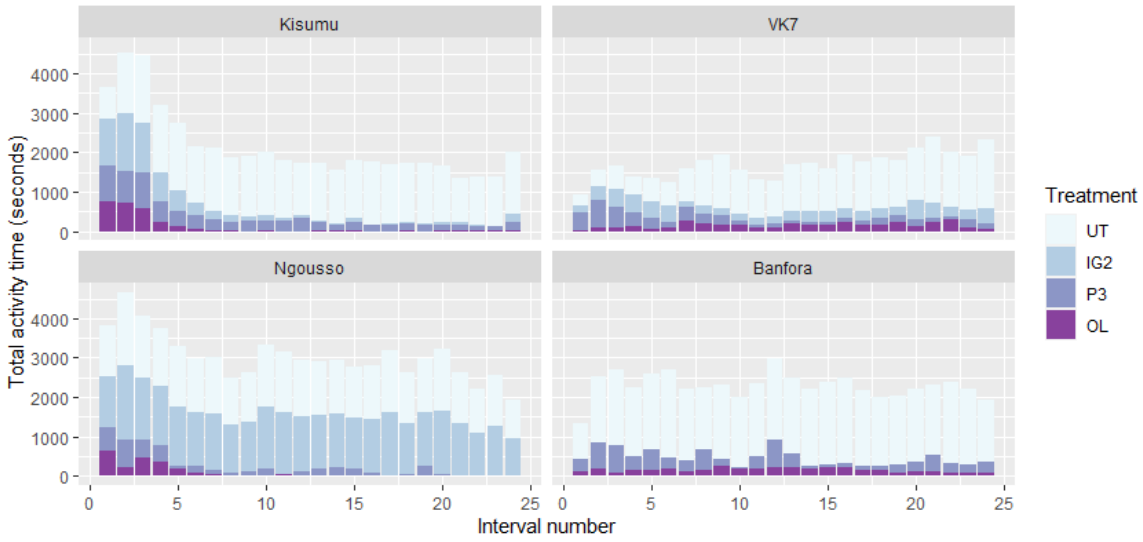


Figure 13. Rates of Anopheles gambiae activity across four behavioural modes, throughout 120minute recording test period. Total activity is shown for untreated net (UT), Olyset Net (OL), PermaNet 3.0 (P3) and Interceptor G2 (IG2) for Kisumu, N'gousso, VK7 and Banfora (Gleave et al., 2022).

All ITNs reduced blood-feeding in resistant strains one-hour post-exposure, with a more pronounced effect seen with OL and P3 than IG2.

Summary interpretation and conclusion

These are the first results to provide an in-depth description of the behaviour of susceptible and resistant *Anopheles gambiae* strains around next-generation bed nets using a room-scale tracking system to capture multiple behaviours. This study indicates that the range of effects on ITNs on mosquito behaviour is consistent, with no major alterations in mosquito responses between differing insecticide resistance levels.

Our results show that despite promising results from experimental hut studies (Bayili *et al*., 2017; Camara *et al*., 2018; Tungu *et al.,* 2021) and a recent clinical trial (Mosha *et al*., 2022) looking at Interceptor G2, both 24hr and 72hr mortality of insecticide-resistant mosquitoes remains low when tested under these conditions. This is concerning as we observed no difference in the number of contacts or the duration of time spent contacting ITNs between susceptible or resistant strains.

Behavioural data, like that collected in this study, could be used to improve insect trapping or non-lethal collection methods in the future. It is, however, important to consider that that this system does not accurately represent the shape of a bed net that would be used in

a realistic setting, and due to our testing being carried out under controlled insectary conditions, we were not able to replicate any potential wind flow or room atmospheric changes which may alter mosquito behaviour over the course of a night.
Leading on from this study, the tracking system is currently being used in Benin to record the behaviour of wild *Anopheles gambiae* around next-generation nets.

Author contribution

KG and AG collected data. KG analysed results and prepared the manuscript.

Chapter 4. The impact of insecticide resistance and parasite infection on mosquito behaviour and longevity

Paper 5 – The effect of temephos, permethrin and malathion selection on the fitness and fecundity of *Aedes aegypti*.

Rationale

Within the major arbovirus vector *Aedes aegypti*, resistance to all four classes of insecticides commonly used in public health has been documented in larval and adult life stages (Montella *et al*., 2007). Resistance mechanisms have been reported to cause significant alterations to key physiological functions in vectors, such as depleting energy recourses (Diniz *et al*., 2015), affecting development time (Martins *et al*., 2012; Rahim *et al*., 2017; Ramos *et al*., 2018), and altering immune functions (Vontas *et al*., 2005), all of which can impact on disease transmission. In addition, there are often multiple interactions occurring between fitness-related phenotypes, so this study aimed to investigate to what extent mosquito fitness may be affected in a colony of *Aedes aegypti* after selection with temephos, permethrin or malathion insecticides by measuring energetic reserves, development time, longevity, reproduction, and flight.

Methods

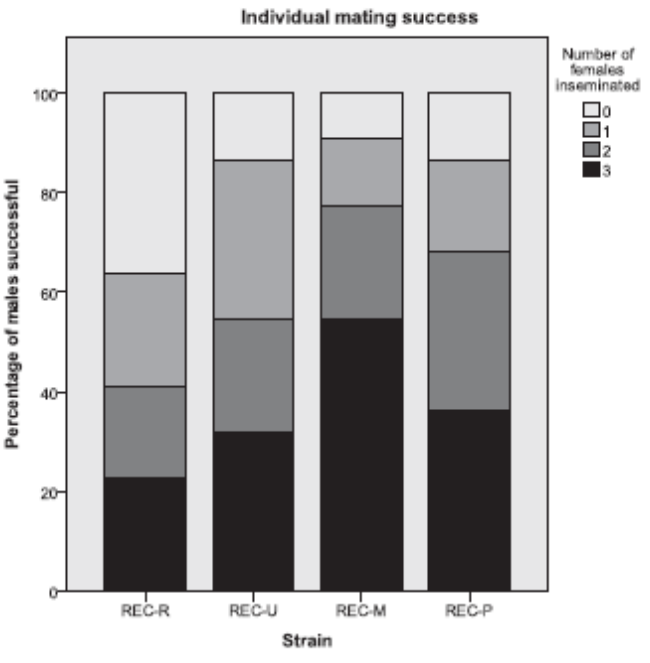
Aedes aegypti colony from Recife, Brazil, was used to create four strains via exposure over ten generations to either: the larval organophosphate temephos (REC-R); the adult pyrethroid permethrin (REC-P), the adult organophosphate malathion (REC-M); or no insecticide exposure (REC-U) (Thornton, Gomes, Ayres, & Reimer, 2020). All strains were established and maintained at the Liverpool School of Tropical Medicine (LSTM) under standard rearing conditions (27 °C and 80% relative humidity, 12:12 light/dark cycle).
We measured immature development, sex ratio, adult longevity, energetic reserves (lipid and glycogen) under different rearing conditions (standard and crowded) and time points (two and eight days post-exposure), ingested bloodmeal volume, mosquito size (wing length), male and female reproductive fitness (sperm number, individual mating success, cross mating success, female egg laying, larval hatch rate) and flight capability (using an insect tethered flight mill) in the unexposed offspring of the four strains (Table 10).

*Table 10. Study objectives and measured endpoints. *denotes wing length measurements were taken. (Gleave, Mechan, & Reimer, 2022)*

Objective	Cohort	Outcome	Measured endpoints
Life traits	Standard density	Immature development	Number successfully pupated and time to pupation and sex ratio
			Number successfully eclosed and time to eclosion and sex ratio
		Adult longevity	Day of death
	Crowded density	Immature development	Time to pupation and sex ratio
			Time to eclosion and sex ratio
Energy reserves	Standard density	Blood meal volume	Haemoglobin content
		Reserves (day 2)	Lipid content (µm/mL)*
			Glycogen content (µm/mL)*
		Reserves (day 8)	Lipid content (µm/mL)*
			Glycogen content (µm/mL)*
	Crowded density	Reserves (day 2)	Lipid content (µm/mL)*
			Glycogen content (µm/mL)*
		Reserves (day 8)	Lipid content (µm/mL)*
			Glycogen content (µm/mL)*
Reproductive fitness	Male	Fertility	Total sperm count per male *
			Sperm number per mm wing length
		Individual mating success	Number females inseminated per male
		Cross mating success	Number females inseminated per male
	Female	Female fecundity	Total egg number per female fed to repletion
			Total L1 per female fed to repletion
Flight capability	Female	Flight distance	Total distance (m)
			Average speed (m/s)
		Flight bursts	Number of bursts over test period

Results

At both rearing densities, REC-R had the highest pupation and eclosion rates; however, for all strains, the time to 50% pupation and eclosion were slower for the crowded rearing density. With a mean female survival of 28.07 days and mean male survival of 35.13days, REC-R lived significantly longer



than REC-U and REC-M. The best fit model for lipid content reported a significant interaction between 'strain' and 'age'. At two DPE, lipid content for REC-R was significantly higher than REC-M and REC-P. In contrast, REC-M lipids were significantly higher than REC-P at day eight. Regarding glycogen content, there was a significant interaction between 'strain' and 'density', showing that mean glycogen content for REC-R was significantly higher than both REC-P and REC-U. At two DPE, REC-R contained significantly more glycogen reserves than all other strains, but no difference was observed at day eight. For reproductive fitness: REC-R contained significantly more sperm per mm of wing length than all other strains; REC-R was significantly poorer at mating three females than REC-U (Figure 14); REC-U produced a larger mean egg batch and had a higher larval hatch rate than REC-R and REC-M however these results were not significant. When we flew mosquitoes on the tethered insect flight mill, REC-P flew a longer distance within an hour than REC-R.

Summary interpretation and conclusion

We found that insecticide selection impacts fitness traits in both female and male mosquitoes, with our results suggesting that continued selection with temephos at larval stages leads to shorter development time and increased longevity but reduces fecundity. However, switching to selection with malathion at the adult stage leads to better reproductive fitness. One explanation for this is that exposure during larval stages can only lead to resource allocation to promote survival rather than be used for reproduction. This suggests that continued exposure to insecticide pressure can lead to trade-offs in life-history phenotypes that could either enhance or limit vectorial capacity. These results have implications for VC programmes targeting the larval stages as mosquito longevity is crucial for vectorial capacity and disease transmission.

Author contribution

KG and corresponding author conceived and designed the study. KG collected and analysed the data. KG prepared initial drafts of manuscript with all authors contributing to the final version.

Paper 6 – Filarial infection influences mosquito behaviour and fecundity.

Rationale

The neglected tropical disease (NTD), lymphatic filariasis (LF), is the second-largest cause of permanent and long-term disability worldwide (WHO, 2010). Three species of filarial nematode are responsible for causing LF: *Wuchereria bancrofti*, *Brugia malayi,* and *Brugia timori* (WHO, 2022), and they can be transmitted with varying degrees of success by mosquitoes from the *Anopheles*, *Culex*, *Aedes* and *Mansonia* genera (Wattam & Christensen, 1992; Erickson *et al*., 2013; WHO, 2022). LF transmission is indirect, with parasites undergoing development within the mosquito vector before passing on to the definitive human host (Paily, Hoti, & Das, 2009). Parasite development within the host takes between 11-14 days and can be highly damaging to the mosquito as microfilariae (mf) penetrate out of the midgut wall and, as larvae, migrate through flight muscles. Previous work in different disease systems has shown that parasite infection can alter vector physiology and behaviour, which are essential for disease transmission. Understanding vector-parasite interactions is becoming critical as we move towards control programme elimination goals, where vector dynamics may differ with reduced transmission pressure. This study aimed to determine how an infection with *Brugia malayi* influences *Aedes aegypti* host-seeking behaviour and fecundity after exposure to low and high densities of microfilaraemic blood.

Methods

Aedes aegypti Liverpool strain (LVP strain) mosquitoes were reared and maintained under standard insectary conditions (27 °C and 80% relative humidity, 12:12 light/dark cycle). We split 4–6-day-old females into separate cohorts and allowed them to feed on either control uninfected blood or blood containing *Brugia malayi* parasites at low (5,450 - 7,750 mf/ml) or high (10,550 - 15,400 mf/ml) densities for 30minutes. Mosquitoes were dissected at different time points to correspond with the development times of *Brugia malayi* within the vector. All filarial worms recovered were included in the study and categorised as mf, developing (L1 and L2) or infective (L3), along with recording body region in which they were found.

To assess the impact of infection on behaviour in the presence of a host, a short-range host assay was carried out on mosquitoes 4-6 days post-exposure (DPE) and at 11-13 DPE, followed by mosquito dissection. Per replicate, we placed ten mosquitoes into a holding chamber which was connected to another chamber of the exact dimensions via a ‘tunnel’ of

48cm (Figure 15). Mosquitoes settled in the holding chamber before we opened a gate, and they had the option to move through the tunnel towards host cues in the second chamber. Those that remained in the holding cage were considered to non-responsive, while those that flew down were responsive to host cues. Fecundity assays were carried out three days after blood-feeding.

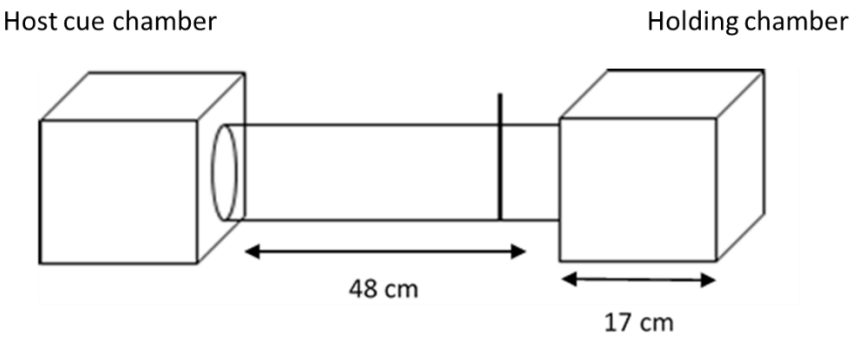


Figure 15. Schematic diagram of short-range host-seeking assay.

Results

We observed a dynamic, stage-specific and density-dependent change in *Aedes aegypti* behaviour towards host cues when exposed to *Brugia malayi* filarial parasites. During filarial larval development (L1/L2), mosquitoes exhibited reduced flight towards host cues compared to controls: however, when infective stage larvae (L3) were present, mosquitoes were five times more likely to fly towards host cues ($p<0.001$) (Figure 16). This observed behaviour was density-dependent, with non-responsive mosquitoes harbouring a more significant burden of L1/L2, while activated mosquitoes contained a greater number of L3 ($p<0.001$). Reductions in fecundity were also density-dependent and extended to mosquitoes exposed to mf but did not support larval development.

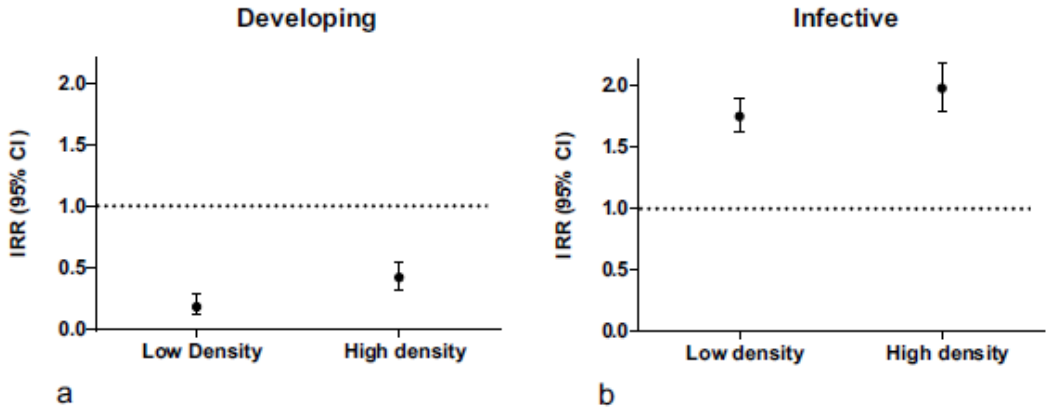


Figure 16. Incidence rate ratio (IRR) of mosquito convergence in the presence of host cues. (a) Converging mosquitoes at the developing stage (4-6 DPE) compared to the control cohort. (b) Converging mosquitoes at the infective stage (11-13 DPE) compared to the control cohort. All observed behaviours were significantly different than the control un-infected mosquitoes at both time points (p<0.0001). Control (n=790), Low Density (n=250), High Density (n=930) (Gleave et al., 2016).

Summary interpretation and conclusion

Many disease transmission models are based on set parameters that describe the same behaviours, physiology and vector-parasite interactions for all mosquitoes regardless of infection state. Here we have explained how these traits can differ depending on whether mosquitoes are uninfected, exposed to mf but do not harbour an infection, or contain developing or infective larvae, all in a density-dependant manner. As current elimination programmes rely on mass drug administration, which reduces community mf prevalence, it is essential to consider what effect this could have on continued parasite transmission and the vectorial capacity of mosquitoes. Mosquitoes that avoid risky behaviour while parasitic worms are in the developing stage, are more likely to survive until they become infective, thus improving their vectorial capacity and ability to transmit disease.

Further work is needed to understand how the complexity of these behavioural changes contributes to transmission dynamics, particularly when considering whether alterations in behaviour towards hosts could impact flight around control interventions such as bed nets. Similarly, we need to consider whether alterations in flight towards host cues could be caused by parasite infection damaging the thoracic muscles. This led the author team to consider the work that ultimately led to paper 7 (Somerville, Gleave, Jones, & Reimer, 2019). We also considered other factors that could contribute to alterations in vectorial capacity and went on to carry out a study investigating the impact that insecticide selection has on mosquito life-history traits. (Gleave, Mechan, & Reimer, 2022) (Paper 5).

Author contribution

KG along with corresponding author conceived and designed the study. KG conducted experiments and carried out data analysis. KG prepared initial drafts of manuscript with all authors contributing to the final version.

Paper 7 – The consequences of *Brugia malayi* infection on the flight and energy resources of *Aedes aegypti* mosquitoes.

Rationale

Previous experimental infection studies have shown that infected mosquitoes exhibit altered host-seeking behaviours, with suppression and activation of these traits dependent on the parasite's developmental stage (Gleave, Cook, Taylor, & Reimer, 2016). The exact cause of this altered host-seeking behaviour remains unclear; however, damage to flight muscles or the impact of infection on mosquito energy reserves could influence vital life-history traits. As any alterations in vector behaviour or physiological functions can significantly impact disease transmission and vectoral capacity (Cator, *et al*., 2014; Killeen *et al*., 2017), future modelling frameworks could benefit from an increased understanding of these interactions (Irvine *et al*., 2015). The primary aim of this work was to determine the influence of filarial infection on a range of mosquito flight parameters and assess whether infection also altered mosquito energy resources.

Methods

Aedes aegypti Liverpool strain (LVP strain) was reared at LSTM under standard rearing conditions (27 °C and 80% relative humidity, 12:12 light/dark cycle). We offered mosquitoes a blood meal containing either microfilaraemic blood (20,000 mf/ml) or uninfected blood, removing all mosquitoes that had not fed to repletion from the study.

We assessed flight ability using a tethered insect flight mill (Figure 17), at 4-6 DPE (when developing L1/L2 were present) or 11-13 DPE (when infective L3 were present). Flight parameters measured are described in Table 11. We split mosquitoes into four treatment cohorts: a one-hour flight mill assay 4-6 DPE followed by dissection to recover larvae; a one-hour flight mill assay 11-13 DPE followed by dissection to recover larvae; a one-hour flight mill assay 9 DPE followed by lipid and glycogen analysis; lipid and glycogen analysis 9 DPE but with no flight. In addition, we measured wing length to determine if there was a correlation with flight activity.

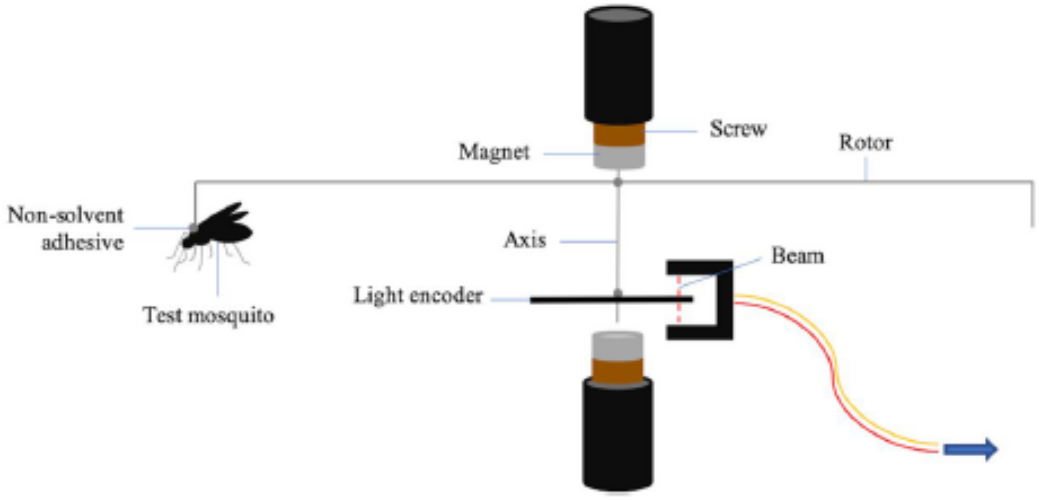


Figure 17. The set-up of a flight mill used during testing in this study, including rotor. Mosquitoes fly around a radius measuring 4cm, causing the light encoder to periodically break a laser beam with measures distance. 1 rotation = 25.13cm. (Image provided by A. Somerville).

Table 11. The definition and rationale for the flight responses measured and analysed using the tethered insect flight mill system (Somerville et al., 2019).

Flight parameter	Unit	Definition	Rationale
Flight distance	Meters (m)	Total distance covered in one hour.	Damage caused to thoracic flight muscles by developing filarial worms is likely to affect flight distance. Previous studies indicate reduced distance from filarial infection.
Average speed	Meters per second (m/s)	Average distance covered per second across one hour.	Damage caused to thoracic flight muscles by developing filarial worms is likely to affect measures of speed.
Maximum speed	Meters per second (m/s)	Highest speed reached within flight testing.	Damage caused to thoracic flight muscles by developing filarial worms is likely to affect measures of speed
Number of flight bursts	-	Any flight attempt that lasts more than 5 seconds and covered a distance of at least 0.25m.	Previous studies indicate reduced flight attempts following filarial infection.

Results

Two hundred and seventeen mosquitoes (123 fed with infected blood and 94 fed with uninfected blood) were flown on the tethered insect flight mill for one hour across three replicate experiments. Dissections performed post-flight on those fed on infected blood found that 63.1% were infected with developing larvae (4-6 DPE), and 50.0% contained infective larvae (11-13 DPE). We split mosquitoes into three groups for analysis: 'exposed' (fed on infected blood but did not contain filarial worms at the time of dissection), 'infected' (fed on infected blood and contained at least one worm at time of dissection, 'control' (fed on uninfected blood).

Generalised Linear Mixed Models (GLMMs) indicated that infection status had a significant effect on flight distance (χ^2 = 10.5, p=0.005), average speed (χ^2 = 10.3, p=0.006), maximum speed (χ^2 = 20.5, p<0.001) and the number of flight bursts (χ^2 = 17.6, p<0.001). Pairwise comparisons found that both exposure and infection lead to a decline in distance and speed of flight and an increase in the number of flight bursts a mosquito makes. We analysed 76 mosquitoes for energy resources, with infected mosquitoes containing significantly less glycogen and lipid content than controls. Flight activity had no significant effect on glycogen levels but did increase lipid content (Figure 18).

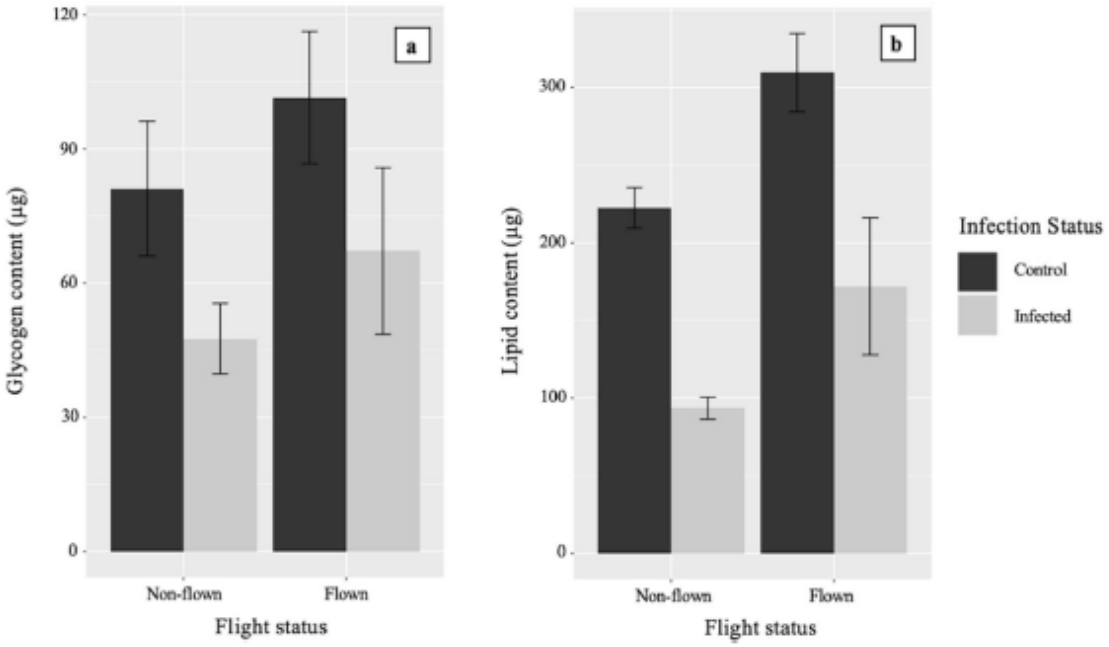


Figure 18. The glycogen and lipid content of Aedes aegypti mosquitoes based on Brugia malayi mf feeding status and flight status. (a) Glycogen, (b) Lipid. All mosquitoes were allowed to fly for a total time of one hour. Mosquitoes are categorised as either control or having fed on infected blood, as confirmation of infection intensity was not possible. Standard error bars are shown (Somerville et al., 2019).

Summary interpretation and conclusion

This work followed a previous study reporting parasite stage-specific alteration in the host-seeking behaviour of *Aedes aegypti*(Gleave *et al*., 2016). Questions arose as to whether the observed changes were parasite-mediated, or due to damage caused by parasite movement through body tissues that would affect flight ability. These results found that exposure to microfilaraemic blood led to a significant decrease in average and maximum flight speeds even in the absence of an established infection. In addition, mosquitoes fed on microfilaraemic blood showed reduced levels of glycogen (−37.9%) and lipids (−49.7%) compared to controls at nine days post-exposure. However, a one-hour period of flight activity caused an increase in lipid content for both infected and control mosquitoes.

Further exploration into the complication dynamics between parasites and vectors is needed. Aedes mosquitoes do not tend to disperse as far as Anopheles mosquitoes do, so any alterations in flight caused by damage or parasite manipulation could lead to smaller flight areas and hence smaller pockets of sustained diseases transmission. However, this work, along with others, has increased our understanding of the interactions between parasite infection, fitness costs, immunity and flight, all of which may explain the heterogeneous distribution of lymphatic filariasis.

Author contribution

KG aided in training, data collection and manuscript preparation.

Discussion

Our reliance on insecticides for vector control means that the rapid spread of insecticide resistance across Africa poses a considerable threat and could halt the progress already made in reducing the clinical incidence of vector-borne diseases such as malaria (Churcher *et al.*, 2016; Hancock *et al*., 2020; Ranson & Lissenden, 2016).

Consequently, the first chapter of this thesis investigated the spread and distribution of resistance in the main malaria vectors in Africa. In response to growing concerns over insecticide resistance, it is crucial to have accurate data available to inform control programmes and aid decision making on which control methods would be best placed in which areas.

The collation and analyses of all available insecticide resistance data from Africa has provided a modelling framework which can be used to analyse spatial and temporal patterns of resistance for different *Anopheles* species. This work includes results from 71 countries and covers resistance to carbamates, organochlorines, organophosphates and pyrethroids. Between paper 1 (Coleman *et al*., 2017) and paper 2 (Moyes *et al*., 2019), we now have information from a geospatial analysis which can be downloaded as standardised datasets on resistance phenotype and genotype for multiple species across multiple locations and time points. One of the challenges of working with large datasets is that information is often collected using various methods, with different assay protocols, different species and sibling species being tested, and different volumes of mosquitoes used. This can make results difficult to interpret, but projects like IR-MAPPER, which give measures of resistance for a representative sample of a population, will aid policymakers, control programmes and researchers in decision making on which vector methods and regimes should be implemented, hopefully prolonging the use of current and new insecticides.

We have previously observed that the introduction of insecticides into countries without prior sufficient insecticide resistant testing and profiling, can have detrimental effects. During South Africa's indoor residual spraying (IRS) control programme, pyrethroids were chosen for use and had a negative effect on disease control, as pyrethroid resistant *Anopheles funestus* were reintroduced into areas and malaria cases increased (Hargreaves et al., 2000). This has also been observed in Burkina Faso where local vectors are now 1000

fold resistant to pyrethroids, meaning that both the personal and community protection of ITNs has been lost (Toé et al., 2014).

Many control programmes use a combination of vector control tools; however, multiple countries are now reporting resistance to two or more classes of insecticide with differing resistance mechanisms. If we are to continue having success in controlling disease vectors, we must use insecticide resistance data alongside new methods to target and kill pyrethroid-resistant mosquitoes. One way to achieve this is by adding the synergist piperonyl-butoxide to pyrethroid nets to inhibit cytochrome p450s and prevent the metabolism of pyrethroids within mosquitoes, thus restoring a net's lethal effect. In 2021 over 40% of all ITNs distributed across Africa were next-generation pyrethroid-PBO nets (The Alliance for Malaria Prevention 2022). However, while these nets are currently widely distributed, there are still gaps in our knowledge about their effectiveness under different conditions and durability after washing over the intended three-year lifespan (Gleave *et al.*, 2021; Kleinschmidt *et al*., 2018; Mosha *et al*., 2022).

Various trials have been completed, but it can generally be challenging to understand the overall impact of these new nets on entomological and epidemiological outcomes. So a Cochrane systematic review was designed and carried out (Gleave *et al.*, 2021). This review assessed the effectiveness of pyrethroid-PBO nets, compared to their pyrethroid-only counterparts, against malaria vectors in Africa in areas of differing pyrethroid resistance. The rigorous examination of entomological and epidemiological data is crucial, having set criteria for trials so that results can be meta-analysed together.

Two cluster randomised controlled trials were available to analyse at the time of this review, one in Tanzania (Protopopoff *et al*., 2018) and one in Uganda (Staedke *et al*., 2020). The trial in Tanzania compared parasite prevalence in children using either Olyset Plus (pyrethroid-PBO net) or Olyset Net (pyrethroid only) in areas where mosquitoes are highly resistant to pyrethroids. They found that pyrethroid-PBO nets reduced malaria prevalence by 60% at the final reported timepoint of 21months. The second cRCT compared two pyrethroid-PBO nets, Olyset Plus and PermaNet.30, against their pyrethroid-only counterparts, Olyset Net and PermaNet 2.0, again in areas of high pyrethroid resistance. Results from this trial reported that pyrethroid-PBO nets also reduced parasite prevalence by 17% at 25months post-deployment. We found the trial methods and results of these studies to be robust and concluded that we are very confident that the intervention's true effect lies close to that of the estimate of the effect (high-certainty evidence).

To best examine entomological data from experimental hut studies, we stratified results by resistance level and found that PBO-nets reduced mosquito blood-feeding rates and increased mosquito mortality in areas of high pyrethroid resistance. However, these results were not sustained when nets had been washed. The results from this review highlight the importance of examining data in this way. We were able to determine that in areas of high pyrethroid resistance, pyrethroid-PBO nets increased mosquito mortality, reduced blood-feeding success and reduced the clinical incidence of malaria. We found no differences in the performance of nets from different manufacturers; however, due to the low number of studies available, we could not compare them all.

Pyrethroid-PBO nets were not superior in moderate or low pyrethroid resistance areas, but they did not perform any worse than standard ITNs, so we present no evidence that they should not be used in all settings if it would be cost-efficient to do so. Performing hut trials alongside cRCTs will aid with measuring the effect of new active ingredients, such as PBO, on resistant populations and would be useful for assessing net durability (Lees *et al*., 2022; Lissenden *et al*., 2022), particularly if the effects on mortality and blood-feeding do not appear to be sustained after washing. A recent study by Mosha *et al*., (2022), undertaken in a high pyrethroid-resistant area of Tanzania, reported that pyrethroid-PBO nets sustained their effectiveness for less time than the nets included in this review. One explanation is that net use declined more rapidly in the study areas due to poor physical integrity and nets becoming more torn than other ITNs.

Most of the available data evaluated in the performance of pyrethroid-PBO nets is against *Anopheles gambiae s.l,* with very little data available for the second major species complex in Africa, *Anopheles funestus*, and none for other minor species. To increase our understanding, more studies need to be carried out in areas where different species are present, as they will have different behaviours and different mechanisms of resistance. This is a critical data gap to fill as it could have implications for net deployment where members of the *An. gambiae* complex are not the primary vector of disease.

The success of next-generation nets relies, in the same way as previous ITNs, on the daily behaviour of mosquito populations (Pates & Curtis, 2005; Killeen *et al*., 2006). If a mosquito does not contact a treated net, then the intended effects (mortality or a reduction in fecundity) will not occur. While there have been multiple field trials for next-generation nets examining their impact on disease reduction and mosquito mortality (N'Guessan *et al*.,

2016; Bayili *et al*., 2017; Camara *et al*., 2018; Protopopoff *et al*., 2018; Tungu *et al*., 2021; Mosha *et al*., 2022), there is little work on how mosquitoes interact with these nets.

A room-scale infrared video tracking system increased our knowledge of mosquito interactions around novel insecticide chemistries and combinations. Previous work using this system (Parker *et al*., 2017) categorised mosquito behaviour into four behavioural modes (swooping, visiting, resting and bouncing) and reported that the majority of mosquito interactions with bednets occurred on the roof above the human torso, which correlates with results from a previous study (Lynd & Mccall, 2013).

The work in this thesis explains the first results using room-scale video tracking to record susceptible and resistant mosquitoes around two different next-generation insecticide-treated nets – PermaNet 3.0 and Interceptor G2 (Gleave *et al*., 2022). Using this system, data was collected and analysed on net bioefficacy, mosquito longevity and blood-feeding success, mosquito activity and behavioural modes, number and duration of contacts made with the net and rates of activity decline over time. Bioefficacy of the standard pyrethroid-only net was low as expected against resistant strains. The pyrethroid-PBO net induced higher mortality but did not manage to kill all mosquitoes (24hour mortality VK7 71%, Banfora 72%), and the efficacy of Interceptor G2 against resistant VK7 was lower, causing 16% mortality. To consider the delayed lethal action of chlorfenapyr, we recorded mortality at 72hours and monitored mosquito longevity until the natural day of death. Resistant strain mortality at 72hours increased to 24%; however, the median survival time for mosquitoes exposed to Interceptor G2 was the same as those exposed to an untreated net (10 days). The low mosquito mortality in this study does not reflect the promising entomological results from experiments carried out in the field (Bayili *et al*., 2017; Camara *et al*., 2018; Tungu *et al.*, 2021). The recent randomised controlled trial in Tanzania (Mosha *et al*., 2022) also reported the high efficacy of chlorfenapyr nets, showing a decrease in malaria incidence in children alongside a decrease in vector abundance and longevity. These results are not mirrored in the room-scale tracking study; however, we did observe a reduction in blood-feeding success with resistant mosquitoes that survived exposure.

Total mosquito activity was higher around untreated nets, comparable to previous work (Parker *et al*., 2017). However, all essential characteristics of responses were the same between the three ITNs for both susceptible and resistant strains, observing no difference in activity, contact number or contact duration. This is promising for next-generation nets as

it suggests that resistant mosquitoes do not interact differently around the novel chemistries used compared to a pyrethroid only net. However, with this style of behavioural study, it is important to note that results must be interpreted carefully. We could not track individual mosquitoes throughout the recording to interpret the effects of insecticide exposure, so contact duration with a net could vary between mosquitoes. This room-scale tracking system is now being implemented in Benin to investigate the behaviour of local wild mosquitoes around the same next-generation nets.

For mosquitoes to be successful disease vectors, they need to survive the parasite extrinsic incubation period (EIP). A substantial part of this thesis was investigating the effect of insecticide selection and parasite infection on mosquito behaviour and physiology and considering how this may impact vectorial capacity. Insecticide resistance mechanisms cause alterations to physiological functions by depleting energy reserves (Diniz *et al*., 2015), affecting development time (Martins *et al*., 2012) and impacting immune responses (Vontas *et al*., 2005). To examine the effects of insecticide selection, we measured multiple life-history parameters after exposure to temephos, malathion and permethrin or after no sustained insecticide exposure on mosquitoes that originated from the same parental colony. This study concluded that insecticide exposure impacted both male and female physiological traits, suggesting that exposure to the larval organophosphate temephos leads to shorter developmental times and increased longevity but reduces fecundity. However, selection with the adulticide malathion leads to improved reproductive fitness. We believe this can be explained by differences in resource allocation that promote survival rather than increasing offspring. Our temephos resistant line had an increased number of sperm per millimetre of wing length compared to the other strains, but the poor insemination success suggests that these males potentially produce a larger ejaculate but at less frequent intervals. Similar results were observed in a different study, were male mating success was inversely proportional to the temephos resistance ratio (Belinato, Martins, & Valle, 2012) and in work by Diniz *et al.,* (2015) who showed that resistance status impacts male mating success. Body size is a well-documented factor in male mating success, with previous studies (A Ponlawat & Harrington, 2007; Alongkot Ponlawat & Harrington, 2009) reporting that *Aedes aegypti* body size was correlated with sperm number. However, our study confirmed that the significant differences in sperm number between strains were not attributable to differences in body size.

Our results on female fecundity were again similar to that of Belinato *et* al., (2012), who showed females from a highly resistant temephos field strain laid fewer eggs than the

susceptible counterpart. While reduced fecundity in resistant strains could lead to lower mosquito densities, adult female longevity is a crucial factor in the vectorial capacity of wild mosquito populations. REC-R female and male mosquitoes survived significantly longer than other strains in this study, however, previous work using a different *Aedes albopictus* strain reported that temephos resistant field strains had a shorter life span than their susceptible counterpart (Rahim et al., 2017). One notable difference between these studies, is that we tested laboratory mosquitoes with an extended history of insecticide pressure, in contrast to a progeny originating from only one round of larviciding. The outcomes of insecticide selection presented here will have different effects on vectorial capacity, either increasing the chance to survive the EIP or reproducing effectively and passing on resistance genes.

Along with increasing our understanding of the effects of resistance, this thesis expands our knowledge on another essential factor in vectorial capacity, the effect of parasite infection. Parasite transmission relies on a vector's ability to successfully locate a host and acquire a blood meal. Previous studies, mainly investigating mosquito-*Plasmodium* interactions, have shown that parasitic infection can alter this behavioural process, with infective vectors more likely to initiate probing, probe for longer and feed to repletion (Anderson et al., 1999; Koella, Rieu, & Paul, 2002; Wekesa et al., 1992). These alterations in host-seeking behaviour appear to be stage specific, with mosquitoes positive for infective sporozoites being more likely to initiate probing, probe for longer and feed to repletion. These results suggest that mosquito behaviour may be altered in order to reduce risky behaviour, such as foraging and blood-feeding, when parasites are still developing, while promoting these behaviours when infective parasites are present. However, subsequent work by Cator and colleagues (Cator et al., 2013, 2015) suggested that the change in receptivity and host-seeking behaviour was a generic response to exposure, that corresponded with *Plasmodium* developmental stages. Paper 6 (Gleave *et al.*, 2016) examines the effects of the filarial nematode *Brugia malayi* on the host-seeking behaviour and fecundity of *Aedes aegypti*. Results demonstrated a stage-specific and density-dependent alteration in host-seeking behaviour during filarial development (L1/L2), with infected females less likely to follow host cues, however at the infective L3 stage, females were five times more likely to host-seek than uninfected controls. These results could suggest a parasite-mediated change in mosquito behaviour through reducing risky behaviour, which could lead to premature vector death, such as host-seeking, during parasite development is beneficial to the parasite for sustained

transmission. Only increasing host-seeking behaviour when the infective stage is present, increases the chance of passing on an infection.

It is vital to consider all interactions within a vector-parasite system. For example, what if the alterations we observed were not due to parasite manipulation but instead caused by the mechanical damage of an active infection, affecting the gut wall, thoracic muscles or mouthparts. This thought led to the study described in paper 7 (Somerville *et al.*, 2019), undertaken to provide a more in-depth understanding of how a filarial infection can affect mosquito flight parameters. It can be complicated to disentangle the effects observed during behavioural studies, which in this case were: are mosquitoes not host-seeking while harbouring developing larvae because of parasite manipulation to reduce risky foraging behaviour or is the damage caused by larvae consuming energy reserves, moulting through life stages and moving through flight muscle prohibiting mosquitoes from being able to fly. *Aedes aegypti* were given the opportunity to feed on blood containing microfilaria, and then at either 4-6 days post-exposure (developing larvae present), or 11-13 days post-exposure (infective larvae present), they were attached to a tethered insect flight mill and flown for 1 hour. Results showed a detrimental impact on flight capacity after exposure to microfilaraemic blood at both time points compared to unexposed controls. This suggests that the decline in flight could be caused by internal damage or energy resource consumption by larvae. It is interesting to note that filarial infection increased the number of flight bursts a mosquito took, suggesting infected vectors may take several smaller, slower flight attempts. This finding lends it support to previous research which has found *Plasmodium* infection is associated with an incapacitation of flight (Rowland & Boersma, 1988; Schiefer, Ward, & Eldridge, 1977), but increased nectar-feeding. Reduced energetic reserves caused by harbouring an infection could support this idea, although further research is needed. This could affect transmission potential if mosquitoes have a reduced flight range to host seek within.

Mosquito behaviour is crucial for disease transmission; hence any alterations need to be considered, whether this is a change in species distribution, a shift in biting time or biting location, alterations in flight activity around a novel bed net to prevent insecticide contact, manipulations by parasites or the effects of resource depletion from harbouring insecticide resistance mechanisms.

The studies in this thesis have contributed significantly to our understanding of how insecticide resistance and parasite infection influence mosquito behaviour. Therefore, when

considering control and elimination programmes, is it essential to have a more holistic understanding of the factors that can affect vector dynamics and to incorporate mosquito physiological parameters into transmission models.

Future work

This thesis found that insecticide resistance and parasite infection affected mosquito behaviour in multiple ways.

Further work which would be beneficial to add to our knowledge and understanding are:

- The development of standardised assays to measure the effects of next-generation nets on mosquito mortality and fecundity and utilising them to continually monitor the efficacy and durability on different species in different locations.
- To build on the work presented here, behavioural studies that consider various interactions would benefit this subject area. Investing the impact of insecticide selection and parasitic infection in the same mosquito cohort would benefit modelling and control programmes. Results already show that resistance and infection can affect life-history parameters positively and negatively, so it will be interesting to examine how multiple factors interact.
- Expanding our knowledge on how mosquitoes interact with new chemistries, using strains with different resistance mechanisms, such as cuticular resistance, to explore further how new nets may perform in Africa.
- Investigating evidence for parasite manipulation on mosquito behaviour requires more studies to attempt to disentangle the multiple interactions at play.

References

Adolfi, A., Poulton, B., Anthousi, A., Macilwee, S., Ranson, H., & Lycett, G. J. (2019). Functional genetic validation of key genes conferring insecticide resistance in the major African malaria vector, Anopheles gambiae. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(51), 25764–25772. https://doi.org/10.1073/pnas.1914633116

Afify, A., & Galizia, C. G. (2015). Chemosensory Cues for Mosquito Oviposition Site Selection. *CHEMICAL ECOLOGY J. Med. Entomol*, *52*(2), 120–130. https://doi.org/10.1093/jme/tju024

Allan, S. A., Day, J. F., & Edman, J. D. (1987). Visual Ecology of Biting Flies. *Annual Review of Entomology*, *32*(1), 297–314. https://doi.org/10.1146/annurev.en.32.010187.001501

Anderson, R. A., Koella, J. C., & Hurd, H. (1999). The effect of Plasmodium yoelii nigeriensis infection on the feeding persistence of Anopheles stephensi Liston throughout the sporogonic cycle. *Proceedings of the Royal Society B: Biological Sciences*, *266*(1430), 1729–1733. https://doi.org/10.1098/rspb.1999.0839

Angarita-Jaimes, N. C., Parker, J. E. A., Abe, M., Mashauri, F., Martine, J., Towers, C. E., … Towers, D. P. (2016). A novel video-tracking system to quantify the behaviour of nocturnal mosquitoes attacking human hosts in the field. *Journal of the Royal Society Interface*, *13*(117). https://doi.org/10.1098/rsif.2015.0974

Balabanidou, V., Kampouraki, A., Maclean, M., Blomquist, G. J., Tittiger, C., Juárez, M. P., … Vontas, J. (2016). Cytochrome P450 associated with insecticide resistance catalyzes cuticular hydrocarbon production in Anopheles gambiae. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(33), 9268–9273. https://doi.org/10.1073/pnas.1608295113

Barreaux, P., Koella, J. C., N'guessan, R., & Thomas, M. B. (2022). Use of Novel Lab-Assays to Examine the Effect of Pyrethroid-Treated Bed Nets on Blood Feeding Success and Longevity of Highly Insecticide-Resistant Anopheles Gambiae S.I. Mosquitoes. *Parasites & Vectors*, 1–9. https://doi.org/10.1186/s13071-022-05220-y

Battle, K. E., Gething, P. W., Elyazar, I. R. F., Moyes, C. L., Sinka, M. E., Howes, R. E., … Hay, S. I. (2012). The Global Public Health Significance of Plasmodium vivax. In *Advances in Parasitology* (Vol. 80). https://doi.org/10.1016/B978-0-12-397900-1.00001-3

Bayili, K., Severin, N., Namountougou, M., Sanou, R., Ouattara, A., Dabiré, R. K., … Diabaté, A. (2017). Evaluation of efficacy of IG2 , a long - lasting insecticide net coated with a mixture of chlorfenapyr and alpha - cypermethrin , against pyrethroid resistant Anopheles gambiae s . l . in Burkina Faso. *Malaria Journal*, 1–9. https://doi.org/10.1186/s12936-017-1846-4

Beenakkers, A. M. T., Horst, D. J. V. D., & Marrewijk, W. J. A. V. (1981). Role of lipids in energy metabolism. In H. Downer, R, G (Ed.), *Energy Metabolism in Insects* (pp. 53–100). New York.

Belinato, T. A., Martins, A. J., & Valle, D. (2012). Fitness evaluation of two Brazilian Aedes aegypti field populations with distinct levels of resistance to the organophosphate temephos. *Memorias Do Instituto Oswaldo Cruz*, *107*(7), 916–922. https://doi.org/10.1590/S0074-02762012000700013

Bhatt, S., Weiss, D. J., Cameron, E., Bisanzio, D., Mappin, B., & Dalrymple, U. (2016). The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. *Nature*, *526*(7572), 207–211. https://doi.org/10.1038/nature15535.The

Bhatt, S., Weiss, D. J., Cameron, E., Bisanzio, D., Mappin, B., Dalrymple, U., … Gething, P. W. (2015). The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. *Nature*, *526*(7572), 207–211. https://doi.org/10.1038/nature15535

Bidlingmayer, W. L. (1994). How mosquitoes see traps: role of visual responses. *Journal of the American Mosquito Control Association*, *10*(2 Pt 2), 272–279.

Black, B. C., Hollingworth, R. M., Ahammadsahib, K. I., Kukel, C. D., & Donovan, S. (1994). Insecticidal action and mitochondrial uncoupling activity of AC-303,630 and related halogenated pyrroles. *Pesticide Biochemistry and Physiology*, Vol. 50, pp. 115–128. https://doi.org/10.1006/pest.1994.1064

Bloomquist, J. R. (1996). Ion channels as targets for insecticides. *Annual Review of Entomology*, *41*, 163–190. https://doi.org/10.1146/annurev.en.41.010196.001115

Bowen, M. F. (1991). The Sensory Physiology of Host-Seeking Behavior in Mosquitoes. *Annual Review of Entomology*, *36*(1), 139–158. https://doi.org/10.1146/annurev.en.36.010191.001035

Camara, S., Phamien, L., Alou, A., Koffi, A. A., Cyntia, Y., & Clegban, M. (2018). *Efficacy of Interceptor G2 , a new long-lasting insecticidal net against wild pyrethroid-resistant Anopheles gambiae s . s . from Côte d ' Ivoire : a semi-field trial*. *42*.

Cardé, R. T. (2015). Multi-Cue Integration: How Female Mosquitoes Locate a Human Host. *Current Biology*, *25*(18), R793–R795. https://doi.org/10.1016/j.cub.2015.07.057

Cator, L. J., George, J., Blanford, S., Murdock, C. C., Baker, T. C., Read, A. F., & Thomas, M. B. (2013). "Manipulation" without the parasite: Altered feeding behaviour of mosquitoes is not dependent on infection with malaria parasites. *Proceedings of the Royal Society B: Biological Sciences*, *280*(1763). https://doi.org/10.1098/rspb.2013.0711

Cator, L. J., Lynch, P. A., Thomas, M. B., & Read, A. F. (2014). Alterations in mosquito behaviour by malaria parasites: Potential impact on force of infection. *Malaria Journal*, *13*(1), 1–11. https://doi.org/10.1186/1475-2875-13-164

Cator, L. J., Pietri, J. E., Murdock, C. C., Ohm, J. R., Lewis, E. E., Read, A. F., … Thomas, M. B. (2015). Immune response and insulin signalling alter mosquito feeding behaviour to enhance malaria transmission potential. *Scientific Reports*, *5*(June), 1–10. https://doi.org/10.1038/srep11947

Chan, H. H., & Zairi, J. (2013). Permethrin resistance in aedes albopictus (Diptera: Culicidae) and associated Fitness Costs. *Journal of Medical Entomology*, *50*(2), 362–370. https://doi.org/10.1603/ME12182

Charlwood, J. D., & Graves, P. M. (1987). The effect of permethrin-impregnated bednets on a population of Anopheles farauti in coastal Papua New Guinea. *Medical and Veterinary Entomology*, *1*(3), 319–327. https://doi.org/10.1111/j.1365-2915.1987.tb00361.x

Charlwood, J. D., Smith, T., Kihonda, J., Heiz, B., Billingsley, P. F., & Takken, W. (1995). Density independent feeding success of malaria vectors (Diptera: Culicidae) in Tanzania. *Bulletin of Entomological Research*, *85*(1), 29–35. https://doi.org/10.1017/S0007485300051981

Churcher, T. S., Lissenden, N., Griffin, J. T., Worrall, E., & Ranson, H. (2016). The impact of pyrethroid resistance on the efficacy and effectiveness of bednets for malaria control in Africa. *Elife*, *5*. https://doi.org/10.7554/eLife.16090

Coetzee, M., & Fontenille, D. (2004). Advances in the study of Anopheles funestus, a major vector of malaria in Africa. *Insect Biochemistry and Molecular Biology*, *34*(7), 599–605. https://doi.org/10.1016/j.ibmb.2004.03.012

Coetzee, Maureen, Hunt, R. H., Wilkerson, R., Della Torre, A., Coulibaly, M. B., & Besansky, N. J. (2013). Anopheles coluzzii and Anopheles amharicus, new members of the Anopheles gambiae complex. *Zootaxa*, *3619*, 246–274.

Coleman, M., Hemingway, J., Gleave, K. A., Wiebe, A., Gething, P. W., & Moyes, C. L. (2017). Developing global maps of insecticide resistance risk to improve vector control. *Malaria Journal*, *16*(1). https://doi.org/10.1186/s12936-017-1733-z

Colón-González, F. J., Sewe, M. O., Tompkins, A. M., Sjödin, H., Casallas, A., Rocklöv, J., … Lowe, R. (2021). Projecting the risk of mosquito-borne diseases in a warmer and more populated world: a multi-model, multi-scenario intercomparison modelling study. *The Lancet Planetary Health*, *5*(7), e404–e414. https://doi.org/10.1016/S2542-5196(21)00132-7

David, M. R., Garcia, G. A., Valle, D., & Maciel-De-Freitas, R. (2018). Insecticide Resistance and Fitness: The Case of Four Aedes aegypti Populations from Different Brazilian Regions. *BioMed Research International*, *2018*. https://doi.org/10.1155/2018/6257860

Davies, T. G. E., Field, L. M., Usherwood, P. N. R., & Williamson, M. S. (n.d.). *Critical Review DDT, Pyrethrins, Pyrethroids and Insect Sodium Channels*. https://doi.org/10.1080/15216540701352042

De Jong, R., & Knols, B. G. J. (1995). Selection of biting sites on man by two malaria mosquito species. *Experientia*, *51*(1), 80–84. https://doi.org/10.1007/BF01964925

Dhadialla, T. S., Carlson, G. R., & Le, D. P. (1998). New insecticides with ecdysteroidal and juvenile hormone activity. *Annual Review of Entomology*, *43*, 545–569. https://doi.org/10.1146/annurev.ento.43.1.545

Dialynas, E., Topalis, P., Vontas, J., & Louis, C. (2009). MIRO and IRbase: IT tools for the epidemiological monitoring of insecticide resistance in mosquito disease vectors. *PLoS Neglected Tropical Diseases*, *3*(6). https://doi.org/10.1371/journal.pntd.0000465

Diniz, D. F. A., Melo-Santos, M. A. V. De, Santos, E. M. D. M., Beserra, E. B., Helvecio, E., De Carvalho-Leandro, D., … Ayres, C. F. J. (2015). Fitness cost in field and laboratory Aedes aegypti populations associated with resistance to the insecticide temephos. *Parasites and Vectors*, *8*(1), 1–15. https://doi.org/10.1186/s13071-015-1276-5

Eisen, L., Coleman, M., Lozano-Fuentes, S., McEachen, N., Orlans, M., & Coleman, M. (2011). Multi-disease data management system platform for vector-borne diseases. *PLoS Neglected Tropical Diseases*, *5*(3), 1–12. https://doi.org/10.1371/journal.pntd.0001016

Elissa, N., Mouchet, J., Riviere, F., Meunier, J. Y., & Yao, K. (1993). Resistance of Anopheles gambiae s.s. to pyrethroids in Côte d'Ivoire. *Annales de La Societe Belge de Medecine Tropicale*, *73*(4), 291–294.

Erickson, S. M., Thomsen, E. K., Keven, J. B., Vincent, N., Koimbu, G., Siba, P. M., … Reimer,

L. J. (2013). Mosquito-Parasite Interactions Can Shape Filariasis Transmission Dynamics and Impact Elimination Programs. *PLoS Neglected Tropical Diseases*, *7*(9), 1–7. https://doi.org/10.1371/journal.pntd.0002433

Garrett-Jones ', C. (1964). The Human Blood Index of Malaria Vectors in Relation to Epidemiological Assessment. *Bull. Org. Mond. Sante*, *30*, 241–261.

Gatton, M. L., Chitnis, N., Churcher, T., Donnelly, M. J., Ghani, A. C., Godfray, H. C. J., … Lindsay, S. W. (2013). The importance of mosquito behavioural adaptations to malaria control in Africa. *Evolution*, *67*(4), 1218–1230. https://doi.org/10.1111/evo.12063

Gibson, G., & Torr, S. J. (1999). Visual and olfactory responses of haematophagous Diptera to host stimuli. *Medical and Veterinary Entomology*, *13*(1), 2–23. https://doi.org/10.1046/j.1365-2915.1999.00163.x

Gillies, M. (1968). The Anophelinae of Africa south of Sahara. In *The South Africa Institute for Medicial Research* (2nd ed.).

Gillies, M., & Coetzee, M. (1987). A supplement to the Anophelinae of Africa South of the Sahara. *South African Institute for Medical Research*, (55). Retrieved from http://www.metoffice.gov.uk/public/weather/climate/u1214qgj0

Gillies, M. T. (1980). The role of carbon dioxide in host-finding by mosquitoes (Diptera: Culicidae): a review. *Bulletin of Entomological Research*, *70*(4), 525–532. https://doi.org/DOI: 10.1017/S0007485300007811

Gleave, K., Cook, D., Taylor, M. J., & Reimer, L. J. (2016). Filarial infection influences mosquito behaviour and fecundity. *Scientific Reports*, *6*. https://doi.org/10.1038/srep36319

Gleave, K., Guy, A., Mechan, F., Matope, A., Emery, M., Murphy, A., … McCall, P. J. (2022). Behaviour of pyrethroid resistant Anopheles gambiae at two dual active-ingredient bed nets, assessed by infrared room-scale video tracking. *Malaria Journal*.

Gleave, K., Lissenden, N., Richardson, M., Choi, L., & Ranson, H. (2021). Piperonyl butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa. *Cochrane Database of Systematic Reviews (Online)*. https://doi.org/10.1002/14651858.CD012776.pub3.www.cochranelibrary.com

Gleave, K., Mechan, F., & Reimer, L. J. (2022). The effects of temephos, permethrin and malathion selection on the fitness and fecundity of Aedes aegypti. *Medical and Veterinary Entomology*, *36*(1), 56–65. https://doi.org/10.1111/mve.12551

Hancock, P.A., Wiebe, A., Gleave, K. A., Bhatt, S., Cameron, E., Trett, A., … Moyes, C. L. (2018). Associated patterns of insecticide resistance in field populations of malaria vectors across Africa. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(23). https://doi.org/10.1073/pnas.1801826115

Hancock, Penelope A., Hendriks, C. J. M., Tangena, J. A., Gibson, H., Hemingway, J., Coleman, M., … Moyes, C. L. (2020). Mapping trends in insecticide resistance phenotypes in African malaria vectors. *PLoS Biology*, *18*(6), 1–23. https://doi.org/10.1371/journal.pbio.3000633

Hargreaves, K., Koekemoer, L. L., Brooke, B. D., Hunt, R. H., Mthembu, J., & Coetzee, M. (2000). Anopheles funestus resistant to pyrethroid insecticides in South Africa. *Medical and Veterinary Entomology*, *14*(2), 181–189. https://doi.org/10.1046/j.1365-2915.2000.00234.x

Harris, C., Lambrechts, L., Rousset, F., Abate, L., Nsango, S. E., Fontenille, D., … Cohuet, A. (2010). Polymorphisms in Anopheles gambiae immune genes associated with natural resistance to plasmodium falciparum. *PLoS Pathogens*, *6*(9). https://doi.org/10.1371/journal.ppat.1001112

Harris, C., Lwetoijera, D. W., Dongus, S., Matowo, N. S., Lorenz, L. M., Devine, G. J., & Majambere, S. (2013). Sterilising effects of pyriproxyfen on Anopheles arabiensis and its potential use in malaria control. *Parasites and Vectors*, *6*(1), 1–8. https://doi.org/10.1186/1756-3305-6-144

Hawley, W. A., Phillips-Howard, P. A., ter Kuile, F. O., Terlouw, D. J., Vulule, J. M., Ombok, M., … Hightower, A. W. (2003). Community-wide effects of permethrin-treated bed nets on child mortality and malaria morbidity in western Kenya. *The American Journal of Tropical Medicine and Hygiene*, *68*(4 Suppl), 121–127.

Hemingway, J. (2017). The way forward for vector control. *Science*, *358*(6366), 998–999. https://doi.org/10.1126/science.aaj1644

Hemingway, J., Ranson, H., Magill, A., Kolaczinski, J., Fornadel, C., Gimnig, J., … Hamon, N. (2016). Averting a malaria disaster: Will insecticide resistance derail malaria control? *The Lancet*, *387*(10029), 1785–1788. https://doi.org/10.1016/S0140-6736(15)00417-1

Howlett, F. M. (1910). The Influence of Temperature upon the Biting of Mosquitoes. *Parasitology*, *3*(4), 479–484. https://doi.org/10.1017/S0031182000002304

Hughes, A., Foster, G. M., Guy, A., Matope, A., Abe, M., Towers, D., & McCall, P. J. (2020). Quantifying late-stage host-seeking behaviour of Anopheles gambiae at the insecticidal net interface using a baited-box bioassay. *Malaria Journal*, *19*(1), 1–11. https://doi.org/10.1186/s12936-020-03213-9

Hughes, A. J., Matope, A., Emery, M., Steen, K., Murray, G. P. D., Ranson, H., … Foster, G. M. (2022). *A closer look at the WHO cone bioassay: video analysis of the hidden effects of a human host on mosquito behaviour and insecticide contact*.

Ingham, V. A., Tennessen, J. A., Lucas, E. R., Elg, S., Yates, H. C., Carson, J., … Ranson, H. (2021). Integration of whole genome sequencing and transcriptomics reveals a complex picture of the reestablishment of insecticide resistance in the major malaria vector Anopheles coluzzii. *PLoS Genetics*, *17*(12), 1–26. https://doi.org/10.1371/journal.pgen.1009970

Irvine, M. A., Reimer, L. J., Njenga, S. M., Gunawardena, S., Kelly-Hope, L., Bockarie, M., & Hollingsworth, T. D. (2015). Modelling strategies to break transmission of lymphatic filariasis -aggregation, adherence and vector competence greatly alter elimination. *Parasites and Vectors*, *8*, 547. https://doi.org/10.1186/s13071-015-1152-3

IVCC. (2020). *New Nets Project (NNP)*. Retrieved from https://www.ivcc.com/market-access/new-nets-project/

Jones, C. M., Machin, C., Mohammed, K., Majambere, S., Ali, A. S., Khatib, B. O., … Kelly-Hope, L. A. (2012). Insecticide resistance in Culex quinquefasciatus from Zanzibar: implications for vector control programmes. *Parasites & Vectors*, *5*. https://doi.org/10.1186/1756-3305-5-78

Killeen, G. F. (2014). Characterizing, controlling and eliminating residual malaria transmission. *Malaria Journal*, *13*(1), 1–22. https://doi.org/10.1186/1475-2875-13-330

Killeen, G. F., Kihonda, J., Lyimo, E., Oketch, F. R., Kotas, M. E., Mathenge, E., … Drakeley, C. J. (2006). Quantifying behavioural interactions between humans and mosquitoes: Evaluating the protective efficacy of insecticidal nets against malaria transmission in rural Tanzania. *BMC Infectious Diseases*, *6*, 1–10. https://doi.org/10.1186/1471-2334-6-161

Killeen, G. F., Marshall, J. M., Kiware, S. S., South, A. B., Tusting, L. S., Chaki, P. P., & Govella, N. J. (2017). Measuring, manipulating and exploiting behaviours of adult mosquitoes to optimise malaria vector control impact. *BMJ Global Health*, *2*(2). https://doi.org/10.1136/bmjgh-2016-000212

Kleinschmidt, I., Bradley, J., Knox, T. B., Mnzava, A. P., Kafy, H. T., Mbogo, C., … Donnelly, M. J. (2018). Implications of insecticide resistance for malaria vector control with long-lasting insecticidal nets: a WHO-coordinated, prospective, international, observational cohort study. *The Lancet Infectious Diseases*. https://doi.org/10.1016/S1473-3099(18)30172-5

Knox, T. B., Juma, E. O., Ochomo, E. O., Pates Jamet, H., Ndungo, L., Chege, P., … Coetzee, M. (2014). An online tool for mapping insecticide resistance in major Anopheles vectors of human malaria parasites and review of resistance status for the Afrotropical region. *Parasites and Vectors*, *7*(1), 1–14. https://doi.org/10.1186/1756-3305-7-76

Koella, J. C., Rieu, L., & Paul, R. E. L. (2002). Stage-specific manipulation of a mosquito's host-seeking behavior by the malaria parasite Plasmodium gallinaceum. *Behavioral Ecology*, *13*(6), 816–820. https://doi.org/10.1093/beheco/13.6.816

Lees, R. S., Armistead, J. S., Azizi, S., Constant, E., Diabaté, A., Fornadel, C., … Oxborough, R. (2022). *Strain Characterisation for Measuring Bioefficacy of ITNs Treated with Two Active Ingredients (Dual-AI ITNs): Develop- ing a Robust Protocol by Building Consensus*. (March). https://doi.org/10.20944/preprints202203.0345.v1

Lissenden, N., Armistead, J. S., Gleave, K., Irish, S. R., Martin, J. L., Messenger, L. A., … Lees, R. S. (2022). Developing consensus standard operating procedures (SOPs) to evaluate new types of insecticide-treated nets. *Insects*, *13*(1), 1–27. https://doi.org/10.3390/insects13010007

Lynd, A., & Mccall, P. J. (2013). Clustering of host-seeking activity of Anopheles gambiae mosquitoes at the top surface of a human-baited bed net. *Malaria Journal*, *12*(1), 1–8. https://doi.org/10.1186/1475-2875-12-267

Macdonald, G., & Director, D. T. M. (1956). EPIDEMIOLOGICAL BASIS OF MALARIA CONTROL. In *Bull. Org. mond. Sante*/ (Vol. 15). Bull. Wid Hith Org.

Martinez-Torres, D., Chandre, F., Williamson, M. S., Darriet, F., Bergeâ, J. B., Devonshire, A. L., … Pauron, D. (1998). Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector Anopheles gambiae s.s. *Insect Molecular Biology*, *7*(2), 179–184.

Martins, A. J., Ribeiro, C. D. e. M., Bellinato, D. F., Peixoto, A. A., Valle, D., & Lima, J. B. P. (2012). Effect of insecticide resistance on development, longevity and reproduction of field or laboratory selected aedes aegypti populations. *PLoS ONE*, *7*(3), 1–9. https://doi.org/10.1371/journal.pone.0031889

McMeniman, C. J., Corfas, R. A., Matthews, B. J., Ritchie, S. A., & Vosshall, L. B. (2014). Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans. *Cell*, *156*(5), 1060–1071.

https://doi.org/10.1016/j.cell.2013.12.044

Mnzava, A. P., Knox, T. B., Temu, E. A., Trett, A., Fornadel, C., Hemingway, J., & Renshaw, M. (2015). Implementation of the global plan for insecticide resistance management in malaria vectors: Progress, challenges and the way forward. *Malaria Journal*, *14*(1), 1–9. https://doi.org/10.1186/s12936-015-0693-4

Moiroux, N., Gomez, M. B., Pennetier, C., Elanga, E., Djènontin, A., Chandre, F., … Corbel, V. (2012). *Changes in Anopheles funestus Biting Behavior Following Universal Coverage of Long-Lasting Insecticidal Nets in Benin*. https://doi.org/10.1093/infdis/jis565

Montella, I. R., Martins, A. J., Viana-Medeiros, P. F., Lima, J. B. P., Braga, I. A., & Valle, D. (2007). Insecticide resistance mechanisms of Brazilian Aedes aegypti populations from 2001 to 2004. *American Journal of Tropical Medicine and Hygiene*, *77*(3), 467–477. https://doi.org/10.4269/ajtmh.2007.77.467

Mosha, J. F., Kulkarni, M. A., Lukole, E., Matowo, N. S., Pitt, C., Messenger, L. A., … Protopopoff, N. (2022). Effectiveness and cost-effectiveness against malaria of three types of dual-active-ingredient long-lasting insecticidal nets (LLINs) compared with pyrethroid-only LLINs in Tanzania: a four-arm, cluster-randomised trial. *The Lancet*, *399*(10331), 1227–1241. https://doi.org/10.1016/s0140-6736(21)02499-5

Moyes, C L, Athinya, D. K., Seethaler, T., Battle, K. E., Sinka, M., Hadi, M. P., … Hancock, P. A. (2020). Evaluating insecticide resistance across African districts to aid malaria control decisions. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(36), 22042–22050. https://doi.org/http://dx.doi.org/10.1073/pnas.2006781117

Moyes, Catherine L., Temperley, W. H., Henry, A. J., Burgert, C. R., & Hay, S. I. (2013). Providing open access data online to advance malaria research and control. *Malaria Journal*, *12*(1), 1–9. https://doi.org/10.1186/1475-2875-12-161

Moyes, Catherine L, Wiebe, A., Gleave, K., Trett, A., Hancock, P. A., Gil Padonou, G., … Coleman, M. (n.d.). *analysis-ready datasets for insecticide resistance phenotype and genotype frequency in african malaria vectors*. https://doi.org/10.1038/s41597-019-0134-2

Müller, P., Warr, E., Stevenson, B. J., Pignatelli, P. M., Morgan, J. C., Steven, A., … Donnelly, M. J. (2008). Field-caught permethrin-resistant Anopheles gambiae overexpress CYP6P3, a P450 that metabolises pyrethroids. *PLoS Genetics*, *4*(11). https://doi.org/10.1371/journal.pgen.1000286

N'Guessan, R. N., Odjo, A., Ngufor, C., Malone, D., & Rowland, M. (2016). *A Chlorfenapyr Mixture Net Interceptor ® G2 Shows High Efficacy and Wash Durability against Resistant Mosquitoes in West Africa*. *5*, 1–14. https://doi.org/10.1371/journal.pone.0165925

Ngufor, C., Agbevo, A., Fagbohoun, J., Fongnikin, A., & Rowland, M. (2020). Efficacy of Royal Guard, a new alpha-cypermethrin and pyriproxyfen treated mosquito net, against pyrethroid-resistant malaria vectors. *Scientific Reports*, *10*(1), 1–15. https://doi.org/10.1038/s41598-020-69109-5

Ngufor, C., N'Guessan, R., Fagbohoun, J., Odjo, A., Malone, D., Akogbeto, M., & Rowland, M. (2014). Olyset Duo® (a pyriproxyfen and permethrin mixture net): An experimental hut trial against pyrethroid resistant Anopheles gambiae and Culex quinquefasciatus in southern Benin. *PLoS ONE*, *9*(4). https://doi.org/10.1371/journal.pone.0093603

Ohashi, K., Nakada, K., Ishiwatari, T., Miyaguchi, J., Shono, Y., Lucas, J. R., & Mito, N. (2012). Efficacy of Pyriproxyfen-Treated Nets in Sterilizing and Shortening the Longevity of Anopheles gambiae (Diptera: Culicidae). *J. Med. Entomol*, *49*(5), 1052–1058. https://doi.org/10.1603/ME12006

Ohm, J. R., Baldini, F., Barreaux, P., Lefevre, T., Lynch, P. A., Suh, E., … Thomas, M. B. (2018). Rethinking the extrinsic incubation period of malaria parasites. *Parasites and Vectors*, *11*(1), 1–9. https://doi.org/10.1186/s13071-018-2761-4

Oxborough, R. M., N'Guessan, R., Jones, R., Kitau, J., Ngufor, C., Malone, D., … Rowland, M. W. (2015). The activity of the pyrrole insecticide chlorfenapyr in mosquito bioassay: Towards a more rational testing and screening of non-neurotoxic insecticides for malaria vector control. *Malaria Journal*, *14*(1). https://doi.org/10.1186/s12936-015-0639-x

Paaijmans, K. P., Blanford, S., Bell, A. S., Blanford, J. I., Read, A. F., & Thomas, M. B. (2010). Influence of climate on malaria transmission depends on daily temperature variation. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(34), 15135–15139. https://doi.org/10.1073/pnas.1006422107

Paily, K. P., Hoti, S. L., & Das, P. K. (2009). A review of the complexity of biology of lymphatic filarial parasites. *Journal of Parasitic Diseases*, *33*(1), 3–12. https://doi.org/10.1007/s12639-009-0005-4

Parker, Angarita-Jaimes, N., Abe, M., Towers, C. E., Towers, D., & McCall, P. J. (2015). Infrared video tracking of Anopheles gambiae at insecticide-treated bed nets reveals rapid decisive impact after brief localised net contact. *Scientific Reports*, *5*(July), 1–14. https://doi.org/10.1038/srep13392

Parker, J. E. A., Angarita Jaimes, N. C., Gleave, K., Mashauri, F., Abe, M., Martine, J., … McCall, P. J. (2017). Host-seeking activity of a Tanzanian population of Anopheles arabiensis at an insecticide treated bed net. *Malaria Journal*, *16*(1). https://doi.org/10.1186/s12936-017-1909-6

Pates, H., & Curtis, C. (2005). Mosquito behaviour and vector control. *Annual Review of Entomology*, *50*, 53–70.

Ponlawat, A, & Harrington, L. C. (2007). Age and body size influence male sperm capacity of the dengue vector Aedes aegypti (Diptera: Culicidae). *Journal of Medical Entomology*, *44*(February), 422–426. https://doi.org/10.1603/0022-2585(2007)44[422:aabsim]2.0.co;2

Ponlawat, Alongkot, & Harrington, L. C. (2009). Factors associated with male mating success of the dengue vector mosquito, Aedes aegypti. *The American Journal of Tropical Medicine and Hygiene*, *80*(3), 395–400. https://doi.org/80/3/395 [pii] ET - 2009/03/10 LA - eng

Protopopoff, N, Mosha, J. F., Lukole, E., Charlwood, J. D., Wright, A., Mwalimu, C. D., … et al. (2018). Effectiveness of a long-lasting piperonyl butoxide-treated insecticidal net and indoor residual spray interventions, separately and together, against malaria transmitted by pyrethroid-resistant mosquitoes: a cluster, randomised controlled, two-by-two fact. *Lancet*, *391*(10130), 1577-1588. https://doi.org/10.1016/S0140-6736%2818%2930427-6

Protopopoff, Natacha, Mosha, J. F., Lukole, E., Charlwood, J. D., Wright, A., Mwalimu, C. D., … Rowland, M. (2018). Effectiveness of a long-lasting piperonyl butoxide-treated

insecticidal net and indoor residual spray interventions, separately and together, against malaria transmitted by pyrethroid-resistant mosquitoes: a cluster, randomised controlled, two-by-two fact. *The Lancet*, *391*(10130), 1577–1588. https://doi.org/10.1016/S0140-6736(18)30427-6

Pryce, J., Richardson, M., & Lengeler, C. (2018). Insecticide-treated nets for preventing malaria. *Cochrane Database of Systematic Reviews*, *2018*(11). https://doi.org/10.1002/14651858.CD000363.pub3

Rahim, J., Ahmad, A. H., & Maimusa, A. H. (2017). Effects of temephos resistance on life history traits of Aedes albopictus (Skuse) (Diptera: Culicidae), a vector of arboviruses. *Revista Brasileira de Entomologia*, *61*(4), 312–317. https://doi.org/10.1016/j.rbe.2017.08.001

Ramos, R. S., Araújo, V. C. R. De, Pereira, R. R., Martins, J. C., Queiroz, O. S., Silva, R. S., & Picanço, M. C. (2018). Investigation of the lethal and behavioral effects of commercial insecticides on the parasitoid wasp Copidosoma truncatellum. *Chemosphere*, *191*, 770–778. https://doi.org/10.1016/j.chemosphere.2017.10.113

Ranson, H, Jensen, B., Vulule, J. M., Wang, X., Hemingway, J., & Collins, F. H. (2000). Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan Anopheles gambiae associated with resistance to DDT and pyrethroids. In *Insect Molecular Biology* (Vol. 9).

Ranson, H, & Lissenden, N. (2016). Insecticide Resistance in African Anopheles Mosquitoes: A Worsening Situation that Needs Urgent Action to Maintain Malaria Control. *Trends in Parasitology*, *32 (3)*, 187–196. https://doi.org/http://dx.doi.org/10.1016/j.pt.2015.11.010

Ranson, Hilary, & Lissenden, N. (2016). Insecticide Resistance in African Anopheles Mosquitoes: A Worsening Situation that Needs Urgent Action to Maintain Malaria Control. *Trends in Parasitology*, *32*(3), 187–196. https://doi.org/10.1016/j.pt.2015.11.010

Robinson, A., Busula, A. O., Voets, M. A., Beshir, K. B., Caulfield, J. C., Powers, S. J., … De Boer, J. G. (2018). Plasmodium-associated changes in human odor attract mosquitoes. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(18), E4209–E4218. https://doi.org/10.1073/pnas.1721610115

Rowland, M., & Boersma, E. (1988). Changes in the spontaneous flight activity of the mosquito Anopheles stephensi by parasitization with the rodent malaria Plasmodium yoelii. *Parasitology*, *97 (Pt 2)*, 221–227. https://doi.org/10.1017/s003118200005842x

Rudolfs, W. (1922). *Chemotropism of mosquitoes* (367th ed.). Bulletin: New Jersey Agricultural Experiment Station.

Saingamsook, J., Yanola, J., Lumjuan, N., Walton, C., & Somboon, P. (2019). Investigation of relative development and reproductivity fitness cost in three insecticide-resistant strains of Aedes aegypti from Thailand. *Insects*, *10*(9), 1–16. https://doi.org/10.3390/insects10090265

Schiefer, B. A., Ward, R. A., & Eldridge, B. F. (1977). Plasmodium cynomolgi: effects of malaria infection on laboratory flight performance of Anopheles stephensi mosquitoes. *Experimental Parasitology*, *41*(2), 397–404. https://doi.org/10.1016/0014-4894(77)90111-4

Service, M. (2012). *Medical Entomology for Students, 5th Edition* (5th ed., Vol. 20). https://doi.org/10.3201/eid2008.131738

Silva, A. P. B., Santos, J. M. M., & Martins, A. J. (2014). Mutations in the voltage-gated sodium channel gene of anophelines and their association with resistance to pyrethroids - A review. *Parasites and Vectors*, *7*(1), 1–14. https://doi.org/10.1186/1756-3305-7-450

Sinka, M. E., Rubio-Palis, Y., Manguin, S., Patil, A. P., Temperley, W. H., Gething, P. W., … Hay, S. I. (2011). Erratum: The dominant Anopheles vectors of human malaria in the Americas: Occurrence data, distribution maps and bionomic précis (Parasit Vectors (2010) 3 (72)). *Parasites and Vectors*, *4*(1), 1–26. https://doi.org/10.1186/1756-3305-4-210

Smallegange, R. C., Verhulst, N. O., & Takken, W. (2011). Sweaty skin: An invitation to bite? *Trends in Parasitology*, *27*(4), 143–148. https://doi.org/10.1016/j.pt.2010.12.009

Smith, D. L., McKenzie, F. E., Snow, R. W., & Hay, S. I. (2007). Revisiting the basic reproductive number for malaria and its implications for malaria control. *PLoS Biology*, *5*(3), 0531–0542. https://doi.org/10.1371/journal.pbio.0050042

Soderlund, D. M., Clark, J. M., Sheets, L. P., Mullin, L. S., Piccirillo, V. J., Sargent, D., … Weiner, M. L. (2002). Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. In *Toxicology* (Vol. 171). Retrieved from www.elsevier.com/locate/toxicol

Somerville, A. G. T., Gleave, K., Jones, C. M., & Reimer, L. J. (2019). The consequences of Brugia malayi infection on the flight and energy resources of Aedes aegypti mosquitoes. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-54819-2

Staedke, S. G., Gonahasa, S., Dorsey, G., Kamya, M. R., Maiteki-Sebuguzi, C., Lynd, A., … Donnelly, M. J. (2020). Effect of long-lasting insecticidal nets with and without piperonyl butoxide on malaria indicators in Uganda (LLINEUP): a pragmatic, cluster-randomised trial embedded in a national LLIN distribution campaign. *Lancet*, *395*(10232), 1292–1303. https://doi.org/10.1016/s0140-6736(20)30214-2

Steele, J. E. (1981). The role of carbohydrate metabolism in physiological function. In *Energy Metabolism in Insects*. Plenum, New York.

Stevenson, B. J., Bibby, J., Pignatelli, P., Muangnoicharoen, S., O'Neill, P. M., Lian, L. Y., … Paine, M. J. I. (2011). Cytochrome P450 6M2 from the malaria vector Anopheles gambiae metabolizes pyrethroids: Sequential metabolism of deltamethrin revealed. *Insect Biochemistry and Molecular Biology*, *41*(7), 492–502. https://doi.org/10.1016/j.ibmb.2011.02.003

Sutcliffe, J F. (1994). Sensory bases of attractancy: morphology of mosquito olfactory sensilla-- a review. *Journal of the American Mosquito Control Association*, *10*(2 Pt 2), 309–315.

Sutcliffe, James F., & Yin, S. (2014). Behavioural responses of females of two anopheline mosquito species to human-occupied, insecticide-treated and untreated bed nets. *Malaria Journal*, *13*(1), 1–19. https://doi.org/10.1186/1475-2875-13-294

Takken, W. (1991). The Role of Olfaction in Host-Seeking of Mosquitoes: A Review. *International Journal of Tropical Insect Science*, *12*(1), 287–295. https://doi.org/10.1017/S1742758400020816

The Alliance for Malaria Prevention. (n.d.). The Alliance for Malaria Prevention. Retrieved from https://allianceformalariaprevention.com/tools-guidance/multi-product-itn-distributio n/

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

Thornton, J., Gomes, B., Ayres, C., & Reimer, L. (2020). Insecticide resistance selection and reversal in two strains of Aedes aegypti. *Wellcome Open Research*.

Tiono, A. B., Ouédraogo, A., Ouattara, D., Bougouma, E. C., Coulibaly, S., Diarra, A., … Lindsay, S. W. (2018). Efficacy of Olyset Duo, a bednet containing pyriproxyfen and permethrin, versus a permethrin-only net against clinical malaria in an area with highly pyrethroid-resistant vectors in rural Burkina Faso: a cluster-randomised controlled trial. *The Lancet*, *392*(10147), 569–580. https://doi.org/10.1016/S0140-6736(18)31711-2

Toé, K. H., Jones, C. M., N'fale, S., Ismai, H. M., Dabiré, R. K., & Ranson, H. (2014). Increased pyrethroid resistance in malaria vectors and decreased bed net effectiveness Burkina Faso. *Emerging Infectious Diseases*, *20*(10), 1691–1696. https://doi.org/10.3201/eid2010.140619

Treacy, M., Miller, T., Black, B., Gard, I., Hunt, D., & Hollingworth, R. M. (1994). Uncoupling activity and pesticidal properties of pyrroles. *Biochemical Society Transactions*, *22*(1), 244–247. https://doi.org/10.1042/bst0220244

Tungu, P. K., Michael, E., Sudi, W., Kisinza, W. W., & Rowland, M. (2021). Efficacy of interceptor ® G2 , a long - lasting insecticide mixture net treated with chlorfenapyr and alpha - cypermethrin against Anopheles funestus : experimental hut trials in north - eastern Tanzania. *Malaria Journal*, 1–15. https://doi.org/10.1186/s12936-021-03716-z

Van Breugel, F., Riffell, J., Fairhall, A., & Dickinson, M. H. (2015). Mosquitoes use vision to associate odor plumes with thermal targets. *Current Biology*, *25*(16), 2123–2129. https://doi.org/10.1016/j.cub.2015.06.046

Vaughan, J. A. (2007). Population dynamics of Plasmodium sporogony. *Trends in Parasitology*, *23*(2), 63–70. https://doi.org/10.1016/j.pt.2006.12.009

VCAG. (2015). Third meeting of the Vector Control Advisory Group, Geneva, Switzerland 12-14 November 2014. *Third Meeting of the Vector Control Advisory Group, Geneva, Switzerland 12-14 November 2014*, (November), xi-pp. Retrieved from http://apps.who.int/iris/bitstream/10665/162861/1/9789241508674_eng.pdf

Venugopal, K., Hentzschel, F., Valkiūnas, G., & Marti, M. (2020). Plasmodium asexual growth and sexual development in the haematopoietic niche of the host. *Nature Reviews Microbiology*, *18*(3), 177–189. https://doi.org/10.1038/s41579-019-0306-2

Vézilier, J., Nicot, A., Gandon, S., & Rivero, A. (n.d.). *Plasmodium infection decreases fecundity and increases survival of mosquitoes*. https://doi.org/10.1098/rspb.2012.1394

Viana-Medeiros, P. F., Bellinato, D. F., Martins, A. J., & Valle, D. (2017). Insecticide resistance,

associated mechanisms and fitness aspects in two Brazilian Stegomyia aegypti (= Aedes aegypti) populations. *Medical and Veterinary Entomology*, *31*(4), 340–350. https://doi.org/10.1111/mve.12241

Voloshin, V., Kröner, C., Seniya, C., Murray, G. P. D., Guy, A., Towers, C. E., … Towers, D. P. (2020). *Diffuse retro-reflective imaging for improved video tracking of mosquitoes at human baited bednets*. https://doi.org/10.1098/rsos.191951

Vontas, J., Blass, ‡ C, Koutsos, A. C., David, J.-P., Kafatos, F. C., Louis, C., … Ranson, H. (2005). Gene expression in insecticide resistant and susceptible Anopheles gambiae strains constitutively or after insecticide exposure. *Insect Molecular Biology*, *14*(5), 509–521. https://doi.org/10.1111/j.1365-2583.2005.00582.x

Wattam, A. R., & Christensen, B. M. (1992). Induced polypeptides associated with filarial worm refractoriness in Aedes aegypti. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(14), 6502–6505. https://doi.org/10.1073/pnas.89.14.6502

Wekesa, J. W., Copeland, R. S., & Mwangi, R. W. (1992). Effect of Plasmodium falciparum on blood feeding behavior of naturally infected Anopheles mosquitoes in western Kenya. *The American Journal of Tropical Medicine and Hygiene*, *47*(4), 484–488. https://doi.org/10.4269/ajtmh.1992.47.484

WHO. (2010). Global Programme to Eliminate Lymphatic Filariasis: Progress Report 2000-2009 and Strategic Plan 2010-2020. *World Health Organization*, 1–93.

WHO. (2016). *Test procedures for insecticide resistance monitoring in malaria vector mosquitoes Second edition*.

WHO. (2017). Conditions for deployment of mosquito nets treated with a pyrethroid and piperonyl butoxide. *Global Malaria Programme, World Health Organization*, *2017*(September), 1–4. Retrieved from http://apps.who.int/iris/bitstream/handle/10665/258939/WHO-HTM-GMP-2017.17-eng.pdf;jsessionid=E82BB1F992D156DD7A961AEF99AC1C48?sequence=1

WHO. (2018). *Global report on insecticide resistance in malaria vectors: 2010-2016*.

WHO. (2019a). WHO | Prequalified Lists: Vector control products. *Who*, (001), 1–5. Retrieved from https://www.who.int/pq-vector-control/prequalified-lists/LOPrequalifiedProducts20190411.pdf?ua=1

WHO. (2019b). World malaria report 2019. In *WHO Regional Office for Africa*. Retrieved from https://www.who.int/news-room/fact-sheets/detail/malaria

WHO. (2021). World Malaria Report 2021. In *Word Malaria report Geneva: World Health Organization. (2021). Licence: CC*.

Wiebe, A., Longbottom, J., Gleave, K., Shearer, F. M., Sinka, M. E., Massey, N. C., … Moyes, C. L. (2017). Geographical distributions of African malaria vector sibling species and evidence for insecticide resistance. *Malaria Journal*, *16*(1). https://doi.org/10.1186/s12936-017-1734-y

Williams, J., Flood, L., Praulins, G., Ingham, V. A., Morgan, J., Lees, R. S., & Ranson, H. (2019). Characterisation of Anopheles strains used for laboratory screening of new vector control products. *Parasites and Vectors*, *12*(1), 1–14. https://doi.org/10.1186/s13071-019-3774-3

Williams, J., Ingham, V. A., Morris, M., Toe, H. K., Hien, A. S., Morgan, J. C., … Ranson, H. (2022). Sympatric populations of the Anopheles gambiae complex in southwest Burkina Faso evolve multiple diverse resistance mechanisms in response to intense selection pressure with pyrethroids. *Insects*.

Wood, O. R., Hanrahan, S., Coetzee, M., Koekemoer, L. L., & Brooke, B. D. (2010). Cuticle thickening associated with pyrethroid resistance in the major malaria vector Anopheles funestus. *Parasites and Vectors*, *3*(1), 1–7. https://doi.org/10.1186/1756-3305-3-67

World Health Organization. (2006). *Guidelines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets*.

World Health Organization. (2013). *Guidelines for laboaratory and field-testing of long-lasting insecticidal nets*.

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

Zaim, M., Aitio, A., & Nakashima, N. (2000). Safety of pyrethroid-treated mosquito nets. *Medical and Veterinary Entomology*, *14*(1), 1–5. https://doi.org/10.1046/j.1365-2915.2000.00211.x

Zwiebel, L. J., & Takken, W. (2004). *Olfactory regulation of mosquito-host interactions*. https://doi.org/10.1016/j.ibmb.2004.03.017

WHO (2019) *List of WHO Prequalified Vector Control Products Date (Updated: 11.04.2019)*.

Available at:

https://extranet.who.int/pqweb/vector-control-products/prequalified-product-list

Appendix 1. Katherine Gleave full bibliography

Gleave, K., Cook, D., Taylor, M. J., Reimer, L. J. (2016) Filarial infection influences mosquito behaviour and fecundity. *Nature Scientific Reports*. 6:36319 DOI: 10.1038/srep36319 [Conceived and designed study with LR, conducted experiments and carried out data analysis]

Coleman, M., Hemingway, J., **Gleave, K**., Wiebe, A., Gething, P. W., Moyes, C. L. (2017) Developing global maps of insecticide resistance risk to improve vector control. *Malaria Journal*. 16:86 DOI 10.1186/s12936-017-1733-z [Compiled insecticide resistance data, geopositioned data, contributed to interpretation and write up of results and manuscript]

Wiebe, A., Longbottom, J., **Gleave, K**., Shearer, F. M., Sinka, M. E., Massey, N. C., Cameron, E., Bhatt, S., Gething, P. W., Hemingway, J., Smith, D. L., Coleman, M., Moyes, C. L. (2017) Geographical distributions of African malaria vector sibling species and evidence for insecticide resistance. *Malaria Journal* 16.85 DOI 10.1186/s12936-017-1734-y [Compiled insecticide resistance data overview, along with other authors processed and geopositioned data, all authors contributed to manuscript]

Parker, J. E. A., Angarita Jaimes, N. C., **Gleave, K**., Mashuari, F., Abe, M., Martine, J., Towers, C. E., Towers, D., McCall, P. J. (2017) Host-seeking activity of a Tanzanian population of *Anopheles arabiensis* at an insecticide treated bed net. *Malaria Journal*. 16;270 https://doi.org/10.1186/s12936-017-1909-6 [Assisted with data extraction and analyses, all authors contributed to final manuscript]

Hancock, P. A., Wiebe, A., **Gleave, K**., Bhatt, S., Cameron, E., Trett, A., Weetman, D., Smith, D. L., Hemingway, J., Coleman, M., Gething, P. W., Moyes, C. L. (2018) Associated patterns of insecticide resistance in field populations of malaria vectors across Africa. *PNAS* 5;115(23):5938-5943 doi: 10.1073/pnas.1801826115 [performed research to extract and analyse data]

Moyes, C. L., Wiebe, A., **Gleave, K**., Trett, A., Hancock, P. A., Padonou, G. G., Chouaibou, M. S., Sovi, A., Abuelmaali, S. A., Ochomo, E., Antonio-Nkondjio, C., Dengela, D., Kawada, H., Dabire, R. K., Donnelly, M. J., Mbogo, C., Fornadel, C., Coleman, M. (2019) Analysis-ready datasets for insecticide resistance phenotype and genotype frequency in African malaria vectors. *Nature Scientific Data*. 6:121 https://doi.org/10.1038/s41597-019-0134-2

[extracted, processed and geopositioned data, extracted recommended sample sizes, doses and exposure durations from protocols, contributed to manuscript]

Somerville, A. G. T., **Gleave, K**., Jones, C. M., Reimer, L. J. (2019) The consequences of *Brugia malayi* infection on the flight and energy resources of *Aedes aegypti* mosquitoes. *Nature Scientific Reports*. 9:18449 https://doi.org/10.1038/s41598-019-54819-2 [Assisted with training, data collection, and manuscript]

Gleave, K., Lissenden, N., Chaplin, M., Choi, L, Ranson, H. (2021) Piperonyl butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa. *Cochrane Database of Systematic Reviews*. DOI: 10.1002/14651858.CD012776.pub3. [Conceived and designed the protocol, conducted trail screening, data extraction and data analysis, wrote manuscript]

Lissenden, K., Armistead, J. S., **Gleave, K**., Irish, S. R., Martin, J. L., Messenger, L. A., Moore, S. J., Ngufor, C., Protopopoff, N., Oxborough, R., Spiers, A., Lees, R. S. (2021) Developing consensus standard operating procedures (SOPs) to evaluate new types of insecticide-treated nets. *Insects* 13(1), 7; https://doi.org/10.3390/insects13010007 [Investigation, methodology development, reviewing and editing manuscript]

Gleave, K., Mechan, F., Reimer, L. J. (2021) The effects of temephos, permethrin and malathion selection on the fitness and fecundity of *Aedes aegypti*. *Medical and Veterinary Entomology*. 36, 56-65 doi: 10.1111/mve.12551 [Conceived and designed the study, collected and analysed the data, manuscript]

Hall, M. L., **Gleave, K**., Hughes, A., McCall, P. J., Towers, C. E., Towers, D. P. (2022) The application of digital holography for accurate three-dimensional localisation of mosquito-bednet interaction. *Light: Advanced Manufacturing* doi: 10.37188/lam.2022.020 [Aided with data collection, knowledge, manuscript]

Gleave, K., Guy, A., Mechan, F., Foster, G., Ranson, H., McCall, P. J. (2022) Behaviour of pyrethroid resistant Anopheles gambiae at the interface of two dual active-ingredient bed nets, assessed by room-scale infrared video tracking. [Data collection, data analysis, manuscript]

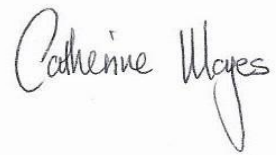
Appendix 2. Signed author statements

Katherine Gleave is submitting the following paper for consideration as part of a PhD by published work in the Vector Biology Department at the Liverpool School of Tropical Medicine.

Coleman, M., Hemingway, J., **Gleave, K**., Wiebe, A., Gething, P. W., Moyes, C. L. (2017) Developing global maps of insecticide resistance risk to improve vector control. *Malaria Journal*. 16:86 DOI 10.1186/s12936-017-1733-z

Statement of contribution by Katherine Gleave:

KG compiled insecticide resistance data from published articles, contacted authors for unpublished or missing data and contacted custodians of unpublished data sets (with AW). KG geopositioned collated data (with AW) and contributed to the interpretation and write up of results (with all authors).

Name	Signature	Date
Moyes, C. L		21 April 2022

Katherine Gleave is submitting the following paper for consideration as part of a PhD by published work in the Vector Biology Department at the Liverpool School of Tropical Medicine.

Moyes, C. L., Wiebe, A., **Gleave, K.**, Trett, A., Hancock, P. A., Padonou, G. G., Chouaibou, M. S., Sovi, A., Abuelmaali, S. A., Ochomo, E., Antonio-Nkondjio, C., Dengela, D., Kawada, H., Dabire, R. K., Donnelly, M. J., Mbogo, C., Fornadel, C., Coleman, M. (2019) Analysis-ready datasets for insecticide resistance phenotype and genotype frequency in African malaria vectors. *Nature Scientific Data*. 6:121 https://doi.org/10.1038/s41597-019-0134-2

Statement of contribution by Katherine Gleave:

KG extracted, processed and geopositioned collected data (with AW). KG extracted recommended sample sizes, insecticide doses and exposure durations from all WHO and CDC protocols. KG contributed to the final manuscript.

Name	Signature	Date
Coleman, M		21/04/2022

Katherine Gleave is submitting the following paper for consideration as part of a PhD by published work in the Vector Biology Department at the Liverpool School of Tropical Medicine.

Gleave, K., Lissenden, N., Chaplin, M., Choi, L, Ranson, H. (2021) Piperonyl butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa. *Cochrane Database of Systematic Reviews*. DOI: 10.1002/14651858.CD012776.pub3.

Statement of contribution by Katherine Gleave:

KG conceived and designed the study (with NL and HR). KG conducted trial screening, data extraction and analysis (with NL). KG prepared the first drafts of the manuscript (with NL) and completed the final version for publication.

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Lissenden, L		08.04.22
Ranson, H		08.04.22

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Statement of contribution by Katherine Gleave:

KG collected room scale tracking data (with AG). KG cleaned and analysed the data. KG wrote and prepared the manuscript for circulation and completed the final version.

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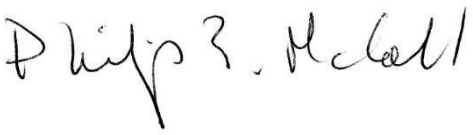
Gleave, K		07.04.22
McCall, P. J		7th April 2022

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KG (with LJR) conceived and designed the study. KG collected the laboratory data and led data analysis (with FM). KG wrote the first version of the manuscript and prepared the final manuscript for publication.

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Gleave, K., Cook, D., Taylor, M. J., Reimer, L. J. (2016) Filarial infection influences mosquito behaviour and fecundity. *Nature Scientific Reports*. 6:36319 DOI: 10.1038/srep36319

Statement of contribution by Katherine Gleave:

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KG performed training, aided in data collection (with AGTS) and contributed to manuscript preparation.

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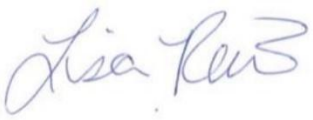
Reimer, L. J		11.04.22

The published works

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Malaria Journal

RESEARCH Open Access

Developing global maps of insecticide resistance risk to improve vector control

Michael Coleman[1*], Janet Hemingway[1], Katherine Ann Gleave[1], Antoinette Wiebe[2], Peter W. Gething[2] and Catherine L. Moyes[2*]

Abstract

Background: Significant reductions in malaria transmission have been achieved over the last 15 years with elimination occurring in a small number of countries, however, increasing drug and insecticide resistance threatens these gains. Insecticide resistance has decreased the observed mortality to the most commonly used insecticide class, the pyrethroids, and the number of alternative classes approved for use in public health is limited. Disease prevention and elimination relies on operational control of *Anopheles* malaria vectors, which requires the deployment of effective insecticides. Resistance is a rapidly evolving phenomena and the resources and human capacity to continuously monitor vast numbers of mosquito populations in numerous locations simultaneously are not available.

Methods: Resistance data are obtained from published articles, by contacting authors and custodians of unpublished data sets. Where possible data is disaggregated to single sites and collection periods to give a fine spatial resolution.

Results: Currently the data set includes data from 1955 to October 2016 from 71 malaria endemic countries and 74 anopheline species. This includes data for all four classes of insecticides and associated resistance mechanisms.

Conclusions: Resistance is a rapidly evolving phenomena and the resources and human capacity to continuously monitor vast numbers of mosquito populations in numerous locations simultaneously are not available. The Malaria Atlas Project-Insecticide Resistance (MAP-IR) venture has been established to develop tools that will use available data to provide best estimates of the spatial distribution of insecticide resistance and help guide control programmes on this serious issue.

Keywords: Insecticide resistance, Malaria, Anopheles, Map

Background

Since the beginning of the century the number of annual deaths attributed to malaria has more than halved due to significant investment in improved case treatment, and insecticide-based vector control [1]. Only through this multifaceted approach will malaria control and elimination succeed. Effective vector control is a key component of this strategy with insecticides playing a central role in most malaria control programmes. The main focus of prevention relies on long-lasting insecticide-treated nets (LLINs) or indoor residual spraying (IRS), with LLINs alone contributing to 68% of all averted cases over the last 15 years [2]. In Africa over 60% of the population at risk are estimated to sleep under a net while 5% are protected by IRS [1]. The efficacy of these interventions may be compromised by both behavioural avoidance and physiological resistance in malaria vectors. Previously the Malaria Atlas Project (MAP) has collated what data is available for vector bionomics, including behaviour [3] for the dominant vectors of human malaria and now MAP aims to address physiological insecticide resistance.

Currently the only insecticides recommended for use on LLINs by the World Health Organization (WHO) are

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pyrethroids [4], as they have low mammalian toxicity and high insecticidal activity [5]. In 2013 nearly two thirds of IRS programmes world-wide also relied on pyrethroids. This, along with pyrethroid use in agriculture, has resulted in a high selection pressure for pyrethroid resistance [6–8]. The pressure has been sufficiently severe that there is increasing evidence of pyrethroid failure, particularly for IRS. Since 2015 the more expensive organophosphate pirimiphos methyl has largely replaced pyrethroids for IRS.

The history of insecticide resistance detection has been reviewed elsewhere [6, 7], as have the tools and methods used in detecting resistance [9, 10]. Of greater concern are the increased reports on the ineffectiveness of current malaria prevention tools [11–15]. Risk in public health is defined as; 'the potential for realization of unwanted, adverse consequences to human life health, property or the environment' [16]. Applying this here, insecticide resistance poses a serious risk to current malaria prevention activities.

In 2012, WHO published the Global Plan for Insecticide Resistance Management (GPIRM) [17] with the aim of raising awareness of insecticide resistance. The goal is that this plan will be supplemented with guidelines, enabling control programmes to develop individually tailored insecticide resistance management strategies. One acute operational difficulty is the lack of nationally representative spatial and temporal comparable data that concurrently measures insecticide resistance and associated mechanisms. This can be attributed to the shortfall of entomologists, lack of appropriate infrastructure and available funding [18].

To date information on the increase in insecticide resistance is rooted in national reporting systems, predominantly driven by the locality of researchers [1, 7]. Previously, two global insecticide resistance databases have been established, IR Mapper collated 4,084 susceptibility data points by 2014 [19] and VectorBase currently provides 5,656 corrected mortality values [20], and WHO has now created a third [21]. These databases all contain differing amounts of resistance data with information, displayed as single points on maps. The online tools provided by each database allow users to visualise information about each data point, such as the species tested or the sample size, but they do not attempt to take account of any of the potential confounding factors within these datasets or the sampling biases that are present. This, combined with under reporting, for example less than half of the malaria endemic countries reported any entomological data last year, highlights the need to take account of potential confounders and biases to produce robust, consistent and comprehensive estimates of resistance that fill the current gaps in the data.

A new global mapping project

MAP-IR will first collate and assess the available field data on insecticide resistance, then develop a modelling framework to analyse spatiotemporal patterns of resistance. Here the dataset collated so far from published and unpublished sources is described and assessed. The strengths and weaknesses of the available data are discussed and an analytical plan is outlined that mitigates the issues associated with using collated data that was not generated from a single, systematic, global sampling design. The ultimate aim of this work is to provide resistance data that can be combined with information on vector species and disease prevalence to increase our understanding of the impact that resistance has on disease control. Future work based on the data and principles outlined here will generate the tools to help better target interventions and aid with the development of insecticide resistance management plans [21, 22] on a global scale.

Methods

Resistance data are obtained from three sources; through published articles, by contacting authors, and by contacting the custodians of unpublished datasets. Published articles are identified using the search terms "insecticide resistance" and "anopheles" in the Web of Science database with no date or language restrictions. Currently all articles published up to the end of 2015 that could be obtained have been reviewed and 684 articles containing bioassay results identified. Of the groups contacted, 15 have so far provided unpublished data.

Where possible received data is disaggregated to single sites and collection periods to provide a fine resolution spatial and temporal dataset. Records reporting less than 100% mortality in the susceptible strain were excluded as were records with control mortality above 20% and results from samples that had been through more than one generation in the laboratory. The data fields extracted cover: mosquito collection methods; mosquito identification methods; bioassay conditions including protocol followed, insecticide concentration, exposure period, mosquito generation tested (wild caught, F1 or mixed), and whether a synergist was used; information about the collection site, and information about the data source. Further details on the exact data fields recorded are given in Additional file 1. Sites covering an area less than 25 km^2 are assigned coordinates in digital degrees using either the coordinates provided with the data, or using contextual information provided about the site to locate it in online gazetteers such as GeoNames and Google Maps. If mosquitoes from multiple sites were pooled for the bioassay, each site is recorded in the database. If an area greater than 25 km^2 is given and it is not possible

to disaggregate this further, the borders of the area are defined using GIS software such as ArcMap or QGIS. In circumstances where the area given is an administrative unit then the borders are taken from the FAO's Global Administrative Unit Layers [23]. In addition, when resistance mechanism data are provided, such as *kdr* allele frequencies and P450/mixed function oxidase (MFO) test results, this information is linked to the mosquito collection fields and when relevant also to the bioassay fields.

Site coordinates linked to each dataset are checked using GIS software to ensure the coordinates fall on land, in the right country, and that the location of sites matches the description given by the data source. All other fields are checked to ensure each value falls within the expected range and to identify any missing data, which are then requested from the data source.

This data collation is still in process but data has been extracted from all available articles published up to the end of 2015 that met the inclusion criteria. The current dataset has been assessed to inform the next stage of the planned analyses.

In order to visualise apparent trends for the most important class of insecticides, the full dataset was filtered to extract all bioassay records that used a pyrethroid insecticide. The current dataset was examined over three time periods which were chosen based on data availability and the introduction of pyrethroids in agriculture and public health. Each location linked to these bioassays was assigned to the first order administrative division, as defined by the Global Administrative Units Layer for 2013, that the coordinates or polygon fell within. Any locations that spanned more than one administrative unit were excluded. Where the collection date was missing, the date was assumed to be two years before the article publication year, based on the trend seen for records that have a collection date. For the purposes of this exercise, if the number of mosquitoes tested was missing then the number was assumed to be 60, which is the lower quartile value from the full set of records that did report the number tested.

Data from each first order administrative unit for each of the three time periods was then combined to obtain the first and last years that mosquitoes were collected in, the total number of bioassay records (each record represents a unique collection site and period from a unique study), the total number of mosquitoes tested, and the average reported mortality across all of the records. The average mortality was then plotted on a map, and the full data fields are given in Additional file 2.

Results

Data availability for standard metrics linked to insecticide resistance

The full current dataset as of October 2016 is summarized in Table 1 and includes insecticide resistance data from 1955 from 71 malaria endemic countries and 74 anopheline species or species complexes. The data includes 1018 survey locations reporting carbamate resistance, 1655 reporting organochlorine resistance, 1056 locations reporting organophosphate resistance and 3127 reporting pyrethroid resistance. These data also cover different insecticides within each class, specifically three carbamates, five organochlorines, eight organophosphates and eight pyrethroids. The methods used to generate these data included CDC bottle assays and ten versions of the WHO bioassay. Figure 1 shows that the data for each of the major insecticide classes are highly clustered, indicating that any analysis of this data needs to account of the clear biases in the location sampled. Temporal bias can also be seen with more data available in more recent years for each class of insecticide.

Mapping of pyrethroid resistance over time

The apparent trends of pyrethroid resistance (Fig. 2) were mapped. The base map layers used show malaria endemicity for each time period. Specifically, the 1980–99 map used the 1990 data from the Malaria Elimination Initiative's time series [24], the 2000–07 map used the WHO's 2004 data [25] and the 2008–15 map used the 2011 data from the WHO's 2012 world malaria report [26].

The purpose of the map presented in Fig. 2 was to assess whether there are apparent trends of potential interest that justify a full analysis. The data visualization presented in Fig. 2 should be treated with caution. This map simply displays the raw data without any correction for spatial bias within administrative divisions or

Table 1 The number of records collated to-date

Data type	No. records	No. point locations	No. polygons
Insecticide resistance data from bioassays	14,951	2057	333
kdr allele frequencies	1475	882	25
P450 enzyme activity and gene expression	104	34	1
Esterase enzyme activity	222	123	4

A record is defined as either susceptibility to a specific insecticide or the results of a test for a specific mechanism of resistance, linked to a field-collected species or complex from a defined place and time

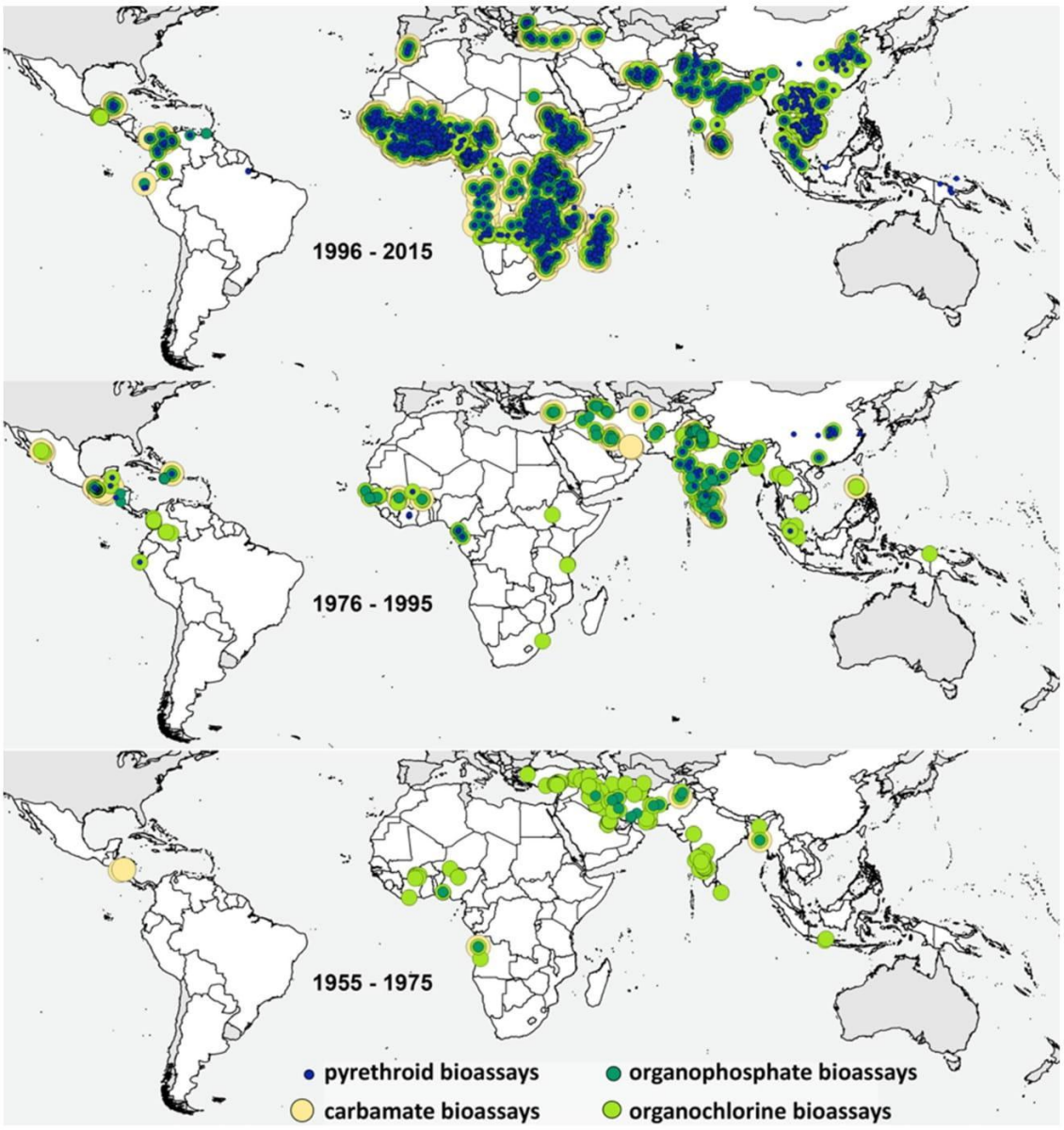

Fig. 1 Distribution of the 13,514 insecticide resistance mortality points collected and geopositioned to date

temporal bias within each time period. The values shown also combine data from multiple species, insecticides and protocols as noted above. The trend of increased reporting of resistance to pyrethroids over the last 25 years is evident, with areas of Africa that traditionally had no data now reporting.

It is important to note that although the colour scale used in Fig. 2 highlights the thresholds defined by the WHO, the full range of mortality values from 0 to 100% are available for the proposed analyses.

Data availability for the mechanisms of resistance

In addition to bioassay data, mechanism data linked to field collections were also extracted. The target site for pyrethroid insecticides is the sodium channel and modification of this, known as *kdr*, can lead to resistance [27].

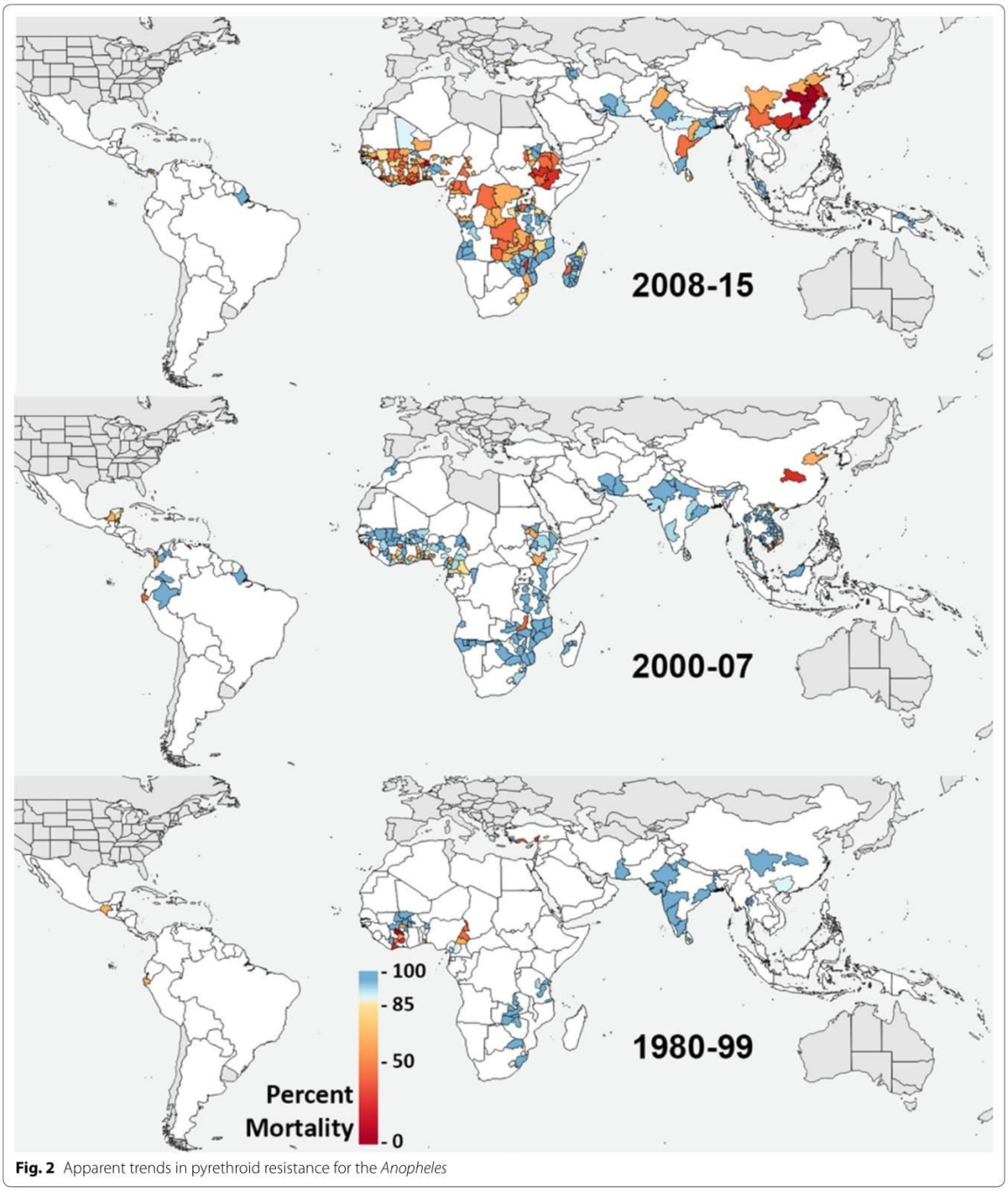

Fig. 2 Apparent trends in pyrethroid resistance for the *Anopheles*

The full current dataset was filtered to extract all records reporting *kdr* allele data including full genotype frequencies (e.g. the number of homozygotes and heterozygotes), individual allele frequencies and resistant/susceptible allele frequencies. Studies that only provided allele frequencies for a non-representative subset of the population (e.g. bioassay survivors only) were excluded. If data for different species were provided separately, these were

combined to give a single value for that site and period. If data for bioassay survivors and dead were provided separately these were combined and weighted by the proportion that had died in the bioassay, to give a single representative value for that site and period. Finally, the susceptible allele frequency was calculated for each record. All frequency values derived from less than 20 mosquitoes tested were excluded. The final dataset current contains 1471 data points at 876 unique locations as shown in Fig. 3.

Mixed function oxidase is one of the key resistance mechanisms for pyrethroids [27] and has been associated with malaria programme failure [17]. The full current dataset was filtered for all records reporting evidence on cytochrome P450/MFO enzyme activity or gene expression. Each record was classified as either showing significantly higher enzyme activity compared to an appropriate control, not showing significantly higher activity, showing significant overexpression of one or more relevant genes, or not showing overexpression. The current dataset provides 331 P450/MFO data points. The locations of each report of overexpression was then plotted on a map layered on top of reports of high enzyme activity, on top of an absence of overexpression, on top of an absence of high activity. That is, evidence for a ramping up of the P450/MFO enzymes was displayed preferentially over a lack of evidence if both classes of evidence were found at the same location in Fig. 4. Unlike the data for insecticide susceptibility and for *kdr* alleles, it was not possible to derive a single metric for P450/MFO upregulation. The gene expression data covers multiple alleles and the enzyme activity data was recorded using a range of different methods that are difficult to compare.

Addressing the limitations of the data

The maps presented here allow us to visualize the availability of data and start to see apparent trends, however, an analysis that addresses multiple potential confounding factors (Table 2) is required to elucidate real trends and relationships. It is clear that the only universal metric with the high global data volumes needed to produce comprehensive maps of resistance is phenotypic susceptibility data from standard bioassays. The bioassays methods used include CDC bottle assays [28] and WHO bioassays linked to ten protocol updates [29, 30] meaning any analysis of this dataset needs to incorporate the protocol used as a variable or standardize these data.

Data volumes available for *kdr* alleles are much lower and this factor is not strongly linked to the variable of most interest, the efficacy of insecticides. Other mechanisms such as P450/MFO upregulation are more strongly linked to insecticide efficacy, or mosquito mortality, but the volumes of data are currently very low. It may be possible to analyse relationships between mechanism data and the spatiotemporal patterns generated using the bioassay data, especially as mechanism data volumes increase, but these data are insufficient to form the mainstay of the currently planned spatiotemporal analyses.

An initial assessment of the data reveals that spatial variation appears to exist and, as expected, temporal trends are apparent. Sampling intensity is, however, biased in both time and space. To understand these trends it will be important to incorporate both spatial and temporal factors in the analysis to avoid one confounding the other. Insecticide resistance appears to be patchy in space. Spatial patchiness is also seen in malaria prevalence and geostatistical methods incorporating

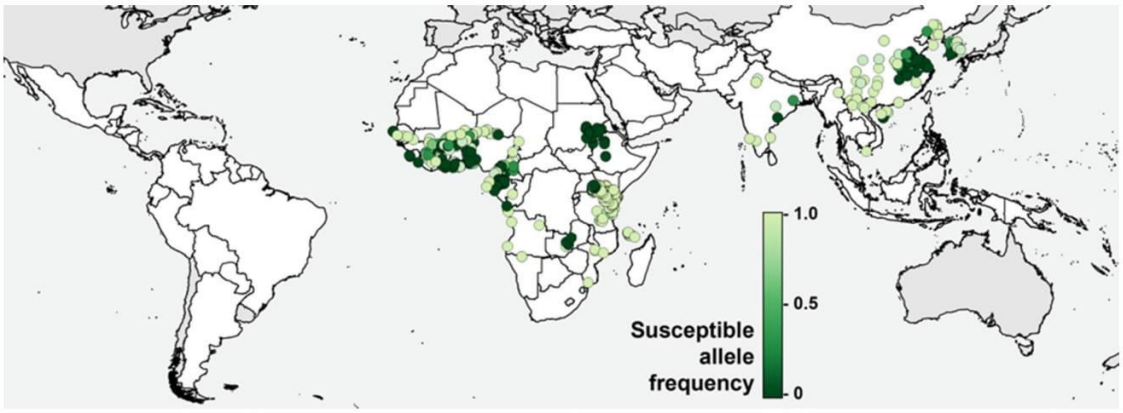

Fig. 3 Geographical distribution of *kdr* reports the susceptible allele frequencies

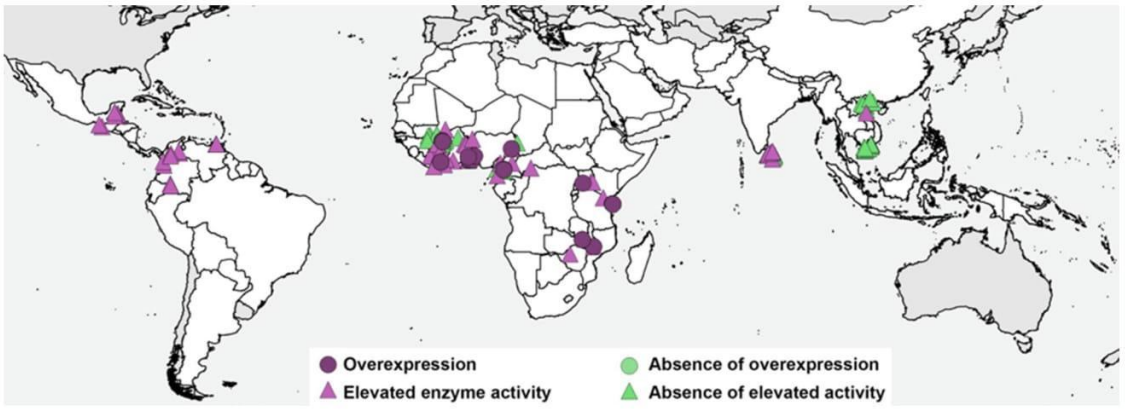

Fig. 4 Location of P450/MFO expression reports

Table 2 Potential confounders, factors and covariates expected to have the largest effect on observed insecticide susceptibility

Variable	Notes
Sampling bias (spatial)	The dataset was not generated using a single systematic sampling design; the data are highly clustered in geographical space
Sampling bias (temporal)	The dataset did not come from a time series that sampled the same locations at regular intervals; each time period incorporates a different set of sites and much higher data volumes are available for more recent years
Species	The full dataset is linked to 74 malaria vector species and species complexes, however, over half of the bioassay records are linked to members of the *An. gambiae* species complex
Insecticide	Within each insecticide class, different insecticides were tested (6 carbamates, 5 organochlorines, 16 organophosphates, and 8 pyrethroids)
Protocol variation	Corrected mortality values were derived from a mixture of WHO bioassays (using 9 updated protocols) and CDC bottle assays
Exposure dose and duration	The exposure dose and duration used in the bioassays varied although the majority of bioassays used standard doses and times
Generation tested	Population samples were maintained in the laboratory for differing periods, however, only results from bioassays using F0 and F1 generations were included

spatial dependence have been shown to provide a robust approach to model these data [31]. These methods have been developed further to incorporate temporal trends and covariates [2], both of which it is expected will to play an important role in insecticide resistance. Specifically, potential drivers of selection such as ITN and IRS use, environmental variables and agricultural use of pesticides will be used as covariates in the model proposed.

The analysis is further complicated by the fact that large numbers of species are represented. Individual anopheline species differ in the likelihood that resistance mechanisms will arise and alleles spread within and between populations so species needs to be included as a factor in the spatiotemporal analyses. The composition of malaria vector species globally forms distinct zones [32] and patterns of resistance may differ among these zones. The planned analysis will therefore consider insecticide resistance within each zone rather than treating this as a single global dataset. Current data volumes are adequate for India, Africa and the Mekong Basin but more data for these areas, particularly historical datasets, will improve the planned analysis and more data for other regions is needed before they can be considered for analysis.

Discussion

The extent of global insecticide resistance reporting has improved over time (Fig. 1). However, there are still extensive malaria endemic areas for which there are no data yet these data are essential for the selection of appropriate tools for vector control and management of the limited number of insecticides available.

Pyrethroids are a key insecticide class in the fight against malaria as they are still the only class recommended for use on LLINs. The expected impact of a high

coverage of LLINs on malaria cases can be lost if efficacy of treated nets on killing resistant mosquitoes is reduced [33]. It has already been noted that the introduction of pyrethroids into South Africa's IRS control programme had a detrimental effect as pyrethroid resistant *Anopheles funestus* were reintroduced and malaria cases increased [14]. Whereas in the Bioko Island Malaria Control Programme, an initial swap from pyrethroids to carbamates was reversed when it was shown that the *kdr* resistance mechanism alone was not having an operational impact and pyrethroids could still be used to control malaria [34]. This trend is also being observed in LLINs, for example, in Burkina Faso, where local vectors are now 1000 fold resistant to pyrethroids, the personal and community impact of ITNs has been lost [11].

Most programmes rely on a combination of vector control tools. However, countries are now reporting resistance to two or more classes of insecticide with differing resistance mechanisms in different vectors [35, 36]. This makes the development of insecticide resistance management plans challenging and there is a need to potentially target different tools and insecticides to different areas of a country, all of which requires spatial maps of vector species and their insecticide resistance profiles at a granular scale.

Alteration of the pyrethroid target site, *kdr,* is widely distributed but has arisen multiple times in all the vector species tested, with the exception of *An. funestus*, where *kdr* has still to be recorded. The 2000–07 data collected here shows that *kdr* is widespread and corresponds to the period shortly after the scale up of pyrethroid impregnated LLINs, but resistance levels conferred are low. The numbers of reports of *kdr* appear to be declining in recent years, but this is probably because it is less easy to get this information published rather than any evidence that *kdr* testing is declining. This highlights the need for a repository that is able to house both published and unpublished data. GPIRM [17] stresses that metabolic resistance to pyrethroids is probably more important in mosquitoes, however, Fig. 3 shows that this is less well studied. This reflects the difficulty in monitoring metabolic resistance directly in the field, when simple PCR based diagnostics are not available.

Map discussion

This work has shown that the data volumes of insecticide susceptibility bioassay results are sufficient to allow an analysis of spatiotemporal trends that will yield regional maps and provide modelled predictions for all locations, at a high resolution. The aim while compiling this dataset is to capture the potential confounding factors in addition to the core measures of resistance, linked to location and time data, in order to incorporate these factors into a robust analysis of spatiotemporal trends. The planned Bayesian geostatistical method has been successfully used to model spatiotemporal variation in the prevalence of *Plasmodium falciparum* infections in malaria [2]. Modelling resistance across the vectors that transmit *P. falciparum* and the other human malaria parasites is potentially more complicated and the data requirements for a Bayesian geostatistical model are high. Progress in building a database to feed into this analysis is well under way as presented here but it is noticeable that not all regions are currently well represented and the decision on which regions to include in the model will depend on data availability.

Data sharing is a cornerstone of this work. MAP-IR and VectorBase regularly share non-confidential datasets to maximize the content of both databases. MAP-IR data is also shared with the WHO providing either (i) the data have previously been published, or (ii) the data owners have provided permission for the data to be shared. MAP-IR will utilize the MAP platform [37, 38], allowing users to obtain modelled insecticide resistance risk maps online. MAP-IR differs from previous attempts at mapping insecticide resistance as it is a global initiative that aims to share data from the outset and the largest dataset available is being assembled. In addition to the modelled maps and data, the database of input data (the bioassay and mechanism records described here) will be released into the public domain via the MAP platform. The expected release date for the input data is 1st September 2017, with data being continuously added post-release.

Conclusions

Insecticide resistance threatens the gains made in malaria control to date. There are currently neither the data nor the resources to generate the information required for control programmes to generate informed decisions regarding vector control policy and insecticide choice. This project will fill some of these gaps which will translate into prolonging the life of old and new insecticides, reduce costs and maintain the gains made in reducing morbidity and mortality in malaria.

Additional files

Additional file 1. Database fields for bioassay records; the data types extracted from each sources are given within a simplified version of the database structure.

Additional file 2. Pyrethroid resistance by subnational area for three time periods; the number of bioassay records for each first order administrative division is given for 1980–1999, 2000–2007, and 2008–2015 together with the actual year range for which data are available in each instance, the number of mosquitoes assayed, and the average mortality as shown in Fig. 2.

Authors' contributions
CLM, MC PWG, and JH designed the study. KG compiled the insecticide resistance data overview. AW and KG geopositioned the data with input from CLM. All authors contributed to the interpretation and write-up of the results, and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The data used to generate the maps presented here will be made available at MAP-IR.

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References

1. WHO. World Malaria report 2015. Geneva: World Health Organization; 2015.
2. Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U, et al. The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. Nature. 2015;526:207–11.
3. Massey NC, Garrod G, Wiebe A, Henry AJ, Huang Z, Moyes CL, et al. A global bionomic database for the dominant vectors of human malaria. Sci Data. 2016;3:160014.
4. WHO. Pesticide evaluation scheme (WHOPES). Geneva: World Health Organization; 2011.
5. Zaim M, Aitio A, Nakashima N. Safety of pyrethroid-treated mosquito nets. Med Vet Entomol. 2000;14:1–5.
6. Ranson H, N'Guessan R, Lines J, Moiroux N, Nkuni Z, Corbel V. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? Trends Parasitol. 2011;27:91–8.
7. Coleman M, Sharp B, Seocharan I, Hemingway J. Developing an evidence-based decision support system for rational insecticide choice in the control of African malaria vectors. J Med Entomol. 2006;43:663–8.
8. Coetzee M, Horne D, Brooke BD, Hunt RH. DDT, dieldrin and pyrethroid insecticide resistance in African malaria vector mosquitoes: an historical review and implications for future malaria control in Southern Africa. S Afr J Sci. 1999;95:215–8.
9. Donnelly MJ, Isaacs AT, Weetman D. Identification, validation, and application of molecular diagnostics for insecticide resistance in malaria vectors. Trends Parasitol. 2016;32:197–206.
10. Bagi J, Grisales N, Corkill R, Morgan JC, N'Fale S, Brogdon WG, et al. When a discriminating dose assay is not enough: measuring the intensity of insecticide resistance in malaria vectors. Malar J. 2015;14:210.
11. Toe KH, Jones CM, N'Fale S, Ismail HM, Dabire RK, et al. Increased pyrethroid resistance in malaria vectors and decreased bed net effectiveness. Burkina Faso. Emerg Infect Dis. 2014;20:1691–6.
12. Ochomo EO, Bayoh NM, Walker ED, Abongo BO, Ombok MO, Ouma C, et al. The efficacy of long-lasting nets with declining physical integrity may be compromised in areas with high levels of pyrethroid resistance. Malar J. 2013;12:368.
13. Edi CV, Koudou BG, Jones CM, Weetman D, Ranson H. Multiple-insecticide resistance in *Anopheles gambiae* mosquitoes, Southern Cote d'Ivoire. Emerg Infect Dis. 2012;18:1508–11.
14. Hargreaves K, Koekemoer LL, Brooke BD, Hunt RH, Mthembu J, Coetzee M. *Anopheles funestus* resistant to pyrethroid insecticides in South Africa. Med Vet Entomol. 2000;14:181–9.
15. Ranson H, Lissenden N. Insecticide resistance in African *Anopheles* mosquitoes. A worsening situation that needs urgent action to maintain malaria control. Trends Parasitol. 2016;32:187–96.
16. Thompson KM, Deisler PF Jr, Schwing RC. Interdisciplinary vision: the first 25 years of the Society for Risk Analysis (SRA), 1980–2005. Risk Anal. 2005;25:53.
17. WHO. Global plan for insecticide resistance management in malaria vectors. Geneva: World Health Organization; 2012.
18. Mnzava AP, Macdonald MB, Knox TB, Temu EA, Shiff CJ. Malaria vector control at a crossroads: public health entomology and the drive to elimination. Trans R Soc Trop Med Hyg. 2014;108:550–4.
19. Knox TB, Juma EO, Ochomo EO, Pates Jamet H, Ndungo L, Chege P, et al. An online tool for mapping insecticide resistance in major Anopheles vectors of human malaria parasites and review of resistance status for the Afrotropical region. Parasit Vect. 2014;7:76.
20. Dialynas E, Topalis P, Vontas J, Louis C. MIRO and IRbase: IT tools for the epidemiological monitoring of insecticide resistance in mosquito disease vectors. PLoS Negl Trop Dis. 2009;3:e465.
21. Mnzava AP, Knox TB, Temu EA, Trett A, Fornadel C, Hemingway J, et al. Implementation of the global plan for insecticide resistance management in malaria vectors: progress, challenges and the way forward. Malar J. 2015;14:173.
22. WHO. Global strategic framework for integrated vector management. Geneva: World Health Organization; 2004.
23. Foof and Agriculture Organization of the United Nations. The global administrative unit layers (GAUL): technical aspects. EC-FAO Food Security Program; 2008.
24. http://events.ucsf.edu/downloads/malaria/map.html.
25. A consideration of the epidemiology, ecology, and physical and social landscapes of infectious diseases. http://www.infectionlandscapes.org/2011/04/malaria-part-4-landscape.html.
26. WHO. World malaria report 2014. Geneva: World Health Organization; 2014.
27. Hemingway J, Hawkes NJ, McCarroll L, Ranson H. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem Mol Biol. 2004;34:653–65.
28. Brogdon WG, Chan A. Guideline for evaluating insecticide resistance in vectors using the CDC Bottle Assay. The Centres for Disease Control and Prevention, Atlanta; 2011.
29. WHO. Test procedures for insecticide resistance monitoring in malaria vectors. Geneva: World Health Organization; 2013.
30. WHO. Test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticides on treated surfaces. WHO/CDS/CPC/MAL/98.12. Geneva: World Health Organization; 1998.
31. Patil AP, Gething PW, Piel FB, Hay SI. Bayesian geostatistics in health cartography: the perspective of malaria. Trends Parasitol. 2011;27:246–53.
32. Sinka ME, Bangs MJ, Manguin S, Rubio-Palis Y, Chareonviriyaphap T, Coetzee M, et al. A global map of dominant malaria vectors. Parasit Vect. 2012;5:69.
33. Gu W, Novak RJ. Predicting the impact of insecticide-treated bed nets on malaria transmission: the devil is in the detail. Malar J. 2009;8:256.
34. Hemingway J, Vontas J, Poupardin R, Raman J, Lines J, Schwabe C, et al. Country-level operational implementation of the Global Plan for Insecticide Resistance Management. Proc Natl Acad Sci USA. 2013;110:9397–402.
35. Riveron JM, Osae M, Egyir-Yawson A, Irving H, Ibrahim SS, Wondji CS. Multiple insecticide resistance in the major malaria vector *Anopheles funestus* in southern Ghana: implications for malaria control. Parasit Vect. 2016;9:504.
36. Wondji CS, Coleman M, Kleinschmidt I, Mzilahowa T, Irving H, Ndula M, et al. Impact of pyrethroid resistance on operational malaria control in Malawi. Proc Natl Acad Sci USA. 2012;109:19063–70.
37. Moyes CL, Temperley WH, Henry AJ, Burgert CR, Hay SI. Providing open access data online to advance malaria research and control. Malar J. 2013;12:161.
38. http://www.map.ox.ac.uk/.

Additional files.

Database field	**Notes**
Sample ID	A unique identifier for a single collection sample used in a single test (or a set of tests conducted on the same mosquitoes) and linked to up to four citations (see below).
Field collection table	
Sample ID	A unique identifier for a single collection sample used in a single test (or a set of tests conducted on the same mosquitoes).
Site ID	A unique identifier for each location.
Capture method	Four fields are provided to list up to four capture methods if mosquitoes caught using different methods were pooled.
Start month	The dates of the field collection for the sample that was tested.
Start year	The dates of the field collection for the sample that was tested.
End month	The dates of the field collection for the sample that was tested.
End year	The dates of the field collection for the sample that was tested.
Field site table	
Site ID	A unique identifier for each location.
Country	
Site name	
Site type	A 'point' location defined as an area <25km2 or a polygon location defined as an area >25km2. If mosquitoes from multiple sites were pooled before they were tested, this is recorded as 'multi-point' or 'multi-polygon' as applicable.
Latitude	Provided for point locations, in decimal degrees. This field is repeated for 'multi-points'.
Longitude	Provided for point locations, in decimal degrees. This field is repeated for 'multi-points'.
GAUL code	An identifier for polygon locations that match a formal administrative division as defined by the UN's Global Administrative Units Layers.
Polygon code	An identifier for polygons that do not match GAUL (see above).
Species identification table part I: all species	
Sample ID	A unique identifier for a single collection sample used in a single test (or a set of tests conducted on the same mosquitoes).
Species or complex name	Taxonomic classification of the sample that was tested.
Identification method	Two fields are provided to list up to two different identification methods.
Subset identified	Classifies the sample that was identified as either 'all' mosquitoes assayed, 'survivors' only, 'dead' only, or a 'mixture' of survivors and dead but not all of those assayed.

Pooled sample	If samples were pooled before mosquitoes were identified, the sample ID for the record linked that has been linked to the identification data is recorded.
No. identified	The number of mosquitoes used in the molecular identification tests.
Percent identified correctly	The percent of mosquitoes identified as the species given under 'species name'.
Species identification table part II:* An. Gambiae *species complex	
% *An. gambiae/coluzzii*	
% *An. coluzzii*	
% *An. gambiae*	
% *An. arabiensis*	
% *An. melas*	
% *An. merus*	
% *An. quadriannulatus*	
No. g/c identified	If a subset of the sample used in a first identification test that did not split out coluzzii and gambiae was then used in a second test to split out coluzzii and gambiae, the number used in the second test is recorded.
% *An. coluzzii / subset*	
% *An. gambiae / subset*	
Bioassay table	
Sample ID	A unique identifier for a single collection sample used in a single test (or a set of tests conducted on the same mosquitoes).
Insecticide	
Insecticide class	
Synergist	
Test method	WHO protocol from a specific year, or CDC bottle assay.
WHO insecticide concentration (%)	
CDC insecticide concentration	
CDC concentration unit	
Synergist concentration	
Synergist concentration unit	
WHO exposure time (min.)	Duration in minutes.
CDC exposure time (min.)	Duration in minutes.
Wild caught	'adults' or 'larvae' or 'both'.
Generation tested	'wild' = F0, or 'F1' or a 'mixture' of F0 and F1.
Lower age (days)	
Upper age (days)	
Fed status	'blood fed' or 'non blood fed'.
Gravid status	
No. mosquitoes tested	
No. mosquitoes dead	
Corrected % mortality	
kdr frequency table	

Sample ID	A unique identifier for a single collection sample used in a single test (or a set of tests conducted on the same mosquitoes).
Subset tested	Defines the overlap with the sample bioassayed as 'all', 'survivors', 'dead', or a 'mixture' of survivors and dead but not all of those assayed.
Test method	Two fields are provided to list up to two kdr test performed on the same sample.
No. tested	
L/L %	Percent homozygous for L allele
L/F %	Percent with L and F alleles
L/S %	Percent with L and S alleles
S/S %	Percent homozygous for S allele
L/C %	Percent with L and C alleles
C/C %	Percent homozygous for C allele
F/S %	Percent with F and S alleles
F/C %	Percent with F and C alleles
Susc/Susc %	Percent homozygous for susceptible allele
Resist/Resist %	Percent with no susceptible allele
Susc/Resist %	Percent heterozygous for susceptible allele
L1014L %	Frequency of the L allele
L1014F %	Frequency of the F allele
L1014S %	Frequency of the S allele
L1014C %	Frequency of the C allele
kdr %	Frequency of resistant alleles
P450/MFO data table I: enzyme activity	
Sample ID	A unique identifier for a single collection sample used in a single test (or a set of tests conducted on the same mosquitoes).
Subset tested	Defines the overlap with the sample bioassayed as 'all', 'survivors', 'dead', or a 'mixture' of survivors and dead but not all of those assayed.
Test method	Two fields are provided to list up to two kdr test performed on the same sample.
No. tested	
Comparison strain	
Evidence for elevated activity	'yes' or 'no' based on significant increase in enzyme activity as defined by the original study.
P450/MFO data table I: expression	
Sample ID	A unique identifier for a single collection sample used in a single test (or a set of tests conducted on the same mosquitoes).
Subset tested	Defines the overlap with the sample bioassayed as 'all', 'survivors', 'dead', or a 'mixture' of survivors and dead but not all of those assayed.
Test method	Two fields are provided to list up to two kdr test performed on the same sample.
No. tested	
Gene	
Comparison strain	

Fold change	
Evidence for elevated expression	'yes' or 'no' based on significantly higher expression as defined by the original study.
Esterase data table I: enzyme activity	
Sample ID	A unique identifier for a single collection sample used in a single test (or a set of tests conducted on the same mosquitoes).
Subset tested	Defines the overlap with the sample bioassayed as 'all', 'survivors', 'dead', or a 'mixture' of survivors and dead but not all of those assayed.
Test method	Two fields are provided to list up to two kdr test performed on the same sample.
No. tested	
Comparison strain	
Evidence for elevated activity	'yes' or 'no' based on significant increase in enzyme activity as defined by the original study.
Source information table	
Citation	Four fields are provided to list up to four sources for the data on that sample and the test(s) performed.
Citation type	'journal article' or 'published report' or 'unpublished report' or 'personal communication'.
Release status	'published' or 'unpublished but permission to release' or 'confidential'.

SCIENTIFIC DATA

DATA DESCRIPTOR

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The impact of insecticide resistance in malaria vectors is poorly understood and quantified. Here a series of geospatial datasets for insecticide resistance in malaria vectors are provided, so that trends in resistance in time and space can be quantified, and the impact of resistance found in wild populations on malaria transmission in Africa can be assessed. Specifically, data have been collated and geopositioned for the prevalence of insecticide resistance, as measured by standard bioassays, in representative samples of individual species or species complexes. Data are provided for the *Anopheles gambiae* species complex, the *Anopheles funestus* subgroup, and for nine individual vector species. Data are also given for common genetic markers of resistance to support analyses of whether these markers can improve the ability to monitor resistance in low resource settings. Allele frequencies for known resistance-associated markers in the Voltage-gated sodium channel (*Vgsc*) are provided. In total, eight analysis-ready, standardised, geopositioned datasets encompassing over 20,000 African mosquito collections between 1957 and 2017 are released.

Current malaria control activities are heavily reliant on vector control using insecticides, which means resistance to these compounds has the potential to derail control efforts[1,2]. Studies have started to investigate the impact of resistance in certain situations[3,4] but a full understanding of impact requires comprehensive quantification of resistance. To quantify the factors that influence vector control generally, data from vector populations are required and a number of vector databases are already available for species distributions, infection prevalence, and bionomic parameters[5–12]. A database for insecticide resistance in malaria vectors, that allows users to

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download analysis-ready datasets, is vital so that the impact of levels of resistance found in wild populations, on malaria transmission, can be assessed. These datasets are also essential to quantify trends in resistance in space and time, filling the gaps in the available data with robust predictions, to aid resistance management and the deployment of interventions designed to counter resistance[13].

Studies of phenotypes in natural populations may be confounded by variation in the environments sampled, including factors linked to climate, land use and malaria control interventions. It is not possible to control for all variables in the natural environment but this issue can, in part, be mitigated by sampling a large number of locations encompassing different combinations of environmental variables. Large, collated datasets do, however, have potential disadvantages. Collated datasets that are a combination of data points representing different types of sample, different measurement methods, different location types and so on, risk undermining any analysis that is performed[6,14].

Each dataset should be constructed to address a specific question or set of questions, and the data within each set needs to be standardised to allow robust analyses. The goal of the current work was to collate data from multiple studies characterising the insecticide resistance phenotype and genotype in communities of malaria vectors at as many locations and times as possible. The first aim was then to generate standardised datasets designed to address specific questions using geospatial analyses. Namely, what are the trends in resistance in time and space in specific vector assemblages, to then assess whether these trends are associated with trends in malaria transmission. The second aim was to provide data that can be used to investigate associations between genetic markers for individual mechanisms of resistance and the insecticide resistance phenotype, to assess whether genetic markers can improve the ability to monitor resistance in low resource settings[15].

Data sources. Data were obtained from published journal articles, published reports, and unpublished datasets. Published journal articles were identified in the Web of Science bibliographic database by using the search terms "insecticide resistance" and "anopheles" together with the name of each malaria endemic country in turn. The Web of Science was chosen because it incorporates many relevant databases including the SciELO Citation Index from 1997 onwards, MEDLINE from 1950 onwards (from the U.S. Library of Medicine), the Data Citation Index from 1993 onwards (provides details of datasets in international data depositories), the BIOSIS Citation Index from 1969 onwards (covers pre-clinical, experimental, and animal research) and the Web of Science's own Core Collection from 1945 to date.

The earliest date was unrestricted, and the search was completed on 31 December 2017. The initial search yielded 3,685 articles published from 1956 to 2017, with the first African paper published in 1957. Data were extracted from each article as outlined below and 342 articles provided data from field samples of mosquitoes collected in malaria endemic African countries for either the insecticide resistance phenotype and/or genotype. If values for some data fields were missing, the authors were contacted. In these instances, either (i) the phenotype/genotype data was given in the article but supplementary information such as the date of sampling or mosquito identification method was missing, or (ii) the genotype/phenotype data were missing, or had been aggregated across sites or years, so the disaggregated data for each site-year were requested. In the latter instance, any genotype/phenotype data received from the authors were treated as unpublished. In total, 81 sets of authors were contacted about 114 journal articles. Of these, 56 sets of authors provided further information; 30 sets of authors confirmed details such as the collection dates and 26 sets of authors provided test results that were not published with the original articles, as well as confirming any missing details of their study.

In addition, agencies reporting on vector surveillance and groups involved in large studies that had not yet published their results, were asked to provide these reports and unpublished datasets. In total, 48 reports and unpublished datasets from African countries were provided. For all unpublished data, permission to include these data in this release was requested. Of a total of 11,057 unpublished data points, permission was received to release 10,834.

Data aggregation/disaggregation. The aim of this work was to provide measures of insecticide resistance for representative samples of a species population (or a species complex or a subgroup) found at a particular time and place, rather than data at the level of an individual mosquito. Replicates from the same mosquito collection sampled at a single "site" and "collection period" were aggregated. The spatial resolution of a "site" was defined by the original field studies and classified by the current study, as described in the data geo-referencing section below. The temporal resolution of a "collection period" was also defined by the original data generators and the duration of each collection period was recorded in the current dataset, as described below. If the reported data had been pooled across multiple sites or collection periods, but was originally obtained at a finer resolution, the disaggregated data for each site-period were requested. For example, if mosquitoes were collected from five sites and bioassayed separately, giving a bioassay result for each site, but only a single average result for the region was published, then the five separate results were requested. The purpose of this disaggregation was to avoid imprecise estimates associated with large areas or long time periods wherever possible. Further details on appropriate methods to analyse data at different resolutions is given in the Usage Notes.

Datasets were constructed based on mosquito samples that represented either a single species or a species complex or subgroup. Species-level data were entered wherever it was available and aggregated to provide data for the species complex later, using the original species composition. If insecticide resistance data were provided for each species but the original species composition was not available for that study, the data points for each individual species were included in the species-level datasets (provided they met the inclusion criteria below) but they were not aggregated to provide data for the species complex. Data are provided for individual species within the *An. gambiae* complex (*An. arabiensis*, *An. coluzzii, An. gambiae*, *An. melas* and *An. quadriannulatus*) and for species within the *An. funestus* subgroup (*An. funestus* and *An. parenesis*) within datasets 2, 7 and 8. Separately,

Number	Title	No. data points
1	Standard WHO susceptibility test results for the *Anopheles gambiae* complex and *Anopheles funestus* subgroup.	13,618
2	Standard WHO susceptibility test results for individual species.	3,525
3	Standard CDC bottle bioassay results for the *An. gambiae* complex and *An. funestus* subgroup.	1,061
4	Paired WHO susceptibility test or CDC bottle bioassay results with and without a synergist (*An. gambiae* complex and *An. funestus* subgroup).	1,013
5	WHO and CDC intensity bioassay results (*An. gambiae* complex and *An. funestus* subgroup).	1,816
6	*Vgsc* allele frequencies for the *An. gambiae* complex and *An. funestus* subgroup.	1,068
7	*Vgsc* allele frequencies for individual species.	1,890
8	Paired *Vgsc* allele frequencies from dead and alive subsamples after an insecticide susceptibility test.	296

Summary of each of the eight data files released.

values for aggregate *An. gambiae* complex samples and aggregate *An. funestus* subgroup samples are provided in datasets 1, 3, 4, 5 and 6. The majority of studies provided results for samples of a complex or subgroup, rather than single species (Table 1).

Inclusion criteria for data extraction. Subgroup-, complex- or species-level insecticide resistance phenotype data generated from either a WHO susceptibility test[16–26] or a CDC bottle bioassay[27] using either the F0 or F1 generation from a field collection of *Anopheles* mosquitoes were included (Supplementary Information).

Susceptibility tests use two types of control; for the first control, a known susceptible mosquito strain is exposed to the insecticide-treated paper to test that the paper is working, and for the second control, a subsample of the mosquito population being tested are not exposed to the insecticide to check the baseline mortality rate. Data were excluded if the susceptible strain control failed, i.e. mortality in the susceptible strain was <100% indicating that the insecticide-treated paper was not effective. If the first control was successful (all of the mosquitoes died) and a baseline mortality rate was obtained from the second control and used in Abbot's formula to correct the mortality for the field sample, this corrected mortality value was entered in the database.

Subgroup-, complex- or species-level data on the resistance variants in the voltage-gated sodium channel (*Vgsc*) gene that were derived from F0 or F1 generations from field collections of *Anopheles* mosquitoes, provided as either genotype or allele frequencies, were included.

Only mosquito samples that were representative of a species complex or subgroup and/or a species were included and any samples that were subject to sub-setting that biased the original sample were excluded. For example, if a mixed species sample was collected but a bioassay result was only reported for the most common species, that bioassay result cannot be considered as representative of the species complex at that time and place. In this example, the data were included in the species-level datasets released here, but these values were not included in the datasets for species complexes. Similarly, if a mixed species sample was collected and then the F1 generation was sorted into single species by identifying the mother of each egg batch, those results cannot be considered representative of the species complex at that time and place. Furthermore, if the allele frequency was calculated for mosquitoes that survived a bioassay, and dead mosquitoes were not tested, this result cannot be considered as representative for either the species complex, or the individual species, and was not included in any dataset. If a mixture of dead and alive mosquitoes from a bioassay were tested to obtain an allele frequency, but the ratio of dead:alive was not representative of the original sample, for example 80% died in the bioassay but the sample tested was 50:50 then these data were also excluded.

Individual data files. The full database was used to generate eight individual data files (Table 1) that address specific questions for defined sets of mosquitoes.

The aim in creating Data Files 1 and 2 was to provide a set of comparable results for each insecticide from bioassays that had used the same insecticide concentration and exposure duration, however, the recommended concentrations and durations vary with WHO protocol versions[18–26]. The protocol version used to define the standard insecticide concentrations and exposure durations in Data Files 1 and 2 was the 1998 WHO test procedures, because the highest volumes of data across all years were available for the concentrations and durations specified by this protocol version[25]. Insecticides that were not covered by the 1998 protocol version were specified in the 2013 version so this later protocol was also used to set the standard values for Data Files 1 and 2[26].

Data fields. The data fields included in this release are described in Tables 2–7. The source data fields (Table 2), the sample collection data fields (Table 3), and the geo-location data fields (Table 4) are provided in all data files. The species identification data fields (Table 5) are provided in Data Files 2, 4, 7 and 8. The bioassay data fields (Table 6) are provided in Data Files 1–5. The *Vgsc* data fields (Table 7) are provided in Data Files 6–8.

All of the data fields were extracted and entered as they were provided by the data source. If any information was missing, no value was entered (see the missing data section below) and the authors were contacted. The only values that were generated after the data were extracted from the sources, were the geo-location values. Full details on how the geo-location data were generated is given in the next section.

Data files are provided for species complexes or subgroups, and for individual species, separately. Bioassay data for individual species were obtained from studies that either sorted egg batches based on mothers' species prior to a bioassay being performed, or disaggregated the results by species after the bioassay was performed,

Title	Data type	Description
Source citation	Text	Citation for the data source. This field is duplicated up to four times to record instances where the full information linked to that data point came from more than one source.
Source type	Category	Each source is categorised as 'journal article', 'personal communication' and so on.

Data source data fields. These data fields are included in all 8 data files.

Title	Data type	Description
Capture method	Category	The method used to capture the mosquito sample. This field is duplicated four times to record instances where samples from different capture methods were pooled before testing.
Start month	Integer	The month when the mosquito collection began.
Start year	Integer	The year when the mosquito collection began.
End month	Integer	The month when the mosquito collection ended.
End year	Integer	The year when the mosquito collection ended.

Sample collection data fields. These data fields are included in all 8 data files.

Title	Data type	Description
Country	Category	The country that the site sampled was in.
Site type	Category	Sites can be a point, a polygon or multiple-points, as described in the geo-positioning section.
Site name	Free text	Name of the field site sampled.
Latitude	Decimal number	For 'point' sites only, the geographical coordinates are given in decimal degrees. This field is duplicated multiple times to record instances where samples from more than one 'point' site were pooled before testing.
Longitude	Decimal number	For 'point' sites only, the geographical coordinates are given in decimal degrees. This field is duplicated multiple times to record instances where samples from more than one 'point' site were pooled before testing.
Admin level	Category	If the site is a 'polygon' that matches an administrative unit, the administrative level (0, 1 or 2) is recorded.
GAUL code	Integer	If the site is a 'polygon' that matches an administrative unit, the identifier from the Global Administrative Units Layer is recorded.

Geo-locations data fields. These data fields are included in all 8 data files and are described further in the data geo-referencing section of the text. GAUL is the Global Administrative Units Layer.

Title	Data type	Description
Identification method	Category	The molecular method used to identify individual species in the original sample. This field is duplicated to record instances where two methods were used.

Species identification data fields. These data fields are included in Data Files 2, 4, 7 and 8.

or instances where all mosquitoes in the original sample were found to be one species after the bioassay was performed.

For Data File 4, an additional identifier (the "matched set ID") is included to allow results from bioassays that used the same mosquito collection and exactly the same bioassay conditions, with or without a synergist, to be identified. The same approach was used for Data File 5 where the "matched set ID" allows results from bioassays that used the same mosquito collection and exactly the same bioassay conditions, but with differing insecticide concentrations and/or exposure durations, to be identified. In total, 453 matched sets are provided in the synergist dataset, 463 in the intensity assay data file, and 148 for *Vgsc* allele frequencies in paired dead and alive subsamples.

Data geo-referencing. In order to use these data in geospatial models at a resolution of ~5 km, each mosquito collection location was classed as either a point (defined as a site located within a 2.5 arc-minute grid cell, i.e. an area of ~5 × ~5 km) or a polygon (defined as a site with an area greater than that of a point).

To determine whether a site should be classified as a point or a polygon, we used all information provided by the data source. This included text describing the site, the site name, and any maps or coordinates provided. If the text described the site as a district, or similar, then we checked that the area of that district matched our definition of a polygon and, if so, used the polygon classification. If coordinates were provided that mapped to the centroid of an administrative unit then we checked whether the site name and text description matched that administrative unit and, if so, classified the site as a polygon (unless the area of the adminstrative unit was less than 5 × 5 km).

For all sites defined as 'points' the following steps were followed. The site name and all contextual information about the location of the site were noted, for example, the district the site was in, its proximity to a major city

Title	Data type	Description
Species	Category	The species that the bioassay result represents.
Complex/subgroup	Category	The species complex or subgroup that the bioassay result represents.
Generation	Category	The mosquito generation tested: F0, F1 or a mix of both.
Test protocol	Category	The WHO or CDC bioassay protocol followed is listed.
Insecticide	Category	The insecticide tested is named.
Concentration (%)	Decimal number	If a WHO protocol was followed, the insecticide concentration is given as a percent.
Concentration (µg/bottle)	Decimal number	If the CDC protocol was followed, the insecticide concentration is given in µg/bottle.
Exposure period (minutes)	Integer	The period of exposure to the insecticide in minutes.
No. mosquitoes tested	Integer	The total number of mosquitoes tested in all replicates.
No. mosquitoes dead	Integer	The total number of mosquitoes that died in all replicates.
Percent mortality	Decimal number	The percent of mosquitoes that died across all replicates, adjusted using Abbot's formula if applicable.

WHO and CDC bioassay data fields. There data fields are included in Data Files 1–5.

Title	Data type	Description
Anophelines tested	Category	The species, or species complex or subgroup, that the genetic result represents.
Method	Category	The molecular method used to identify alleles. This field is duplicated three times to record instances where up to three different methods were used on the same sample.
Generation	Category	The mosquito generation tested: F0, F1 or a mix of both.
No. mosquitoes tested	Integer	The total number of mosquitoes tested.
Genotype frequencies		
L/L (no.)	Integer	The number of mosquitoes homozygous for the wildtype, susceptible allele (1014L).
L/L (%)	Decimal number	The percent of mosquitoes homozygous for the wildtype, susceptible allele (1014L).
L/F (no.)	Integer	The number of mosquitoes heterozygous for the wildtype, susceptible allele (1014L) and the 1014F resistance allele.
L/F (%)	Decimal number	The percent of mosquitoes heterozygous for the wildtype, susceptible allele (1014L) and the 1014F resistance allele.
L/S (no.)	Integer	The number of mosquitoes heterozygous for the wildtype, susceptible allele (1014L) and the 1014S resistance allele.
L/S (%)	Decimal number	The percent of mosquitoes heterozygous for the wildtype, susceptible allele (1014L) and the 1014S resistance allele.
F/F (no.)	Integer	The number of mosquitoes homozygous for the 1014F resistance allele.
F/F (%)	Decimal number	The percent of mosquitoes homozygous for the 1014F resistance allele.
F/S (no.)	Integer	The number of mosquitoes heterozygous for the 1014F and 1014S resistance alleles.
F/S (%)	Decimal number	The percent of mosquitoes heterozygous for the 1014F and 1014S resistance alleles.
S/S (no.)	Integer	The number of mosquitoes homozygous for the 1014S resistance allele.
S/S (%)	Decimal number	The percent of mosquitoes homozygous for the 1014S resistance allele.
Allele frequencies		
L1014L	Decimal number	The allele frequency (%) for the 1014L wildtype, susceptible allele.
L1014F	Decimal number	The allele frequency (%) for the 1014F resistance allele.
L1014S	Decimal number	The allele frequency (%) for the 1014s resistance allele.

Vgsc gene data fields. These data fields are included in Datasets 6–8.

or other geographical features, and so on. If the data source provided coordinates, then these were converted to decimal degrees. If no coordinates were provided, the site name was searched in at least two online gazetteers (Google Maps, GeoNames, OpenStreetMap, WikiMapia and so on). All options identified by this search were cross-checked against the contextual information. If only one option matched the contextual information, the coordinates were extracted from the online gazeteer and added to the database. If more than one option matched the contextual information, or no options were found that matched the contextual information, the individuals who published or provided the data were contacted. In these instances, no coordinates were entered without external confirmation. After all possible coordinates were obtained for a study, they were plotted on a map to ensure the data spread for that study matched any information available on the authors' overarching sampling strategy.

For all sites defined as 'polygons', any contextual information was noted, such as the province that the district was in. The name of the area in question was searched in the FAO's Global Administrative Unit Layers (GAUL, http://www.fao.org/geonetwork/srv/en/metadata), using fuzzy matching to allow for different spellings or transliteration, and checked against any available contextual information. If a single administrative unit in GAUL matched the area name and contextual information, the GAUL code (=a unique identifier for that area/polygon),

Protocol	Min. no. mosquitoes	Duration (minutes)[a]	Duration for fenitrothion (minutes)	Duration for DDT (minutes)
WHO 1963	60	60		60
WHO 1970	60	60	60	60
WHO 1975	60	60	60	60
WHO 1976	60	60	60	60
WHO 1980	60	60	120	60
WHO 1981	60	60	120	60
WHO 1986	60	60	120	60
WHO 1992	60	60	120	60
WHO 1998	80	60	120	60
WHO 2013	80	60	120	60
WHO 2016	80	60	120	60
CDC bottle bioassay	100	30	30	45

Minimum recommended number of mosquitoes and duration of exposure specified by published protocols for the WHO susceptibility test and CDC bottle bioassay. [a]The exposure duration values from the WHO protocols apply to dieldrin, malathion, fenthion, propoxur, chlorphoxim, permethrin, deltamethrin, λ-cyhalothrin, bendiocarb, etofenprox, pirimiphos-methyl, carbosulfan, cyfluthrin, chlorfenapyr, fipronil and α-cypermethrin. The CDC protocol values apply to bendiocarb, cyfluthrin, cypermethrin, deltamethrin, fenitrothion, λ-cyhalothrin, malathion, permethrin and pirimiphos-methyl. Full details can be found in the published protocols.

Protocol	DDT	deltamethrin	permethrin	bendiocarb	λ-cyhalothrin	primiphos-methyl
WHO 1963	M.C.					
WHO 1970	M.C.					
WHO 1975	M.C.					
WHO 1976	M.C.					
WHO 1980	4%	0.025%	0.25%			
WHO 1981	4%	0.025%	0.25%	0.1%		
WHO 1986	4%	0.025%	0.25%	0.1%		
WHO 1992	4%	0.025%	0.25%	0.1%	0.1%	
WHO 1998	4%	0.05%	0.75%	0.1%	0.05%	
WHO 2013	4%	0.05%	0.75%	0.1%	0.05%	0.25%
WHO 2016	4%	0.05%	0.75%	0.1%	0.05%	0.25%
CDC bottle bioassay	100 µg/bottle	12.5 µg/bottle	21.5 µg/bottle	12.5 µg/bottle	12.5 µg/bottle	20 µg/bottle

Insecticide concentrations specified by published protocols for the WHO susceptibility test and CDC bottle bioassay. M.C. denotes that multiple concentrations were recommended so the actual concentration used in any particular bioassay cannot be inferred from the protocol version.

and administrative level for that unit, were extracted and entered in the database. If an administrative unit within GAUL could not be identified, no code was entered.

If an individual site could not be located, or could not be precisely located within a 2.5 arc-minute grid cell, then the data point was linked to the second order administrative division that the site falls within. The administrative division was identified using the same method as for polygons above.

If multiple point locations were sampled and the mosquitoes were pooled before being tested (or only the pooled results were available), the site type was classified as a 'multi-point' and the coordinates for all of the individual point locations were linked to the test result.

Missing data. If data for a particular field was missing from the original data source, the value was recorded as NR, i.e. not reported. For values that were not applicable, rather than missing, NA was used. For example, if only one capture method was used, the value entered for the second capture method was NA. If the geographical coordinates for a site could not be identified (see above), NF was entered, i.e. not found.

If a study did not explicitly state the insecticide concentration, exposure period and/or minimum number of mosquitoes used, but did specify the protocol followed, it may be possible to obtain the missing information from the relevant protocol[16–27]. Protocol values for the most commonly used insecticides are provided in Tables 8 and 9, and the values for all insecticides are given in the Supplementary Information, to allow data users to fill these data gaps if they wish.

Species, complexes and subgroups tested	Complex/subgroup
An. arabiensis	*An. gambiae* complex
An. coluzzii	*An. gambiae* complex
An. gambiae	*An. gambiae* complex
An. melas	*An. gambiae* complex
An. quadriannulatus	*An. gambiae* complex
An. gambiae complex	not applicable
An. funestus	*An. funestus* subgroup
An. parensis	*An. funestus* subgroup
An. funestus subgroup	not applicable
An. rivulorum	*An. funestus* group
An. mascarensis	*An. mascarensis* group
An. pharoensis	not applicable

List of the the species, complexes and subgroups that were tested and are included in the datasets for release.

Insecticides tested
α-cypermethrin
bendiocarb
carbosulfan
chlorpyrifos-methyl
cyfluthrin
DDT
deltamethrin
dieldrin
etofenprox
fenitrothion
fenthion
λ-cyhalothrin
malathion
permethrin
pirimiphos-methyl
propoxur

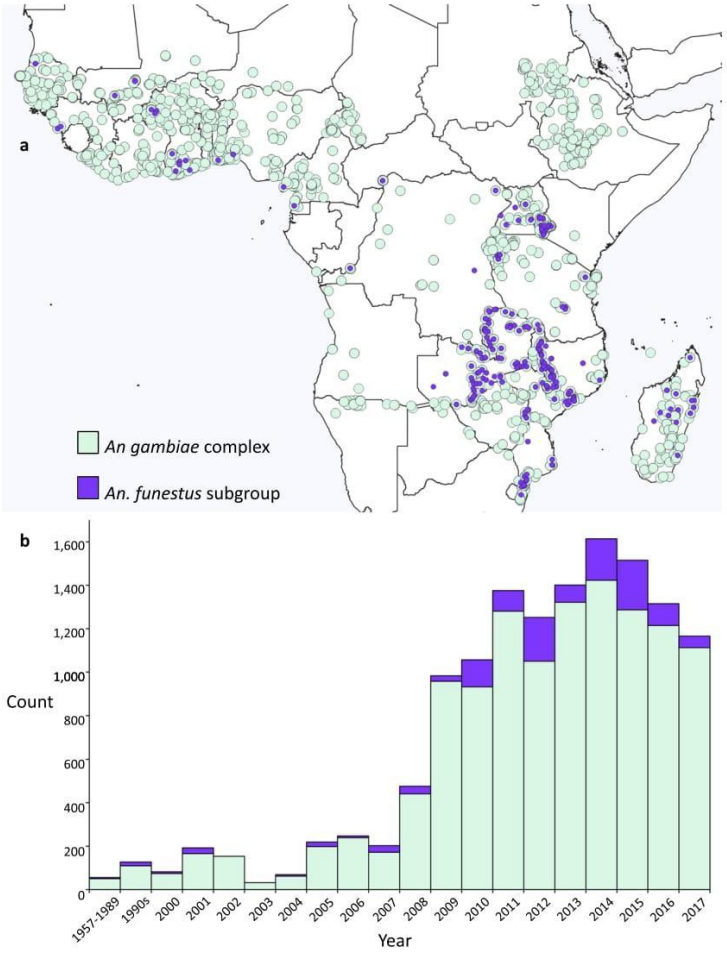
List of the insectides that were tested and are included in the datasets for release.

Data duplication. The data extracted came from several hundred different sources, which introduced the possibility that individual results had been entered into the database more than once. To identify duplicates the following data fields were used: original sample; species tested; date fields; no. mosquitoes tested; no. mosquitoes dead; percent mortality; site name; coordinates. Fuzzy matching was used for all fields to identify duplicates where different levels of aggregation had been used, or different data values were missing, or names were spelled differently. All partial matches were examined to identify genuine duplicates. Duplicate data points were removed, and the source details linked to the single data point that was retained. In total, 3,483 duplicated data points were removed. The final lists of species and insecticides that were included are given in Tables 10 and 11.

The data are available for download from the Dryad Digital Repository[28]. The spatial and temporal distributions of Data File 1, standard WHO susceptibility test results for the *Anopheles gambiae* complex and *Anopheles funestus* subgroup, are shown in Fig. 1. The spatial and temporal distributions of Data File 7, *Vgsc* allele frequencies for individual species, are shown in Fig. 2.

Data File 1 is the largest dataset but all eight have similar spatial distributions with clustered sampling in the east and west of Africa and sparse data points in the centre and southwest. They also share similar temporal distributions with phenotypic data volumes increasing throughout the time period particularly from 2008 onwards, and the genotypic data volumes peaking in 2005 and 2010. The genotype data were almost exclusively extracted from published papers and there is typically a lag of around two years between mosquito collection and the publication of a paper containing the test results.

In addition to the data extracted for *Vgsc* allele frequencies, data were also identified for *Ace-1* allele frequencies and metabolic mechanisms of resistance including cytochrome P450s, esterases and glutathione-*S*-transferases. The volumes of genetic and biochemical data currently available for these mechanisms of resistance did not meet



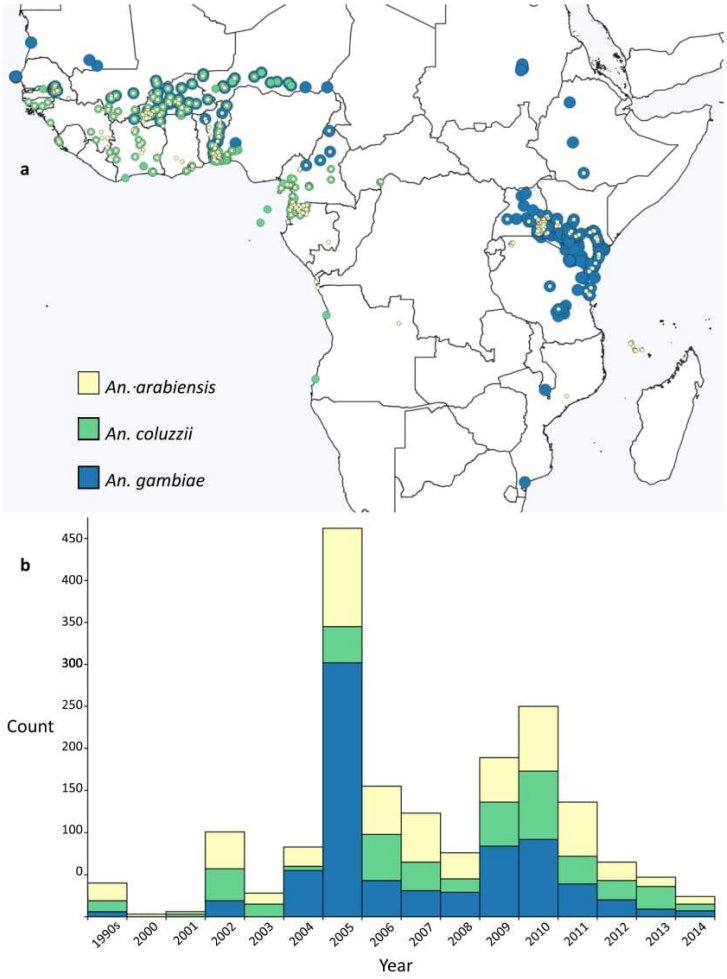
Spatial and temporal distributions of Data File 1. (**a**) The locations of mosquito collections of the *An. gambiae* complex and the *An. funestus* subgroup that were used in standard WHO susceptibility tests. (**b**) The number of data points available for each year for the *An. gambiae* complex and the *An. funestus* subgroup.

our aim of providing standardised data for a large number of locations across Africa, so no collated datasets for these mechanisms were generated. Dataset 4 consists of results from synergist bioassays so it does, therefore, provide data linked to P450-mediated mechanisms of resistance.

Many studies performed both bioassays and genetic tests. If links between the different tests performed on the same sample of mosquitoes were provided by the original study, and providing any subsamples tested were not biased, then it was possible to extract pairs of phenotypic and genotypic measures of resistance for samples from a specific time and place. Unfortunately, however, when instances of paired phenotypic and genotypic results for an individual species from a single time and place were extracted, only sixty pairs were identified. This volume of data did not meet our aim of providing standardised data for a large number of locations across Africa. The same was true for paired phenotypic and genotypic results for a species complex or subgroup from a single time and place.

In addition, the data volumes available for species-level CDC bottle bioassay results, species-level paired bioassays with and without a synergist, and species-level intensity bioassays, were too low to meet our aim of providing standardised data for a large number of locations across Africa.

Data were checked for internal consistency to ensure (i) all coordinates for point locations fell on land and in the right country, as defined by GAUL, (ii) mortality and allele frequencies never exceeded 100%, (iii) the collection end date was never earlier than the collection start date, and (iv) the species name tallied with the identification methods listed. A matrix of species identification methods and species identified by each method was prepared in order to complete this check (Supplementary Information). In addition, a second person reviewed the geographical coordinates in accordance with the geo-locations protocol outlined above.



Spatial and temporal distributions of Data File 7. (**a**) The locations of mosquito collections of *An. arabiensis*, *An. coluzzii* and *An. gambiae* that were used to calculate *Vgsc* allele frequencies. (**b**) The number of data points available for each year.

Each data file released has been designed to provide results for a representative sample of a species complex or subgroup, or an individual species, so users can be confident of what each set of results represents. Older versions of the collated datasets of WHO susceptibility test results and the *Vgsc* allele frequency have been used in a prior geostatistical analysis that aimed to identify associations among resistance to different insecticides in the *An. gambiae* species complex[15].

The data files have been designed for use in geospatial analyses and, in such analyses, the precise location for each data point is important for two reasons. First, because this allows accurate calculation of the Euclidian distances between points for analyses that exploit spatial correlations in the data[15,29]. Secondly, precise location information allows accurate matching of the data to a wide range of environmental variables, such as climatic, socio-economic and intervention variables, to exploit relationships between the biological data and these environmental variables[30,31]. The use of data linked to wider areas is a current area of research aimed at improving model predictions in circumstances where data linked to precise locations are particularly sparse[32,33]. For any kind of spatial analysis, it is essential to know whether the geographical coordinates provided represent a precise location or wider area, what the definition of a precise location is, and where the boundaries of the wider areas lie. The data points released here are linked to a mixture of precise locations and wider areas, the precise locations (referred to here as points) are defined as an area within a 2.5 arc-minute grid cell (approx. 5×5 km), and links to the boundaries of wider areas (referred to here as polygons) are given.

The data files released here are not the result of one single, continent-wide study that used a standard sampling design. It is a compilation of many studies that used many designs and incorporates obvious sampling bias. Sites that are more easily accessed or closer to research centres may be more likely to be sampled. Sites where high

levels of resistance are expected may also be more likely to be sampled, as might sites where insecticide-based interventions are planned as a result of a combination of related variables. Geostatistical models can, however, be used to model sampling intensity to check these biases before proceeding further[34].

Other data resources for insecticide resistance in malaria vector are available. Data on insecticide resistance in the *Anopheles* vectors of malaria are available from VectorBase, however, VectorBase's aim and scope are much broader than those of the current data release and the data volumes for insecticide resistance in *Anopheles* vectors are smaller than those provided here[35]. Furthermore, these data have not been configured specifically for use in mathematical analyses including geospatial analyses. Insecticide resistance data can also be viewed on interactive maps using the IR Mapper and Malaria Threats websites but these are data visualisation tools[36]. The data shown on these sites were not collated in support of mathematical analyses and are not available for download. There are overlaps in all of these databases, including overlaps with the data being released here. The data released here includes data that were provided to the World Health Organization to support the establishment of the Malaria Threats website[37]. In addition, the data being released here were shared with the group behind the IR Mapper site so that both groups could cross-check each other's sources to identify publications that had been missed.

A geo-database of insecticide resistance in the *Aedes* vectors of arboviruses has previously been released but this has much smaller data volumes for Africa, and encompasses a much greater range of insecticides tested at a greater range of concentrations on both adults and larvae[38]. In contrast, the data released here provide sufficient volumes of standardised values to support a range of analyses of insecticide resistance in malaria vectors in Africa and are freely available to all. In addition to the current data release, these data have been shared with the Pan Africa Mosquito Control Association to support the establishment of an Africa -led and –managed data resource. The datasets released here will also be available for download from the IR Mapper website [www.irmapper.com]. In addition, predicted values for the prevalence of resistance (i.e. mortality in a standard WHO susceptibility test) at every location in a ~5 km resolution grid, for each year from 2005 to 2017, will be modelled and released in the coming months.

1. Hemingway, J. *et al.* Averting a malaria disaster: will insecticide resistance derail malaria control? *Lancet* **387**, 1785–1788, https://doi.org/10.1016/s0140-6736(15)00417-1 (2016).
2. Raoult, D. & Abat, C. Developing new insecticides to prevent chaos: the real future threat. *Lancet Infectious Diseases* **17**, 804-+, https://doi.org/10.1016/s1473-3099(17)30395-x (2017).
3. Cook, J. *et al.* Implications of insecticide resistance for malaria vector control with long-lasting insecticidal nets: trends in pyrethroid resistance during a WHO-coordinated multi-country prospective study. *Parasites & Vectors* **11**, https://doi.org/10.1186/s13071-018-3101-4 (2018).
4. Kleinschmidt, I. *et al.* Implications of insecticide resistance for malaria vector control with long-lasting insecticidal nets: a WHO-coordinated, prospective, international, observational cohort study. *Lancet Infectious Diseases* **18**, 640–649, https://doi.org/10.1016/s1473-3099(18)30172-5 (2018).
5. Eisen, L. *et al.* Multi-Disease Data Management System Platform for Vector-Borne Diseases. *Plos Neglected Tropical Diseases* **5**, https://doi.org/10.1371/journal.pntd.0001016 (2011).
6. Kiware, S. S. *et al.* A generic schema and data collection forms applicable to diverse entomological studies of mosquitoes. *Source Code for Biology and Medicine* **11**, https://doi.org/10.1186/s13029-016-0050-1 (2016).
7. Massey, N. C. *et al.* A global bionomic database for the dominant vectors of human malaria. *Scientific Data* **3**, https://doi.org/10.1038/sdata.2016.14 (2016).
8. Mitsakakis, K. *et al.* Converging Human and Malaria Vector Diagnostics with Data Management towards an Integrated Holistic One Health Approach. *International Journal of Environmental Research and Public Health* **15**, https://doi.org/10.3390/ijerph15020259 (2018).
9. Moyes, C. L., Temperley, W. H., Henry, A. J., Burgert, C. R. & Hay, S. I. Providing open access data online to advance malaria research and control. *Malaria Journal* **12**, https://doi.org/10.1186/1475-2875-12-161 (2013).
10. Pfeffer, D. A. *et al.* MalariaAtlas: an R interface to global malariometric data hosted by the Malaria Atlas Project. *Malaria Journal* **17**, https://doi.org/10.1186/s12936-018-2500-5 (2018).
11. Browne, A. J. *et al.* The contemporary distribution of *Trypanosoma cruzi* infection in humans, alternative hosts and vectors (vol 4, 170050, 2017). *ScientificData* **4**, https://doi.org/10.1038/sdata.2017.71 (2017).
12. Ceccarelli, S. *et al.* DataTri, a database of American triatomine species occurrence. *Scientific Data* **5**, https://doi.org/10.1038/sdata.2018.71 (2018).
13. Coleman, M. *et al.* Developing global maps of insecticide resistance risk to improve vector control. *Malaria Journal* **16**, https://doi.org/10.1186/s12936-017-1733-z (2017).
14. Killeen, G. F., Chaki, P. P., Reed, T. E., Moyes, C. L. & Govella, N. J. In *Towards Malaria Elimination* (eds Manguin, S. & Dev, V.) 403–429 (InTechOpen, 2018).
15. Hancock, P. A. *et al.* Associated patterns of insecticide resistance in field populations of malaria vectors across Africa. *Proceedings of the National Academy of Sciences of the United States of America* **115**, 5938–5943, https://doi.org/10.1073/pnas.1801826115 (2018).
16. WHO Division of Malaria and Other Parasitic Diseases. Manual on practical entomology in malaria: part II methods and techniques. 197 (Geneva, 1975).
17. WHO Global Malaria Programme. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes, 2nd edition. 55 (Geneva, 2016).
18. World Health Organization. Insecticide resistance and vector control: thirteenth report of the WHO Expert Committee on Insecticides. 106 (Geneva, 1963).
19. World Health Organization. Insecticide resistance and vector control: seventeenth report of the WHO Expert Committee on Insecticides. 94 (Geneva, 1970).
20. World Health Organization. Resistance of vectors and reservoirs of disease to pesticides: twenty second report of the WHO expert committee on insecticides. 88 (Geneva, 1976).
21. World Health Organization. Fifth report of the WHO Expert Committee on Vector Biology and Control: resistance of vectors of disease to pesticides. 82 (Geneva, 1980).
22. World Health Organization. Instructions for determining the susceptibility or resistance of adult mosquito to organochlorine, organophosphate and carbamate insecticides-diagnostic tests. (Geneva, 1981).
23. World Health Organization. Tenth report of the WHO Expert Committee on Vector Biology and Control: resistance of vectors and reservoirs of disease to pesticides. 88 (Geneva, 1986).

24. World Health Organization. Fifteenth report of the WHO Expert Committee on Vector Biology and Control: vector resistance to pesticides. 68 (Geneva, 1992).
25. World Health Organization. Test procedures for insecticide resistance monitoring in malaria vectors: bio-efficacy and persistance of insecticides on treated surfaces. 46 (World Health Organization, Geneva, 1998).
26. World Health Organization. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. 40 (Geneva, 2013).
27. Brogdon, W. & Chan, A. Guideline for evaluating insecticide resistance in vectors using the CDC bottle bioassay. 28 (Atlanta, 1998).
28. Moyes, C. L. *et al*. Data from: Analysis-ready datasets for insecticide resistance phenotype and genotype frequency in African malaria vectors. *Dryad Digital Repository*, https://doi.org/10.5061/dryad.dn4676s (2019).
29. Giorgi, E., Diggle, P. J., Snow, R. W. & Noor, A. M. Geostatistical Methods for Disease Mapping and Visualisation Using Data from Spatio-temporally Referenced Prevalence Surveys. *International Statistical Review* **86**, 571–597, https://doi.org/10.1111/insr.12268 (2018).
30. Wiebe, A. *et al*. Geographical distributions of African malaria vector sibling species and evidence for insecticide resistance. *Malaria Journal* **16**, https://doi.org/10.1186/s12936-017-1734-y (2017).
31. Moyes, C. L. *et al*. Predicting the geographical distributions of the macaque hosts and mosquito vectors of *Plasmodium knowlesi* malaria in forested and non-forested areas. *Parasites & Vectors* **9**, https://doi.org/10.1186/s13071-016-1527-0 (2016).
32. Gaughan, A. E., Stevens, F. R., Linard, C., Patel, N. N. & Tatem, A. J. Exploring nationally and regionally defined models for large area population mapping. *International Journal of Digital Earth* **8**, 989–1006, https://doi.org/10.1080/17538947.2014.965761 (2015).
33. Golding, N. *et al*. Mapping under-5 and neonatal mortality in Africa, 2000-15: a baseline analysis for the Sustainable Development Goals. *Lancet* **390**, 2171–2182, https://doi.org/10.1016/s0140-6736(17)31758-0 (2017).
34. Pati, D., Reich, B. J. & Dunson, D. B. Bayesian geostatistical modeling with informative sampling locations. *Biometrika* **98**, 35–48 (2010).
35. Lawson, D. *et al*. VectorBase: a data resource for invertebrate vector genomics. *Nucleic Acids Research* **37**, D583–D587, https://doi.org/10.1093/nar/gkn857 (2009).
36. Knox, T. B. *et al*. An online tool for mapping insecticide resistance in major *Anopheles* vectors of human malaria parasites and review of resistance status for the Afrotropical region. *Parasites & Vectors* **7**, https://doi.org/10.1186/1756-3305-7-76 (2014).
37. Mnzava, A. P. *et al*. Implementation of the global plan for insecticide resistance management in malaria vectors: progress, challenges and the way forward. *Malaria Journal* **14**, https://doi.org/10.1186/s12936-015-0693-4 (2015).
38. Moyes, C. L. *et al*. Contemporary status of insecticide resistance in the major *Aedes* vectors of arboviruses infecting humans. *Plos Neglected Tropical Diseases* **11**, https://doi.org/10.1371/journal.pntd.0005625 (2017).

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C.L.M. devised the analysis-ready datasets for release. C.L.M. and A.W. drafted the data extraction and geopositioning protocols. A.W., K.G. and A.T. extracted, processed and geopositioned the data with guidance from C.L.M. and M.C. A.W. classified the species identification methods. K.G. extracted recommended sample sizes, doses and exposure durations from the WHO and CDC protocols. A.T. and A.W. identified duplicates in the data. C.L.M. checked the above work and reviewed the data. C.F., C.M., M.J.D., R.K.D., H.K., D.D., A.-N.C., E.O., S.A.A., A.S., C.S.M. and G.G.P. provided large volumes of unpublished data and provided advice on the use and format of these data. P.H., M.C. and C.L.M. contributed to the potential dataset uses.

Supplementary Information is available for this paper at https://doi.org/10.1038/s41597-019-0134-2.

Competing Interests: The authors declare no competing interests.

Supplementary Information

- Inclusion criteria
- Recommended doses and exposure periods
- Species identification methods

Inclusion criteria for data on the prevalence of the insecticide resistance phenotype

1. Was a WHO susceptibility test used?

a. If yes, was 100% mortality in the susceptible strain achieved after exposure to the treated paper?

i. If yes, see question 2.

ii. If no, exclude.

b. If no, was a CDC bottle bioassay used?

i. If yes, see question 2.

ii. If no, exclude.

2. Were F0 or F1 generation mosquitoes derived from a field collection used?

a. If yes, see question 3.

b. If no, exclude.

3. Are the results disaggregated to species?

a. If no, include in the dataset for the relevant complex/subgroup only.

b. If yes, can the species results be combined to provide an unbiased result for the original complex/subgroup sample?

i. If yes, include each species result in the species dataset and include the combined result in the complex/subgroup dataset.

ii. If no, include each species result in the species dataset only.

Inclusion criteria for data on *Vgsc* allele frequencies

1. Were *Vgsc* alleles tested for?

a. If yes, see question 2.

b. If no, exclude.

2. Were F0 or F1 generation mosquitoes derived from a field collection used?

a. If yes, see question 3.

b. If no, exclude.

3. Are the results disaggregated to species and/or to dead/alive mosquitoes?

a. If no, include in the dataset for the relevant complex/subgroup only.

b. If disaggregated to species only, can the species results be combined to provide an unbiased result for the original complex/subgroup sample?

i. If yes, include each species result in the species dataset and include the combined result in the complex/subgroup dataset.

ii. If no, include each species result in the species dataset only.

c. If disaggregated to dead/alive mosquitoes only, can the dead/alive results be combined to provide an unbiased result for the original complex/subgroup sample?

i. If yes, include in the dataset for the relevant complex/subgroup.

ii. If no, exclude.

d. If disaggregated to both species and to dead/alive, can the dead/alive results for a species be combined to provide an unbiased result for that species?

i. If no, exclude.

ii. If yes, can the species results then be combined to provide an unbiased result for the original complex/subgroup sample? I. If yes, include each species result in the species dataset and include the combined result in the complex/subgroup dataset.
II. If no, include each species result in the species dataset only.

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Gleave K, Lissenden N, Chaplin M, Choi L, Ranson H.
Piperonyl butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa.
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[Intervention Review]

Piperonyl butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa

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ABSTRACT

Background

Pyrethroid long-lasting insecticidal nets (LLINs) have been important in the large reductions in malaria cases in Africa, but insecticide resistance in *Anopheles* mosquitoes threatens their impact. Insecticide synergists may help control insecticide-resistant populations. Piperonyl butoxide (PBO) is such a synergist; it has been incorporated into pyrethroid-LLINs to form pyrethroid-PBO nets, which are currently produced by five LLIN manufacturers and, following a recommendation from the World Health Organization (WHO) in 2017, are being included in distribution campaigns. This review examines epidemiological and entomological evidence on the addition of PBO to pyrethroid nets on their efficacy.

Objectives

To compare effects of pyrethroid-PBO nets currently in commercial development or on the market with effects of their non-PBO equivalent in relation to:

1. malaria parasite infection (prevalence or incidence); and
2. entomological outcomes.

Search methods

We searched the Cochrane Infectious Diseases Group (CIDG) Specialized Register, CENTRAL, MEDLINE, Embase, Web of Science, CAB Abstracts, and two clinical trial registers (ClinicalTrials.gov and WHO International Clinical Trials Registry Platform) up to 25 September 2020. We contacted organizations for unpublished data. We checked the reference lists of trials identified by these methods.

Selection criteria

We included experimental hut trials, village trials, and randomized controlled trials (RCTs) with mosquitoes from the *Anopheles gambiae* complex or the *Anopheles funestus* group.

Data collection and analysis

Two review authors assessed each trial for eligibility, extracted data, and determined the risk of bias for included trials. We resolved disagreements through discussion with a third review author. We analysed data using Review Manager 5 and assessed the certainty of evidence using the GRADE approach.

Main results

Sixteen trials met the inclusion criteria: 10 experimental hut trials, four village trials, and two cluster-RCTs (cRCTs). Three trials are awaiting classification, and four trials are ongoing.

Two cRCTs examined the effects of pyrethroid-PBO nets on parasite prevalence in people living in areas with highly pyrethroid-resistant mosquitoes (< 30% mosquito mortality in discriminating dose assays). At 21 to 25 months post intervention, parasite prevalence was lower in the intervention arm (odds ratio (OR) 0.79, 95% confidence interval (CI) 0.67 to 0.95; 2 trials, 2 comparisons; moderate-certainty evidence).

In highly pyrethroid-resistant areas, unwashed pyrethroid-PBO nets led to higher mosquito mortality compared to unwashed standard-LLINs (risk ratio (RR) 1.84, 95% CI 1.60 to 2.11; 14,620 mosquitoes, 5 trials, 9 comparisons; high-certainty evidence) and lower blood feeding success (RR 0.60, 95% CI 0.50 to 0.71; 14,000 mosquitoes, 4 trials, 8 comparisons; high-certainty evidence). However, in comparisons of washed pyrethroid-PBO nets to washed LLINs, we do not know if PBO nets had a greater effect on mosquito mortality (RR 1.20, 95% CI 0.88 to 1.63; 10,268 mosquitoes, 4 trials, 5 comparisons; very low-certainty evidence), although the washed pyrethroid-PBO nets did decrease blood-feeding success compared to standard-LLINs (RR 0.81, 95% CI 0.72 to 0.92; 9674 mosquitoes, 3 trials, 4 comparisons; high-certainty evidence).

In areas where pyrethroid resistance is moderate (31% to 60% mosquito mortality), mosquito mortality was higher with unwashed pyrethroid-PBO nets compared to unwashed standard-LLINs (RR 1.68, 95% CI 1.33 to 2.11; 1007 mosquitoes, 2 trials, 3 comparisons; moderate-certainty evidence), but there was little to no difference in effects on blood-feeding success (RR 0.90, 95% CI 0.72 to 1.11; 1006 mosquitoes, 2 trials, 3 comparisons; moderate-certainty evidence). For washed pyrethroid-PBO nets compared to washed standard-LLINs, we found little to no evidence for higher mosquito mortality or reduced blood feeding (mortality: RR 1.07, 95% CI 0.74 to 1.54; 329 mosquitoes, 1 trial, 1 comparison, low-certainty evidence; blood feeding success: RR 0.91, 95% CI 0.74 to 1.13; 329 mosquitoes, 1 trial, 1 comparison; low-certainty evidence).

In areas where pyrethroid resistance is low (61% to 90% mosquito mortality), studies reported little to no difference in the effects of unwashed pyrethroid-PBO nets compared to unwashed standard-LLINs on mosquito mortality (RR 1.25, 95% CI 0.99 to 1.57; 1580 mosquitoes, 2 trials, 3 comparisons; moderate-certainty evidence), and we do not know if there was any effect on blood-feeding success (RR 0.75, 95% CI 0.27 to 2.11; 1580 mosquitoes, 2 trials, 3 comparisons; very low-certainty evidence). For washed pyrethroid-PBO nets compared to washed standard-LLINs, we do not know if there was any difference in mosquito mortality (RR 1.39, 95% CI 0.95 to 2.04; 1774 mosquitoes, 2 trials, 3 comparisons; very low-certainty evidence) or on blood feeding (RR 1.07, 95% CI 0.49 to 2.33; 1774 mosquitoes, 2 trials, 3 comparisons; low-certainty evidence).

In areas where mosquito populations are susceptible to insecticides (> 90% mosquito mortality), there may be little to no difference in the effects of unwashed pyrethroid-PBO nets compared to unwashed standard-LLINs on mosquito mortality (RR 1.20, 95% CI 0.64 to 2.26; 2791 mosquitoes, 2 trials, 2 comparisons; low-certainty evidence). This is similar for washed nets (RR 1.07, 95% CI 0.92 to 1.25; 2644 mosquitoes, 2 trials, 2 comparisons; low-certainty evidence). We do not know if unwashed pyrethroid-PBO nets had any effect on the blood-feeding success of susceptible mosquitoes (RR 0.52, 95% CI 0.12 to 2.22; 2791 mosquitoes, 2 trials, 2 comparisons; very low-certainty evidence). The same applies to washed nets (RR 1.25, 95% CI 0.82 to 1.91; 2644 mosquitoes, 2 trials, 2 comparisons; low-certainty evidence).

In village trials comparing pyrethroid-PBO nets to LLINs, there was no difference in sporozoite rate (4 trials, 5 comparisons) nor in mosquito parity (3 trials, 4 comparisons).

Authors' conclusions

In areas of high insecticide resistance, pyrethroid-PBO nets have greater entomological and epidemiological efficacy compared to standard LLINs, with sustained reduction in parasite prevalence, higher mosquito mortality and reduction in mosquito blood feeding rates 21 to 25 months post intervention. Questions remain about the durability of PBO on nets, as the impact of pyrethroid-PBO nets on mosquito mortality was not sustained over 20 washes in experimental hut trials, and epidemiological data on pyrethroid-PBO nets for the full intended three-year life span of the nets is not available. Little evidence is available to support greater entomological efficacy of pyrethroid-PBO nets in areas where mosquitoes show lower levels of resistance to pyrethroids.

PLAIN LANGUAGE SUMMARY

Pyrethroid-PBO nets to prevent malaria

Background

Bed nets treated with pyrethroid insecticides are an effective way to reduce malaria transmission and have been deployed across Africa. However, mosquitoes that spread malaria are now developing resistance to this type of insecticide. One way to overcome this resistance is to add another chemical - piperonyl butoxide (PBO) - to the net. PBO is not an insecticide, but it blocks the substance (an enzyme) inside the mosquito that stops pyrethroids from working.

What is the aim of this review?

The aim of this Cochrane Review was to find out if pyrethroid-PBO nets provide additional protection against malaria when compared to standard pyrethroid-only nets.

Key messages

Pyrethroid-PBO nets were more effective than standard pyrethroid-only nets in killing mosquitoes and preventing blood feeding in areas where mosquito populations are very resistant to pyrethroid insecticides (high-certainty evidence). Pyrethroid-PBO nets reduced the number of malaria infections in areas of high pyrethroid resistance (moderate-certainty evidence), although further studies are needed to measure clinical outcomes for the full lifetime of the net.

What was studied in the review?

We included 16 trials conducted between 2010 and 2020 that compared standard pyrethroid nets to pyrethroid-PBO nets. These consisted of 10 experimental hut trials that measured the impact of pyrethroid-PBO nets on a wild population of mosquitoes, four village trials, and two cRCTs. The two cRCTs measured the impact of pyrethroid-PBO nets on malaria infection in humans; all other studies recorded their impact on mosquito populations. We analysed hut and village studies to determine whether pyrethroid-PBO nets were better for killing mosquitoes and preventing them from blood feeding. For both cRCT trials, we examined whether pyrethroid-PBO nets reduced the number of malaria infections. As the benefit of adding PBO to nets is likely to depend on the level of pyrethroid resistance in the mosquito population, we performed separate analyses for studies conducted in areas of high, medium, and low levels of pyrethroid resistance.

What are the main results of the review?

When mosquitoes show high levels of resistance to pyrethroids, pyrethroid-PBO nets perform better than standard pyrethroid-only nets for killing mosquitoes and preventing them from blood feeding. As expected, this effect is not seen in areas where mosquitoes show low or no resistance to pyrethroid-only insecticides. Two trials looked at the impact of using pyrethroid-PBO nets on the number of people infected with the malaria parasite. These trials, involving 10,603 participants in total and conducted in an area where mosquitoes are very resistant to pyrethroids, found that fewer people were infected with malaria when the population used pyrethroid-PBO nets than when standard pyrethroid-only nets were used.

How up-to-date is this review?

We searched for all studies and trials that had been published up to 25 September 2020.

SUMMARY OF FINDINGS

Summary of findings 1. Summary of findings table 1

Pyrethroid-piperonyl butoxide (PBO) nets compared to long-lasting insecticidal nets (LLINs) for malaria control when insecticide resistance is high

Patient or population: adults and childen living in malaria-endemic areas, *Anopheles gambiae* complex or *Anopheles funestus* group
Setting: areas of high insecticide resistance
Intervention: pyrethroid-PBO nets
Comparison: LLIN

Outcomes	Anticipated absolute effects* (95% CI)		Relative effect (95% CI)	Number of participants, (trials)	Certainty of the evidence (GRADE)	Comments
	Risk with LLIN	Risk with pyrethroid-PBO nets				
Parasite prevalence (4- to 6-month follow-up)	254 per 1000[a]	201 per 1000 (174 to 233)[a]	OR 0.74 (0.62 to 0.89)	11,582 people (2 trials, 2 comparisons, 61 PBO clusters, 64 non-PBO clusters)	⊕⊕⊕⊕ HIGH	Pyrethroid-PBO nets at 4- to 6-month follow-up reduce parasite prevalence in areas of high insecticide resistance
Parasite prevalence (9- to 12-month follow-up)	224 per 1000[a]	172 per 1000 (150 to 199)[a]	OR 0.72 (0.61 to 0.86)	11,370 people (2 trials, 2 comparisons, 61 PBO clusters, 64 non-PBO clusters)	⊕⊕⊕⊖ MODERATE[b] *due to inconsistency*	Pyrethroid-PBO nets at 9- to 12-month follow-up reduce parasite prevalence in areas of high insecticide resistance
Parasite prevalence (16- to 18-month follow-up)	248 per 1000[a]	225 per 1000 (196 to 255)[a]	OR 0.88 (0.74 to 1.04)	11,822 people (2 trials, 2 comparisons, 61 PBO clusters, 64 non-PBO clusters)	⊕⊕⊕⊖ MODERATE[b] *due to inconsistency*	Pyrethroid-PBO nets at 16- to 18-month follow-up reduce parasite prevalence in areas of high insecticide resistance
Parasite prevalence (21- to 25-month follow-up)	350 per 1000[a]	298 per 1000 (265 to 338)[a]	OR 0.79 (0.67 to 0.95)	10,603 people (2 trials, 2 comparisons, 54 PBO clusters, 60 non-PBO clusters)	⊕⊕⊕⊖ MODERATE[b] *due to inconsistency*	Pyrethroid-PBO nets at 21- to 25-month follow-up reduce parasite prevalence in areas of high insecticide resistance
Mosquito mortality (unwashed nets)	238 per 1000[a]	438 per 1000 (381 to 503)[a]	RR 1.84 (1.60 to 2.11)	14,620 mosquitoes (5 trials, 9 comparisons)	⊕⊕⊕⊕ HIGH[c]	Mosquito mortality is higher with unwashed pyrethroid-PBO nets compared to standard unwashed LLINs in areas of high insecticide resistance
Mosquito mortality (washed nets)	201 per 1000[a]	242 per 1000 (177 to 328)[a]	RR 1.20 (0.88 to 1.63)	10,268 mosquitoes (4 trials, 5 comparisons)	⊕⊖⊖⊖ VERY LOW[d,e] *due to imprecision and inconsistency*	We do not know whether pyrethroid-PBO nets have an effect on mosquito mortality in areas of high insecticide resistance when the nets have been washed
Blood-feeding success (unwashed nets)	438 per 1000[a]	263 per 1000 (241 to 311)[a]	RR 0.60 (0.50 to 0.71)	14,000 mosquitoes (4 trials, 8 comparisons)	⊕⊕⊕⊕ HIGH[c]	Mosquito blood-feeding success is decreased with unwashed pyrethroid-PBO nets compared to standard unwashed LLINs in areas of high insecticide resistance
Blood-feeding success (washed nets)	494 per 1000[a]	400 per 1000 (356 to 454)[a]	RR 0.81 (0.72 to 0.92)	9674 mosquitoes (3 trials, 4 comparisons)	⊕⊕⊕⊕ HIGH[c]	Mosquito blood-feeding success is decreased with washed pyrethroid-PBO nets compared to standard washed LLINs in areas of high insecticide resistance

***The risk in the intervention group** (and its 95% CI) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI).
CI: confidence interval; LLINs: long-lasting insecticidal nets; OR: odds ratio; PBO: pyrethroid-piperonyl butoxide; RR: risk ratio.

GRADE Working Group grades of evidence.
High certainty: we are very confident that the true effect lies close to that of the estimate of the effect.
Moderate certainty: we are moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
Low certainty: our confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect.
Very low certainty: we have very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of effect.

[a]Original numbers were used in this table; however in pooled analysis, events and total numbers were generated from cluster-adjusted results, which use the effective sample size. Note that cluster adjustments do not change the point estimate of the effect size - just the standard error.
[b]Downgraded by one for inconsistency.
[c]Not downgraded for imprecision: both best- and worst-case scenarios in this situation are important effects.
[d]Downgraded by one for imprecision due to wide CIs.
[e]Downgraded by two for inconsistency due to unexplained qualitative heterogeneity.

Summary of findings 2. Summary of findings table 2

Pyrethroid-piperonyl butoxide (PBO) nets compared to long-lasting insecticidal nets (LLINs) for malaria control when insecticide resistance is moderate

Patient or population: *Anopheles gambiae* complex or *Anopheles funestus* group
Setting: areas of moderate insecticide resistance
Intervention: pyrethroid-PBO nets
Comparison: LLIN

Outcomes	Anticipated absolute effects* (95% CI)		Relative effect (95% CI)	Number of mosquitoes (experimental hut trials)	Certainty of the evidence (GRADE)	Comments
	Risk with LLIN	Risk with pyrethroid-PBO nets				
Mosquito mortality (unwashed nets)	180 per 1000[a]	303 per 1000 (259 to 411)[a]	RR 1.68 (1.33 to 2.11)	1007 (2 trials, 3 comparisons)	⊕⊕⊕⊖ MODERATE[b] *due to imprecision*	Mosquito mortality is probably higher with unwashed pyrethroid-PBO nets compared to standard unwashed LLINs in areas of moderate insecticide resistance
Mosquito mortality (washed nets)	287 per 1000[a]	307 per 1000 (213 to 443)[a]	RR 1.07 (0.74 to 1.54)	329 (1 trial, 1 comparison)	⊕⊕⊖⊖ LOW[b,c,d] *due to imprecision and indirectness*	There may be little to no difference in the effect of washed pyrethroid-PBO nets on mosquito mortality compared to standard washed LLINs (washed) in areas of moderate insecticide resistance
Blood-feeding success (unwashed nets)	258 per 1000[a]	232 per 1000 (197 to 304)[a]	RR 0.90 (0.72 to 1.11)	1006 (2 trials, 3 comparisons)	⊕⊕⊕⊖ MODERATE[b] *due to imprecision*	There is probably little to no difference in the effect of pyrethroid-PBO nets (unwashed) on mosquito blood-feeding success compared to standard LLINs in areas of moderate insecticide resistance
Blood-feeding success (washed nets)	586 per 1000[a]	533 per 1000 (434 to 662)[a]	RR 0.91 (0.74 to 1.13)	329 (1 trial, 1 comparison)	⊕⊕⊖⊖ LOW[b,c,d] *due to imprecision and indirectness*	There may be little to no difference in the effect of washed pyrethroid-PBO nets on mosquito blood-feeding success compared to standard washed LLINs in areas of moderate insecticide resistance

***The risk in the intervention group** (and its 95% CI) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI).
CI: confidence interval; LLIN: long-lasting insecticidal net; PBO: pyrethroid-piperonyl butoxide; RR: risk ratio.

GRADE Working Group grades of evidence.
High certainty: we are very confident that the true effect lies close to that of the estimate of the effect.
Moderate certainty: we are moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
Low certainty: our confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect.

Very low certainty: we have very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of effect.

[a]Original numbers are used in this table; however for the pooled analysis, we generated events and total numbers from cluster-adjusted results, which used the effective sample size. Note that cluster adjustments do not change the point estimate of the effect size, just the standard error.
[b]Downgraded by one for imprecision due to wide CIs.
[c]Not downgraded for inconsistency, as only one trial measured this outcome in this setting.
[d]Downgraded by one for indirectness: the outcome is highly context-specific, and only one trial is included.

Summary of findings 3. Summary of findings table 3

Pyrethroid-piperonyl butoxide (PBO) nets compared to long-lasting insecticidal nets (LLINs) for malaria control when insecticide resistance is low

Patient or population: *Anopheles gambiae* complex or *Anopheles funestus* group
Setting: areas of low insecticide resistance
Intervention: pyrethroid-PBO nets
Comparison: LLINs

Outcomes	Anticipated absolute effects* (95% CI)		Relative effect (95% CI)	Number of mosquitoes (experimental hut trials)	Certainty of the evidence (GRADE)	Comments
	Risk with LLINs	Risk with pyrethroid-PBO nets				
Mosquito mortality (unwashed nets)	527 per 1000[a]	659 per 1000 (613 to 972)[a]	RR 1.25 (0.99 to 1.57)	1580 (2 trials, 3 comparisons)	⊕⊕⊕⊖ MODERATE[b] *due to imprecision*	There is probably little to no difference in the effect of unwashed pyrethroid-PBO nets on mosquito mortality compared to standard unwashed LLINs in areas of low insecticide resistance
Mosquito mortality (washed nets)	394 per 1000[a]	547 per 1000 (437 to 938)[a]	RR 1.39 (0.95 to 2.04)	1774 (2 trials, 3 comparisons)	⊕⊖⊖⊖ VERY LOW[c,d] *due to imprecision and inconsistency*	We do not know if pyrethroid-PBO nets have an effect on mosquito mortality in areas of low insecticide resistance when the nets have been washed
Blood-feeding success (unwashed nets)	201 per 1000[a]	151 per 1000 (58 to 456)[a]	RR 0.75 (0.27 to 2.11)	1580 (2 trials, 3 comparisons)	⊕⊖⊖⊖ VERY LOW[c,d] *due to imprecision and inconsistency*	We do not know if unwashed pyrethroid-PBO nets have an effect on mosquito blood-feeding success in areas of low insecticide resistance
Blood-feeding success (washed nets)	161 per 1000[a]	172 per 1000 (122 to 578)[a]	RR 1.07 (0.49 to 2.33)	1774 (2 trials, 3 comparisons)	⊕⊕⊖⊖ LOW[d]	Mosquito blood-feeding success may decrease with washed pyrethroid-PBO nets compared to standard washed LLINs in areas of low insecticide resistance

					due to inconsistency	

*The risk in the intervention group (and its 95% CI) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI).
CI: confidence interval; LLIN: long-lasting insecticidal net; PBO: pyrethroid-piperonyl butoxide; RR: risk ratio.

GRADE Working Group grades of evidence.
High certainty: we are very confident that the true effect lies close to that of the estimate of the effect.
Moderate certainty: we are moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
Low certainty: our confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect.
Very low certainty: we have very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of effect.

[a]Original numbers are used in this table; however for the pooled analysis, events and total numbers were generated from cluster-adjusted results, which use the effective sample size. Note that cluster adjustments do not change the point estimate of the effect size, just the standard error.
[b]Downgraded by one for imprecision due to wide CIs.
[c]Downgraded by one for inconsistency due to unexplained heterogeneity.
[d]Downgraded by two for imprecision due to extremely wide CIs.

Summary of findings 4. Summary of findings table 4

Pyrethroid-piperonyl butoxide (PBO) nets compared to long-lasting insecticidal nets (LLINs) for malaria control when mosquitoes are susceptible

Patient or population: *Anopheles gambiae* complex or *Anopheles funestus* group
Setting: areas of insecticide-susceptible mosquitoes

Intervention: pyrethroid-PBO nets
Comparison: LLINs

Outcomes	Anticipated absolute effects* (95% CI)		Relative effect (95% CI)	Number of mosquitoes (experimental hut trials)	Certainty of the evidence (GRADE)	Comments
	Risk with LLINs	Risk with pyrethroid-PBO nets				
Mosquito mortality (unwashed nets)	392 per 1000[a]	471 per 1000 (251 to 887)[a]	RR 1.20 (0.64 to 2.26)	2791 (2 trials, 2 comparisons)	⊕⊕⊝⊝ LOW[b] *due to imprecision*	There may be little to no difference in the effect of unwashed pyrethroid-PBO nets on mosquito mortality compared to standard unwashed LLINs in areas of no insecticide resistance
Mosquito mortality (washed nets)	457 per 1000[a]	489 per 1000 (420 to 571)[a]	RR 1.07 (0.92 to 1.25)	2644 (2 trials, 2 comparisons)	⊕⊕⊝⊝ LOW[b] *due to imprecision*	There may be little to no difference in the effect of washed pyrethroid-PBO nets on mosquito mortality compared to standard washed LLINs in areas of no insecticide resistance
Blood-feeding success (unwashed nets)	57 per 1000[a]	29 per 1000 (6 to 132)[a]	RR 0.52 (0.12 to 2.22)	2791 (2 trials, 2 comparisons)	⊕⊝⊝⊝ VERY LOW[b,c] *due to imprecision and inconsistency*	We do not know if unwashed pyrethroid-PBO nets have an effect on mosquito blood-feeding success in areas of no insecticide resistance
Blood-feeding success (washed nets)	64 per 1000[a]	82 per 1000 (52 to 131)[a]	RR 1.25 (0.82 to 1.91)	2644 (2 trials, 2 comparisons)	⊕⊝⊝⊝ VERY LOW[b,c] *due to imprecision and inconsistency*	We do not know if washed pyrethroid-PBO nets have an effect on mosquito blood-feeding success in areas of no insecticide resistance

*The risk in the intervention group (and its 95% CI) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI).
CI: confidence interval; LLINs: long-lasting insecticidal nets; PBO: pyrethroid-piperonyl butoxide; RR: risk ratio.

GRADE Working Group grades of evidence.
High certainty: we are very confident that the true effect lies close to that of the estimate of the effect.
Moderate certainty: we are moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
Low certainty: our confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect.
Very low certainty: we have very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of effect.

[a]Original numbers are used in this table; however for the pooled analysis, events and total numbers were generated from cluster-adjusted results, which use the effective sample size. Note that cluster adjustments do not change the point estimate of the effect size, just the standard error.
[b]Downgraded by two for imprecision due to extremely wide CIs.
[c]Downgraded by one for inconsistency due to unexplained heterogeneity.

BACKGROUND

Description of the condition

Substantial progress has been made in reducing the burden of malaria in the 21st century. It is estimated that the clinical incidence of *Plasmodium falciparum* malaria in Africa dropped by 40% between 2000 and 2015, equating to prevention of 663 million cases (Bhatt 2015; WHO-GMP 2015). However progress has stalled in recent years (WHO 2019a). Targeting the mosquito vector has proved to be the most effective method of malaria prevention in Africa, with over two-thirds of malaria cases averted in the first 15 years of this century attributed to scale-up in the use of long-lasting insecticidal nets (LLINs) (Bhatt 2015). This method of malaria prevention is particularly effective in Africa, where the major malaria vectors *Anopheles gambiae* and *Anopheles funestus* are largely endophagic (feed indoors) and endophilic (rest indoors after blood feeding).

Currently all LLINs contain pyrethroids; pyrethroids have the required dual properties of low mammalian toxicity and rapid insecticidal activity (Zaim 2000), and their repellent or contact irritant effects may enhance the personal protection of LLINs. Unfortunately, resistance to pyrethroids is now widespread in African malaria vectors (Ranson 2016). This may be the result of mutations in target-site proteins (target-site resistance) (Ranson 2011; Ridl 2008), which result in reduced sensitivity to the insecticide or increased activity of detoxification enzymes (metabolic resistance) (Mitchell 2012; Stevenson 2011), or other as yet poorly described resistance mechanisms, or a combination of all or some of these factors. The evolution of insecticide resistance and its continuing spread threaten the operational success of malaria vector control interventions. The current impact of this resistance on malaria transmission is largely unquantified and varies depending on level of resistance, malaria endemicity, and proportion of the human population using LLINs (Churcher 2016). A multi-country trial found no evidence that pyrethroid resistance reduced the personal protection provided by the use of LLINs (Kleinschmidt 2018). However, it is generally accepted that resistance will eventually erode the efficacy of pyrethroid-only LLINs, and that innovation in the LLIN market is essential to maintain the efficacy of this preventative measure (MPAC 2016).

Description of the intervention

One way of controlling insecticide-resistant mosquito populations is through the use of insecticide synergists. Synergists are generally non-toxic and act by enhancing the potency of insecticides. Piperonyl butoxide (PBO) is a synergist that inhibits specific metabolic enzymes within mosquitoes and has been incorporated into pyrethroid-treated LLINs to form PBO-combination nets (hereafter referred to as pyrethroid-PBO nets). Insecticide-synergist combination nets represent a new product class with the capacity to affect insecticide-resistant populations. In 2017, the World Health Organization (WHO) gave pyrethroid-PBO nets an interim endorsement as a new vector control class and recommended that countries consider deploying these nets in areas where pyrethroid resistance has been confirmed among main malaria vectors (WHO-GMP 2017a).

Currently six pyrethroid-PBO nets are in production: Olyset® Plus; PermaNet® 3.0; Veeralin® LN; Tsara Plus (previously DawaPlus 3.0); Tsara Boost (previously DawaPlus 4.0); and DuraNet Plus. Olyset Plus, which is manufactured by Sumitomo Chemical Company Ltd., is a polyethylene net treated with permethrin (20 g/kg ± 25%) and PBO (10 g/kg ± 25%) across the whole net (Sumitomo 2013). PermaNet 3.0, which is manufactured by Vestergaard Frandsen, is a mixed polyester (sides) polyethylene (roof) net treated with deltamethrin and PBO; PBO is found only on the roof of the net (25 g/kg ± 25%), and the concentration of deltamethrin varies depending on location (roof: 4.0 g/kg ± 25%) and yarn type (sides: 75-denier (thickness) yarn with 70-cm lower border 2.8 g/kg ± 25%, 100-denier yarn without border 2.1 g/kg ± 25%; Vestergaard 2015). Veeralin LN, manufactured by Vector Control Innovations Private Ltd., is a polyethylene net treated with alpha-cypermethrin (6.0 g/kg) and PBO (2.2 g/kg) across the whole net (WHOPES 2016). Tsara Plus and Tsara Boost are manufactured by NRS Moon Netting FZE. Tsara Plus is treated with deltamethrin (3 g/kg) and PBO (11 g/kg) on the roof, and with deltamethrin only (2.5 g/kg) on its sides. Tsara Boost is treated with deltamethrin (120 mg/m^2) and PBO (440 mg/m^2) on all panels. DuraNet Plus, manufactured by Shobikaa Impex Private Limited, is a polyethylene net treated with alpha-cypermethrin (6.0 g/kg) and PBO (2.2 g/kg) across the whole net.

How the intervention might work

PBO inhibits metabolic enzyme families, in particular the cytochrome P450 enzymes that detoxify or sequester pyrethroids. Increased production of P450s is thought to be the most potent mechanism of pyrethroid resistance in malaria vectors, and pre-exposure to PBO has been shown to restore susceptibility to pyrethroids in laboratory bioassays on multiple pyrethroid-resistant vector populations (Churcher 2016).

Widespread use of conventional LLINs provides both personal and community protection from malaria (Bhatt 2015; Lengeler 2004). In areas where mosquito populations are resistant to pyrethroids, experimental hut trials (as described in the Types of studies section) have shown that mosquito mortality rates and protection from blood feeding are substantially reduced when conventional LLINs are used (Abílio 2015; Awolola 2014; Bobanga 2013; N'Guessan 2007; Riveron 2015; Yewhalaw 2012). The addition of PBO to pyrethroids in LLINs can restore the killing effects of LLINs in areas where this has been eroded by insecticide resistance. LLINs that contain PBO have been evaluated in multiple experimental hut trials across Africa (Adeogun 2012; Bayili 2017; Corbel 2010; Koudou 2011; Menze 2020; Moore 2016; N'Guessan 2010; Oumbouke 2019; Pennetier 2013; Toé 2018; Tungu 2010). In most settings, pyrethroid-PBO nets resulted in higher rates of mosquito mortality and greater blood-feeding inhibition than conventional LLINs, although the magnitude of this effect was variable. Village trials have measured the impact on sporozoite infection rates in mosquitoes with mixed results (Awolola 2014; Cisse 2017; Mzilahowa 2014; Stiles-Ocran 2013). Recently, two separate cluster-randomized trials (cRCTs) in Tanzania and Uganda demonstrated that use of pyrethroid-PBO nets can reduce parasite prevalence in children (Protopopoff 2018; Staedke 2020).

Why it is important to do this review

All LLINs approved by the WHO Prequalification Team (formerly the WHO Pesticide Evaluation Scheme (WHOPES)) contain pyrethroids. Six bed nets that contain PBO have received WHO pre-qualification and have been recognized as a new product class by WHO (WHO-GMP 2017a). As pyrethroid-PBO nets are generally more expensive than conventional LLINs, it is important to determine if they are

superior to conventional LLINs, and under what circumstances, to enable cost-effectiveness trials to be performed to inform procurement decisions.

An Expert Review Group (ERG) commissioned by the WHO has recommended pyrethroid-PBO nets be considered for use in areas where the major malaria vectors are resistant to pyrethroids (WHO-GMP 2017a). This guidance has been adopted by some net providers, for example, the President's Malaria Initiative (PMI) (PMI 2018). The WHO recommendation was largely based on a single randomized controlled trial (RCT) of one pyrethroid-PBO net type conducted in Tanzania (Protopopoff 2018), but it was also supported by a meta-analysis of performance of pyrethroid-PBO nets in experimental hut trials, which was used to parameterize a malaria transmission model to predict the public health benefit of pyrethroid-PBO nets (Churcher 2016). The WHO recommendation is that countries should consider deployment of this new product class in areas with intermediate levels of pyrethroid resistance, but it calls for further evidence, including data from a second clinical trial (WHO 2019b). Results of a second RCT evaluating the epidemiological impact of pyrethroid-PBO nets in Uganda were published in 2020, and this review has been updated to include these data (Staedke 2020).

In an attempt to assess evidence of effectiveness of pyrethroid-PBO nets against African malaria vectors in areas with differing levels of insecticide resistance, we have conducted a systematic review of all relevant trials and examined both epidemiological and entomological endpoints. We appreciate that evaluation of PBO will depend on trials in which the background insecticide and dose are the same in both intervention and control groups; we are aware that most trials have evaluated pyrethroid-PBO nets against pyrethroid-only LLINs with different background insecticides and doses, which confounds the effects.

OBJECTIVES

To compare effects of pyrethroid-PBO nets currently in commercial development or on the market with effects of their non-PBO equivalent in relation to:

1. malaria parasite infection (prevalence or incidence); and
2. entomological outcomes

METHODS

Criteria for considering studies for this review

Types of studies

We included:

1. randomized trials that measured epidemiological outcomes, entomological outcomes, or both; and
2. experimental hut trials.

See Table 1 for detailed WHOPES definitions.

Types of participants

Mosquitoes

Anopheles gambiae complex or *Anopheles funestus* group. Included trials had to test a minimum of 50 mosquitoes per trial arm. We examined the insecticide resistance level (measured by phenotypic resistance) during data analysis.

Humans

Adults and children living in malaria-endemic areas.

Types of interventions

Intervention

Bed nets treated with both PBO and a pyrethroid insecticide. Nets must have received a minimum of interim-WHO approval (Table 2), and LLINs had to be treated with a WHO-recommended dose of pyrethroid (Table 3).

Control

Conventional LLINs that contain pyrethroid only. Nets could be treated with the same insecticide at different doses from the intervention net to allow critical appraisal of all pyrethroid-PBO nets currently in development or on the market. For both intervention and control arms, nets could be unholed, holed, unwashed, or washed, provided the trials adhered to WHO guidelines (WHO 2013).

Types of outcome measures

Trials had to include at least one of the following primary outcomes to be eligible for inclusion.

Primary outcomes

Epidemiological

1. Parasite prevalence: presence of malaria parasites detected through microscopy of blood or rapid diagnostic tests (RDTs)
2. Incidence of clinical malaria: clinical diagnosis based on participants' symptoms and on physical findings at examination

Entomological

1. Mosquito mortality: immediate death or delayed death (up to 24 hours), or both, measured as a proportion of total mosquito number. A mosquito is classified as dead if it is immobile, cannot stand or fly, or shows no sign of life
2. Mosquito knock-down: mosquito 'mortality' recorded one hour post insecticide exposure, termed 'knock-down', as some mosquitoes may recover during the 24-hour recovery period before mosquito mortality is recorded at 24 hours post exposure
3. Blood-feeding success: number of mosquitoes that have blood-fed (alive or dead)
4. Sporozoite rate: percentage of mosquitoes with sporozoites in the salivary glands

Secondary outcomes

Entomological

1. Deterrence: the number of mosquitoes that enter a hut that is using a pyrethroid-PBO net relative to the number of mosquitoes found in a control hut that is using a standard LLIN (experimental hut trials only)
2. Exophily: the proportion of mosquitoes found in exit/veranda traps of a hut that is using a pyrethroid-PBO net relative to the control hut that is using a standard LLIN (experimental hut trials only)

3. Mosquito density: measured by all standard methods, such as window exit traps, indoor resting collections, floor sheet collections, pyrethrum spray catch, and light traps (village trials)
4. Parity rate: percentage of parous mosquitoes detected by mosquito ovary dissections (village trials)

Search methods for identification of studies

We identified all relevant trials regardless of language or publication status (published, unpublished, in press, and in progress). We have presented the search strategies in Appendix 1.

Electronic searches

Vittoria Lutje, the Cochrane Infectious Diseases Group (CIDG) Information Specialist, searched the following databases on 25 September 2020 using the search terms and strategy described in Appendix 1: the CIDG Specialized Register; the Cochrane Central Register of Controlled Trials (CENTRAL; 2018, Issue 8), included in the Cochrane Library; MEDLINE (PubMed); Embase (OVID); Web of Science Core Collection; and CAB Abstracts. She also searched for trials in progress at the WHO International Clinical Trials Registry Platform (WHO ICTRP; www.who.int/ictrp/en/) and ClinicalTrials.gov (clinicaltrials.gov/ct2/home).

Searching other resources

We contacted the following organizations for unpublished data: the PMI; the Innovative Vector Control Consortium (IVCC); Vestergaard Frandsen; Sumitomo Chemical Company Ltd.; Vector Control Innovations Private Ltd.; Endura SpA; and WHOPES. We checked the reference lists of trials identified by the above methods.

Data collection and analysis

All analyses were stratified by trial design and mosquito insecticide resistance level when possible. We performed analyses for the primary outcomes stratified by follow-up time (4 to 6 months, 9 to 12 months, 16 to 18 months, and 21 to 25 months).

We determined whether mosquito populations are susceptible or resistant to pyrethroid insecticides based on WHO definitions (WHO 2016; Table 4). We used 24-hour mosquito mortality to determine resistance status; however if this had been unavailable, we intended to use knock-down 60 minutes after the end of the assay. We stratified resistant populations into low-, moderate-, and high-prevalence resistance groups (Table 5), by dividing resistant mosquitoes (i.e. those with < 90% mortality) into three equal groups, with the lower third being most resistant and the upper third most susceptible.

Selection of studies

Two review authors (KG and NL or LC) independently screened titles and abstracts of all retrieved references based on the inclusion criteria (Table 6). We resolved any inconsistencies between review authors' selections by discussion. If we were unable to reach an agreement, we consulted a third review author (HR). We retrieved full-text trial reports for all potentially relevant citations. Two review authors independently screened the full-text articles and identified trials for inclusion, and identified and recorded reasons for exclusion of ineligible trials in a Characteristics of excluded studies table. We resolved any disagreements through discussion or, if required, we consulted a third review author (HR). We identified and excluded duplicates and collated multiple reports of the same trial, so that each trial, rather than each report, was the unit of interest in the review. We recorded the selection process in sufficient detail to complete a PRISMA flow diagram (Moher 2009).

Data extraction and management

After selection, we summarized all included trials according to the tables in Appendix 2. Two review authors (KG and NL or LC) independently extracted data from included trials using the pre-designed data extraction form (Appendix 3). If data were missing from an included trial, we contacted the trial authors to ask for further information. We entered data into Review Manager 5 (RevMan 5) (Review Manager 2014).

Assessment of risk of bias in included studies

Two review authors (KG and NL or LC) independently assessed the risk of bias of each included trial using a set of predetermined criteria specific to each trial type adapted from Strode 2014 (Appendix 4). We assigned a classification of low, high, or unclear risk of bias for each component. For all included trials, we assessed whether any trial authors had submitted any conflicts of interest that may have biased trial methods or results.

Randomized trials and village trials

We assessed 12 criteria for village and RCTs: recruitment bias, comparability of mosquitoes between LLIN/pyrethroid-PBO net households (e.g. species composition), collectors blinded, household blinded, treatment allocation, allocation concealment, incomplete outcome data, raw data reported, clusters lost to follow-up, selective reporting, adjustment for data clustering, and trial authors' conflicting interests.

Experimental hut trials

For experimental hut trials, we assessed 11 criteria: comparability of mosquitoes between LLIN/pyrethroid-PBO net arms (e.g. species composition), collectors blinded, sleepers blinded, sleeper bias accounted for, treatment allocation, treatment rotation, standardized hut design, hut cleaning between treatments, incomplete outcome data, raw data reported, and trial authors' conflicting interests.

Measures of treatment effect

For dichotomous data, we preferentially presented the risk ratio (RR). For the outcome of parasite prevalence from cRCTs, we used the odds ratio (OR) as the measure of effect, as one study presented adjusted ORs that could not be converted to adjusted RRs using the standard formula presented in the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins 2011). We found no continuous or count data; however if we had, we would have used mean differences (MDs) and rate ratios, respectively. We have presented all results with 95% confidence intervals (CIs).

Unit of analysis issues

For trials randomized by hut or village, we used the adjusted measure of effect reported in the paper if available. For the outcome of parasite prevalence from cRCTs, we converted adjusted RRs presented in one study - Staedke 2020 - to adjusted ORs using the standard formula presented in the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins 2011), so that this study could be pooled with Protopopoff 2018.

When adjusted measures of effect were not reported, we used an intracluster correlation coefficient (ICC) and average cluster size to adjust the data ourselves (Higgins 2011 Section 16.3.4). If the included trial did not report the ICC value, we estimated the ICC value and performed sensitivity analyses to investigate the impact of estimating the ICC. When ICCs have been used to adjust results for clustering, forest plots for both hut and village trials show the effective number of events and the number of mosquitoes after adjustments for clustering.

To adjust results of experimental hut trials for clustering, we treated each 'hut and night' combination as the unit of randomization, as each hut was tested with each type of net over a series of nights. Sleepers inside the huts were rotated each night, so by using "hut/night" as the unit of randomization, sleeper effects were also accounted for. We calculated effective sample sizes by estimating an ICC and a corresponding design effect. We divided both the number of mosquitoes and the number experiencing the event by this design effect.

Dealing with missing data

In the case of missing data, we contacted trial authors to request this information. If we had identified trials in which participants were lost to follow-up, we would have investigated the impact of missing data via imputation using a best/worst-case scenario analysis.

When information on mosquito insecticide resistance was not collected at the time of the trial, review authors determined a suitable proxy. Proxy resistance data had to be taken from the same area and conducted within three years of the trial, and the same insecticide, dose, and mosquito species had to be used. More than 50 mosquitoes per insecticide should have been tested against an appropriate control. When no resistance data were available, we determined that resistance status was unclassified.

Assessment of heterogeneity

We presented the results of included trials in forest plots, which we inspected visually, to assess heterogeneity (i.e. non-overlapping CIs generally signify statistical heterogeneity). We used the Chi^2 test with a P value less than 0.1 to indicate statistical heterogeneity. We quantified heterogeneity by using the I^2 statistic (Higgins 2003), and we interpreted a value greater than 75% to indicate considerable heterogeneity (Deeks 2017).

Assessment of reporting biases

To analyse the possibility of publication bias, we intended to use funnel plots if 10 trials with epidemiological endpoints were included in any of the meta-analysis. However, no analyses included 10 or more trials, so this plan was not applicable.

Data synthesis

When appropriate, we pooled the results of included trials using meta-analysis. We stratified results by type of trial, mosquito resistance status, and net type (i.e. by product, e.g. Olyset Plus).

Four review authors (KG, NL, LC, and MC) analysed the data using RevMan 5 (Review Manager 2014), using the random-effects model (if we detected heterogeneity; or if the I^2 statistic value was greater than 75%) or the fixed-effect model (for no heterogeneity; or if the I^2 statistic value was less than 75%). The exception to this is that for the primary outcome of parasite prevalence from cluster trials, we pooled results using the fixed-effect model, although heterogeneity between study results was substantial. For additional information, see 'Effects of Interventions: Epidemiological results'. We would have refrained from pooling trials in meta-analysis if it was not clinically meaningful to do so, due to clinical or methodological heterogeneity.

Subgroup analysis and investigation of heterogeneity

We performed subgroup analyses according to whether nets were washed or unwashed.

Sensitivity analysis

We intended to perform sensitivity analyses to determine the effect of exclusion of trials that we considered to be at high risk of bias; however this approach was not applicable, as no trials were deemed at high risk. We would have performed a sensitivity analysis for missing data during imputation with best/worst-case scenarios, but again this was not applicable.

We performed sensitivity analyses to investigate the impact of estimating an ICC to adjust trial results for clustering. We performed analyses using ICCs of 0.01, 0.05, and 0.1. Because results were robust to these adjustments, we used the most conservative ICC (0.1), and we adjusted all results from unadjusted cluster trials using this ICC. We have not presented analyses using the smaller ICCs (0.01 and 0.05).

Summary of findings and assessment of the certainty of the evidence

We assessed the certainty of evidence using the GRADE approach (Schünemann 2013). We constructed 'Summary of findings' tables using GRADEpro Guideline Development Tool (GDT) software (GRADEpro GDT 2015).

RESULTS

Description of studies

Results of the search

We identified 389 records through our searches. We removed duplicates, leaving 347 records, and we screened all articles for possible inclusion. After abstract and title screening, we excluded 322 ineligible trials. We assessed 25 full-text articles for eligibility and excluded nine articles for the following reasons: three trials did not share full data sets, two were laboratory studies, and four are ongoing. Sixteen trials met the inclusion criteria (Figure 1).

Figure 1. Study flow diagram.

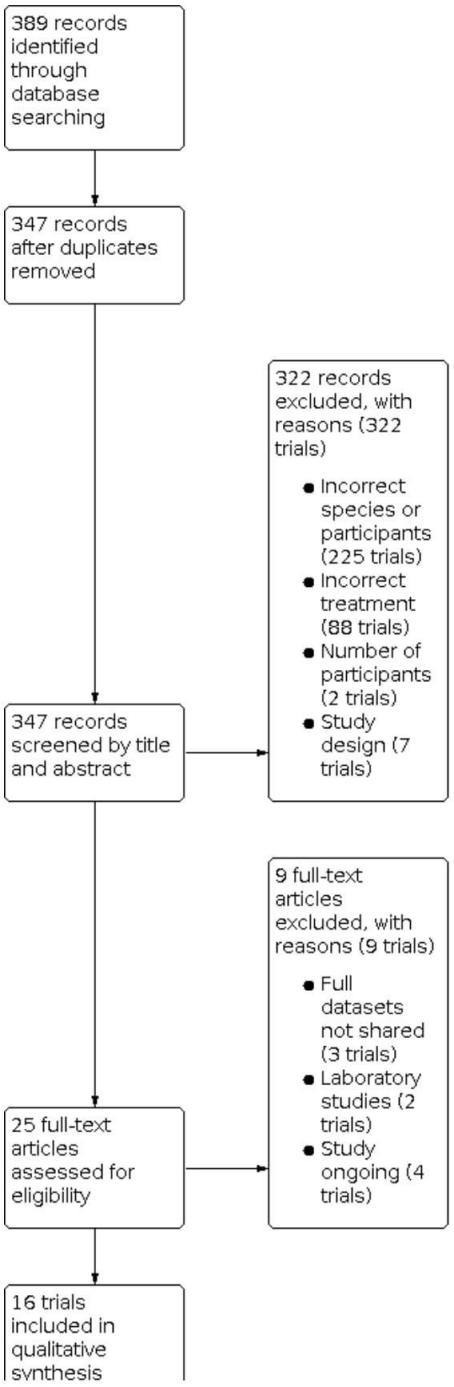
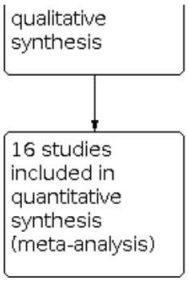


Figure 1. (Continued)



Included studies

Sixteen trials met the inclusion criteria; we have described them in the Characteristics of included studies tables. Ten trials were experimental hut trials (Bayili 2017 (Burkina Faso); Corbel 2010 (Burkina Faso, Benin, Cameroon); Koudou 2011 (Côte d'Ivoire); Menze 2020 (Cameroon); Moore 2016 (Tanzania); N'Guessan 2010 (Benin); Oumbouke 2019 (Côte d'Ivoire); Pennetier 2013 (Benin); Toé 2018 (Burkina Faso); Tungu 2010 (Tanzania)). Four trials were village trials (Awolola 2014 (Nigeria); Cisse 2017 (Mali); Mzilahowa 2014 (Malawi); Stiles-Ocran 2013 (Ghana)). Two were cRCTs (Protopopoff 2018 (Tanzania); Staedke 2020 (Uganda)). All trials were conducted in Africa.

Interventions

Six trials compared Permanet 2.0 to Permanet 3.0 (Awolola 2014; Corbel 2010; Koudou 2011; N'Guessan 2010; Stiles-Ocran 2013; Tungu 2010); two trials compared Olyset Net to Olyset Plus (Pennetier 2013; Protopopoff 2018); two trials compared MAGNet LN to Veeralin LN (Moore 2016; Oumbouke 2019); five trials compared both Olyset Net to Olyset Plus and Permanet 2.0 to Permanet 3.0 (Cisse 2017; Menze 2020; Mzilahowa 2014; Staedke 2020; Toé 2018); and one trial compared DawaPlus 2.0 to DawaPlus 3.0 and DawaPlus 4.0 (Bayili 2017).

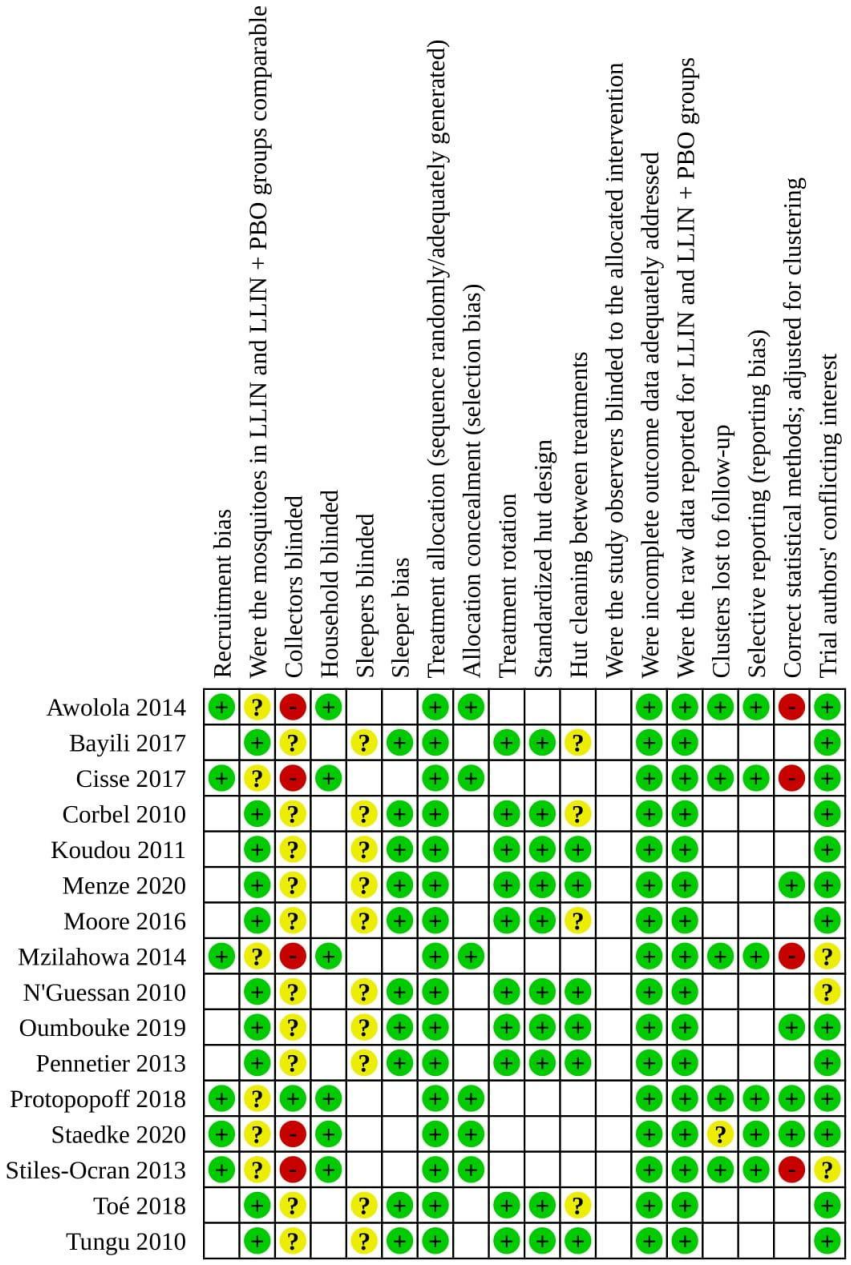
Excluded studies

We assessed 25 full-text articles for eligibility and excluded nine articles for the following reasons: three trials are awaiting classification because we were unable to obtain the full data sets after we contacted trial authors (see Characteristics of studies awaiting classification table); four trials are ongoing (see Characteristics of ongoing studies section); and two trials included only laboratory data (Darriet 2011; Darriet 2013).

Risk of bias in included studies

We have provided a 'Risk of bias' assessment summary in Figure 2. The criteria we used to assess risk of bias are provided in Appendix 5 (experimental hut trials) and in Appendix 6 (village trials).

Figure 2. 'Risk of bias' summary: review authors' judgements about each risk of bias item for each included study.



Allocation

Recruitment bias

We assessed all four village trials as having low risk of recruitment bias, as recruitment bias is related to human participants and so is not applicable to this review (Awolola 2014; Cisse 2017; Mzilahowa 2014; Stiles-Ocran 2013). We assessed the two cRCTs as having low risk, as no participants were recruited after clusters had been randomized (Protopopoff 2018; Staedke 2020).

Mosquito group comparability

We judged all 10 experimental hut trials to be at low risk (Bayili 2017; Corbel 2010; Koudou 2011; Menze 2020; Moore 2016; N'Guessan 2010; Oumbouke 2019; Pennetier 2013; Toé 2018; Tungu 2010), as the huts were situated in the same trial area and therefore were accessible to the same mosquito populations. We judged all four village trials and both cRCTs to be at unclear risk, as for six trials, species composition and resistance status varied slightly between treatment arms (Awolola 2014; Cisse 2017; Menze 2020; Oumbouke 2019; Protopopoff 2018; Stiles-Ocran 2013); for one trial, species and resistance data were not separated by village (Mzilahowa 2014); and for one trial, the size of the area covered made it difficult to classify resistance status in all areas (Staedke 2020).

Blinding

We assessed the 10 hut trials to be at unclear risk, as they did not specify whether observers, collectors and sleepers (hut trials) were blinded (Bayili 2017; Corbel 2010; Koudou 2011; Menze 2020; Moore 2016; N'Guessan 2010; Oumbouke 2019; Pennetier 2013; Toé 2018; Tungu 2010). This is not standard protocol for these trial designs and is thought unlikely to affect the results. We judged four village trials to be at high risk of bias, as it was not stated whether collectors were blinded, and this may have affected searching efforts during collection (Awolola 2014; Cisse 2017; Mzilahowa 2014; Stiles-Ocran 2013). We judged one cRCT as having high risk, as it was stated that LLIN allocation was not masked to collectors (Staedke 2020), and the other as having low risk because collectors were masked to treatment (Protopopoff 2018). For household blinding, we judged all four village trials and both cRCTs to be at low risk of bias. Four village trials and one cRCT did not state whether households were blind to the intervention; however this was unlikely to influence the results (Awolola 2014; Cisse 2017; Mzilahowa 2014; Stiles-Ocran 2013; Staedke 2020). We judged one cRCT as having low risk, as inhabitants and field collectors were blinded to intervention arms (Protopopoff 2018).

Sleeper bias

We assessed the 10 hut trials to be at low risk for sleeper bias, as sleepers were rotated between huts according to a Latin square design (Bayili 2017; Corbel 2010; Koudou 2011; Menze 2020; Moore 2016; N'Guessan 2010; Oumbouke 2019; Pennetier 2013; Toé 2018; Tungu 2010).

Treatment allocation, rotation, and concealment

We assessed the 10 hut trials to be at low risk for treatment allocation and rotation, as treatments were rotated between huts according to a Latin square design (Bayili 2017; Corbel 2010; Koudou 2011; Menze 2020; Moore 2016; N'Guessan 2010; Oumbouke 2019; Pennetier 2013; Toé 2018; Tungu 2010). We assessed all four village trials and both cRCTs to be at low risk for treatment allocation (Awolola 2014; Cisse 2017; Mzilahowa 2014; Protopopoff 2018; Staedke 2020; Stiles-Ocran 2013), as villages were randomly assigned to treatment arms. We assessed all four village trials and both cRCTs as having low risk of bias for allocation concealment (Awolola 2014; Cisse 2017; Mzilahowa 2014; Protopopoff 2018; Staedke 2020; Stiles-Ocran 2013).

Hut design

We assessed all 10 hut trials to be at low risk of bias, as huts were built to standard West or East African specifications (Bayili 2017; Corbel 2010; Koudou 2011; Menze 2020; N'Guessan 2010; Oumbouke 2019; Pennetier 2013; Toé 2018; Tungu 2010), or they used modified but standardized designs (Moore 2016).

Cleaning

We assessed four hut trials to be at unclear risk, as they did not state whether huts were cleaned between treatment arms (Bayili 2017; Corbel 2010; Moore 2016; Toé 2018). We assessed six to be at low risk, as cleaning was conducted between treatment rotations (Koudou 2011; Menze 2020; N'Guessan 2010; Oumbouke 2019; Pennetier 2013; Tungu 2010).

Incomplete outcome data

We assessed all hut trials - Bayili 2017; Corbel 2010; Koudou 2011; Menze 2020; Moore 2016; N'Guessan 2010; Oumbouke 2019; Pennetier 2013; Toé 2018; Tungu 2010, village trials - Awolola 2014; Cisse 2017; Mzilahowa 2014; Stiles-Ocran 2013, and cRCTs - Protopopoff 2018; Staedke 2020 - to be at low risk for both incomplete outcome data and raw data reporting, as there were no incomplete outcome data, or missing data were later provided by trial authors. In cases when raw data were not reported, we were able to calculate them from the percentages and sample sizes given. When these data were not available, we did not include the trials.

Clustering bias

Staedke 2020 lost 14 clusters to follow-up at the latest time point and was therefore assessed as having unclear risk of bias. In the other village and cRCT trials, no clusters were lost to follow-up, and these trials were assessed as having low risk (Awolola 2014; Cisse 2017; Mzilahowa 2014; Protopopoff 2018; Staedke 2020; Stiles-Ocran 2013). We assessed four village trials as having high risk of bias for statistical methods used, as they did not adjust for clustering (Awolola 2014; Cisse 2017; Mzilahowa 2014; Stiles-Ocran 2013). We assessed the two cRCTs as having low risk of bias, as they took clustering into account and adjusted for it in their statistical methods (Protopopoff 2018; Staedke 2020).

Selective reporting

We assessed all village trials and cRCTs as having low risk of bias regarding selective reporting, as they appear to have reported all measured outcomes (Awolola 2014; Cisse 2017; Mzilahowa 2014; Protopopoff 2018; Staedke 2020; Stiles-Ocran 2013).

Other potential sources of bias

Conflicting interests

We judged nine hut trials - Bayili 2017; Corbel 2010; Koudou 2011; Menze 2020; Moore 2016; Oumbouke 2019; Pennetier 2013; Toé

2018; Tungu 2010, two village trials - Awolola 2014; Cisse 2017, and both cRCTs - Protopopoff 2018; Staedke 2020 - as having low risk, as trial authors reported no conflicting interests. We assessed one hut trial to be at unclear risk (N'Guessan 2010), as trial authors stated that they had received funding from LLIN manufacturers when conducting the trials, and the same funders provided comments on the manuscript. We assessed one village trial as having unclear risk, as trial authors did not state whether there were conflicting interests (Mzilahowa 2014), and another trial as having unclear risk, as the trial was conducted to form part of the manufacturer's product dossier (Stiles-Ocran 2013).

Effects of interventions

See: **Summary of findings 1** Summary of findings table 1; **Summary of findings 2** Summary of findings table 2; **Summary of findings 3** Summary of findings table 3; **Summary of findings 4** Summary of findings table 4

We compared the effects of pyrethroid-PBO nets currently in commercial development or on the market with their non-PBO equivalent in relation to malaria infection and entomological outcomes. This review is based on results from 16 trials.

Epidemiological results

Two trials examined the effects of pyrethroid-PBO nets (Olyset Plus and PermaNet 3.0) on parasite prevalence (Protopopoff 2018; Staedke 2020). Pooling the latest endpoint after the intervention from both trials revealed that parasite prevalence was decreased in the intervention arm (Olyset Plus and PermaNet 3.0) (OR 0.79, 95% CI 0.67 to 0.95; 2 trials, 2 comparisons; Analysis 1.1).

There was little variation of effect from the earliest time point (4 to 6 months after: OR 0.74, 95% CI 0.62 to 0.89) to the latest time point (21 to 25 months after: OR 0.79, 95% CI 0.67 to 0.95) (Analysis 1.2).

We used a fixed-effect model to pool data from the two studies. Although heterogeneity between study results was considerable, both studies demonstrated clear beneficial effects with PBO nets. Performing random-effects meta-analysis accounted for differences between study results to the extent that identified benefits disappeared in the pooled analysis, indicating failure of the random-effects model.

Entomological results

Experimental hut trials

Ten experimental hut trials (phase 2 trials) examined the effects of pyrethroid-PBO nets on mosquito mortality, blood feeding, exophily, and deterrence (Bayili 2017; Corbel 2010; Koudou 2011; Menze 2020; Moore 2016; N'Guessan 2010; Oumbouke 2019; Pennetier 2013; Toé 2018; Tungu 2010). We subgrouped the data by net washing into unwashed and washed groups. All washed nets were washed 20 times according to WHO specifications (WHO 2013). We pooled the results initially and then stratified them by insecticide resistance level and by net type. Two trials did not wash their nets and so did not report any data for the washed subgroup (Menze 2020 Toé 2018). One trial did not introduce holes into the nets and so did not report blood-feeding success data (Koudou 2011).

Pooled analysis

Pooled analysis of all experimental hut trials using both unwashed nets - Bayili 2017; Corbel 2010; Koudou 2011; Menze 2020; Moore 2016; N'Guessan 2010; Oumbouke 2019; Pennetier 2013; Toé 2018; Tungu 2010 - and washed nets - Bayili 2017; Corbel 2010; Koudou 2011; Moore 2016; N'Guessan 2010; Oumbouke 2019; Pennetier 2013; Tungu 2010 - revealed that pyrethroid-PBO nets significantly increased mosquito mortality by 43% (risk ratio (RR) 1.43, 95% confidence interval (CI) 1.26 to 1.62) and reduced blood-feeding success by 25% (RR 0.75, 95% CI 0.66 to 0.85). The magnitude of the effect was reduced by net washing. Unwashed pyrethroid-PBO nets increased mosquito mortality by 63% compared to unwashed LLINs (RR 1.63, 95% CI 1.29 to 2.05; 10 trials, 18 comparisons; Analysis 2.1); when nets were washed, this effect was decreased to 19% (RR 1.19, 95% 1.04 to 1.38; 8 trials, 12 comparisons; Analysis 2.1). Unwashed pyrethroid-PBO nets reduced mosquito blood-feeding success by 32% (RR 0.68, 95% CI 0.57 to 0.80; 9 trials, 17 comparisons; Analysis 2.2; Bayili 2017; Corbel 2010; Moore 2016; N'Guessan 2010; Pennetier 2013; Toé 2018; Tungu 2010); however this effect was lost when nets were washed (7 trials, 11 comparisons; Analysis 2.2; Bayili 2017; Corbel 2010; Moore 2016; N'Guessan 2010; Pennetier 2013; Tungu 2010). There was no effect on mosquito exophily in either unwashed (10 trials, 17 comparisons; Analysis 2.3) or washed groups (8 trials, 12 comparisons; Analysis 2.3). Mosquito deterrence data were presented relative to an untreated control and hence are not included as a forest plot. There was considerable variation in deterrence rates but no clear relationship with resistance level, net type, or washing status (Table 7).

Heterogeneity in this pooled analysis was considerable, particularly for estimates of mortality. We therefore performed a pre-specified, stratified analysis, dividing the results into trials conducted in areas of low, moderate, or high resistance in the *Anopheles* population.

Stratified analysis: mosquito resistance status

We used WHO and Centers for Disease Control and Prevention (CDC) definitions of mosquito mortality from WHO tube assays or CDC bottle tests to classify mosquito resistance (Table 4). Both tests define mosquitoes as resistant when mortality is less than 90%. We further stratified resistance based on the following mortality levels: < 30%, high resistance; 31% to 60%, moderate resistance; and 61% to 90%, low resistance (Table 5). When resistance data were not collected at the time of the trial, we identified a suitable proxy based on previously described criteria (see Dealing with missing data section); when we could not identify a suitable proxy, we deemed the trial as 'unclassified' and did not include it in the resistance stratification.

Five trials were conducted in four areas where mosquito populations exhibited high resistance to pyrethroids (Bayili 2017; Corbel 2010; Koudou 2011; Pennetier 2013; Toé 2018). Under these conditions, unwashed pyrethroid-PBO nets increased mosquito mortality by 84% in comparison to unwashed LLINs (RR 1.84, 95% CI 1.60 to 2.11; 5 trials, 9 comparisons; Analysis 2.4); however this effect was lost when nets were washed (4 trials, 5 comparisons; Analysis 2.4; Bayili 2017; Corbel 2010; Koudou 2011; Pennetier 2013). Blood-feeding success was reduced by 40% in unwashed pyrethroid-PBO net groups compared to unwashed LLIN groups (RR 0.60, 95% CI 0.50 to 0.71; 4 trials, 8 comparisons; Analysis 2.5; Bayili 2017; Corbel 2010; Pennetier 2013; Toé 2018), and was reduced by 19% when nets were washed (RR 0.81, 95% CI 0.72 to

0.92; 3 trials, 4 comparisons; Analysis 2.5; Bayili 2017; Corbel 2010; Pennetier 2013).

Two trials at three different sites were conducted in areas with moderate insecticide resistance (Menze 2020; N'Guessan 2010). With unwashed nets, mosquito mortality was increased by 68% in comparison to mosquito mortality with unwashed LLINs (RR 1.68, 95% CI 1.33 to 2.11; 2 trials, 3 comparisons; Analysis 2.6); however there was minimal effect on blood-feeding success. No effect on mosquito mortality (1 trial, 1 comparison; Analysis 2.6) or on blood-feeding success (1 trial, 1 comparison; Analysis 2.7) was observed with washed treatments.

Two trials at three different sites were conducted in areas with low insecticide resistance (Corbel 2010; Oumbouke 2019). A small effect on mosquito mortality was observed with unwashed nets (RR 1.25, 95% CI 0.99 to 1.57; 2 trials, 3 comparisons; Analysis 2.8) and was also seen with washed nets (RR 1.39, 95% CI 0.95 to 2.04; 2 trials, 3 comparisons; Analysis 2.8). No effect on blood-feeding success was noted (2 trials, 3 comparisons; Analysis 2.9).

At susceptible sites (Moore 2016; Tungu 2010), no effect on mosquito mortality (2 trials, 2 comparisons; Analysis 2.10) nor on blood-feeding success (2 trials, 2 comparisons; Analysis 2.11) was observed.

Stratified analysis: net type

After stratifying by resistance status, we performed a secondary analysis stratified according to net type. Due to the limited number of trials, we performed this analysis only for trials using PermaNet 3.0 or Olyset Plus. Although additional trials utilising Veeralin LN, DawaPlus 3.0, and DawaPlus 4.0 have been conducted, not all data were made available to us for the purposes of this Cochrane Review. Futhermore, the analysis was restricted to trials conducted in areas of high resistance, as this analysis indicated an impact of only pyrethroid-PBO nets in these settings. Three trials compared PermaNet 2.0 (LLIN) to PermaNet 3.0 (pyrethroid-PBO nets), and two compared Olyset Nets (LLIN) to Olyset Plus (pyrethroid-PBO nets).

In the PermaNet group, in high-resistance settings, unwashed PermaNet 3.0 increased mosquito mortality by 81% compared to PermaNet 2.0 (RR 1.81, 95% CI 1.56 to 2.10; 3 trials, 4 comparisons; Analysis 2.12; Corbel 2010; Koudou 2011; Toé 2018). After washing, there was no significant increase in mortality in the PermaNet 3.0 arm (2 trials, 2 comparisons; Analysis 2.12; Corbel 2010; Koudou 2011). Blood-feeding success was reduced by 47% when unwashed PermaNet 3.0 was used (RR 0.53, 95% CI 0.40 to 0.69; 2 trials, 3 comparisons; Analysis 2.13; Corbel 2010; Toé 2018); only one trial was available for washed nets (Corbel 2010), and in this trial, PermaNet 3.0 also reduced blood-feeding success (RR 0.76, 95% 0.61 to 0.93; 1 trial, 1 comparison; Analysis 2.13).

In high-resistance settings, Olyset Plus increased mosquito mortality by 72% when nets were unwashed (RR 1.72, 95% CI 1.48 to 1.99; 2 trials, 3 comparisons; Analysis 2.14; Pennetier 2013; Toé 2018). Only one trial compared washed Olyset Plus with washed Olyset (Pennetier 2013); in this trial, enhanced mortality (81%) was still observed in the Olyset Plus arm after washing (RR 1.81, 95% CI 1.25 to 2.61; 1 trial, 1 comparison; Analysis 2.14). There was no impact on blood-feeding success when unwashed Olyset Plus was compared with Olyset (2 trials, 3 comparisons; Analysis 2.15); the single trial that looked at washed Olyset Plus showed decreased blood feeding compared to Olyset (RR 0.50, 95% 0.27 to 0.93; 1 trial, 1 comparison; Analysis 2.15).

Village trials

In the village trials, there was no decrease in sporozoite rate in trial arms receiving pyrethroid-PBO nets (RR 0.82, 95% CI 0.24 to 2.75; 4 trials, 5 comparisons; Analysis 1.3; Awolola 2014; Cisse 2017; Protopopoff 2018; Stiles-Ocran 2013). Mosquito parity was not reduced in pyrethroid-PBO villages (3 trials, 4 comparisons; Analysis 1.4; Cisse 2017; Mzilahowa 2014; Stiles-Ocran 2013). It was not possible to stratify these data by resistance status due to the variability in resistance levels between villages within the same trial. Mosquito density was measured by a variety of methods and was summarized in different ways (e.g. mean number caught per house, mean number caught per village). When baseline data were collected, we calculated a percentage reduction. Higher reductions in mosquito densities were observed in pyrethroid-PBO net villages compared to LLIN villages (Table 8).

DISCUSSION

See Summary of findings 1, Summary of findings 2, Summary of findings 3, and Summary of findings 4.

Summary of main results

Two cluster-randomized controlled trials (cRCTs) were performed on pyrethroid-piperonyl butoxide (PBO) nets. The first trial, which compared parasite prevalence in children using Olyset Plus nets with that in children using Olyset nets, in a region of Tanzania where mosquito vectors are highly resistant to pyrethroids, found that pyrethroid-PBO nets reduced parasite prevalence by 60% at the final time point (21 months) (Protopopoff 2018). The second cRCT compared parasite prevalence in children using Olyset Plus or Permanet 3.0 nets with that in children using Olyset or Permanet 2.0 nets across East and West Uganda, where mosquito vectors are also highly resistant to pyrethroids, and found that pyrethroid-PBO nets reduced parasite prevalence by 17% at the latest time point (25 months) (Staedke 2020).

All other trials included in this review measured entomological endpoints. Four village trials measured sporozoite rates in mosquitoes collected from houses using pyrethroid-PBO nets and standard pyrethroid long-lasting insecticidal nets (LLINs), but the results were highly heterogeneous and no evidence suggests that pyrethroid-PBO nets reduced the mosquito infection rate derived from this pooled analysis (Awolola 2014; Cisse 2017; Protopopoff 2018; Stiles-Ocran 2013). Similarly, the proportion of parous mosquitoes (i.e. mosquitoes that have survived past one gonotrophic cycle; used as an indirect measure of longevity) was not significantly affected by the presence of pyrethroid-PBO nets (Cisse 2017; Mzilahowa 2014; Stiles-Ocran 2013).

When we pooled the results from 10 experimental hut trials (Bayili 2017; Corbel 2010; Koudou 2011; Menze 2020; Moore 2016; N'Guessan 2010; Oumbouke 2019; Pennetier 2013; Toé 2018; Tungu 2010), data showed improved performance of pyrethroid-PBO LLINs over standard LLINs in both increasing mosquito mortality and reducing blood feeding, but these results were highly heterogeneous. Stratifying experimental hut data by resistance levels in this population reduced heterogeneity. In areas where mosquitoes are highly resistant to pyrethroids, pyrethroid-PBO nets will reduce mosquito blood-feeding rates (i.e. users will be

better protected against mosquito bites by using pyrethroid-PBO nets). This impact on blood feeding is reduced when nets have been through the standard 20 washes recommended by the World Health Organization (WHO) to assess chemical durability, but it remains significant (high-certainty evidence). When resistance is high and new unwashed nets are used, mosquito mortality is substantially higher when the nets contain PBO compared to pyrethroid only (high-certainty evidence). However this effect on mosquito mortality, which is important for the community-level protection afforded by LLIN usage (Hawley 2003; Maxwell 2002), is not sustained when nets have been washed multiple times. In this Cochrane Review, we classified mosquitoes as highly resistant if less than 30% were killed in a standard bioassay. When mortality rates exceeded 30%, we found little evidence to suggest that pyrethroid-PBO nets provided greater personal protection or resulted in greater mosquito mortality than standard pyrethroid-only nets. This result is not unexpected, given that in areas where resistance is uncommon or absent, exposure to pyrethroids alone would be expected to negatively affect the mosquito; it is only in areas where the efficacy of pyrethroids has been eroded by the development of high levels of resistance that the addition of a synergist might be needed.

We found no evidence for any difference in the performance of pyrethroid-PBO nets from different manufacturers against highly pyrethroid-resistant mosquitoes. We stratified results by net type only for trials that were conducted in areas of high resistance. We have not reported comparisons for DawaPlus-PBO and Veeralin-PBO nets in this sub-analysis, as there was only a single data point for these net types. We did not stratify data from the cRCTs by net type, as one trial used only one net type (Protopopoff 2018), and the second was not powered to detect differences between nets from different manufacturers and assigned an uneven number of clusters to each net type (Staedke 2020). Unwashed PermaNet 3.0 and Olyset Plus resulted in similar increases in mosquito mortality compared to pyrethroid-only LLINs from the same manufacturer, although this effect on mortality was not always sustained after washing (Corbel 2010; Koudou 2011; Pennetier 2013; Toé 2018). A significant improvement in personal protection for unwashed pyrethroid-PBO nets was observed only for PermaNet 3.0 (Corbel 2010; Toé 2018), but after washing, pyrethroid-PBO nets from both manufacturers provided greater personal protection than the equivalent pyrethroid-only nets (Corbel 2010; Pennetier 2013). Results from comparisons between pyrethroid-PBO nets from different manufacturers should be taken with great caution, given the very limited number of data points available, particularly for washed nets. Further trials, in which nets from different manufacturers are directly compared in the same trial, are needed to address the issue of equivalence between different pyrethroid-PBO nets.

Certainty of the evidence

We appraised the certainty of evidence using the GRADE approach (Summary of findings 1 Summary of findings 2 Summary of findings 3 Summary of findings 4). The two cRCTs provided moderate-certainty evidence that pyrethroid-PBO nets reduced parasite prevalence for the duration of the trial (high-certainty evidence after four to six months) (Protopopoff 2018; Staedke 2020).

This result was obtained from two independent studies, conducted in different locations and settings; therefore the evidence adheres to the WHO recommendation that at least two cRCTs must be completed to demonstrate public health value (WHO-GMP 2017b).

The certainty of evidence from trials using entomological endpoints varied. Data from village trials were difficult to assess, as there was considerable heterogeneity in the level of pyrethroid resistance and presumably also in the resistance mechanisms, both within and between trials. Analysis of data from experimental hut trials yielded high-certainty evidence for superior performance of pyrethroid-PBO nets in areas of high resistance, but evidence from trials conducted in other settings was of low or very low certainty.

Overall completeness and applicability of evidence

All trials included in this review compared pyrethroid-PBO nets with the nearest equivalent pyrethroid-only LLINs. Further changes to net specifications were often included when manufacturers incorporated the synergist. For example, the pyrethroid-PBO net manufactured by Vestergaard (PermaNet 3.0) contains higher levels of deltamethrin and yarn of a different denier (thickness) compared to the pyrethroid-only equivalent, PermaNet 2.0; the pyrethroid in Olyset Plus (Sumitomo Chemical Co. Ltd.) is released from the yarn at a different rate than that in the Olyset nets. These additional variations in chemical or physical composition, or both, of the nets make it difficult to directly assess the added value of the addition of PBO. Furthermore, the concentration of PBO and its site of application differ markedly between nets received from different manufacturers. Two of the currently available pyrethroid-PBO nets (PermaNet 3.0 and Tsara Plus 3.0) contain PBO only on the roof of the netting, exploiting the behavioural patterns of host-seeking mosquitoes to attempt to reach the net user by approaching from above (Parker 2015), whilst the remaining pyrethroid-PBO nets contain the synergist on all sides of the net. The amount of PBO contained within the net differs by a factor of 25-fold. It is not known how net manufacturers selected the doses of PBO applied to the netting.

With currently available data, it is not possible to draw any conclusions on which strategy for producing pyrethroid-PBO nets will prove the most effective under field conditions. The optimum PBO:pyrethroid ratio will likely differ depending on the level of resistance in the mosquito and underpinning resistance mechanisms. Data from experimental hut trials suggest that the PBO component of pyrethroid-PBO nets is lost after repeated washing, as enhanced mortality caused by the synergist nets is not maintained after 20 washes. As yet, no trials on the durability of pyrethroid-PBO nets under operational conditions have been published, although monitoring is under way. It is encouraging to note that both RCTs of pyrethroid-PBO nets found that the superior protective efficacy of Olyset Plus compared to standard Olyset nets was maintained at 21 months of use; the trial in Tanzania is being extended to establish whether this effect lasts the full duration of an LLIN's intended 36-month life span. No plans are under way to continue monitoring in the Uganda trial past the 25-month collections (Staedke 2020).

Most available data evaluated the performance of pyrethroid-PBO LLINs against *Anopheles gambiae* s.l., with very limited data available for the second major species complex in Africa, *An funestus*, and none for other minor vector species. As different mosquito species may differ in their behaviour and in the strength and underpinning mechanisms of pyrethroid resistance, this represents an important data gap that may have implications for

practice in areas where *An gambiae* complex is not the predominant malaria vector.

Potential biases in the review process

As the addition of PBO to pyrethroid LLINs is expected to enhance their performance only in areas where mosquitoes are resistant to pyrethroid insecticides, it was important to stratify the results by resistance status. To do this, we used the WHO definition of resistance as mosquito populations with less than 90% mortality in a discriminating dose assay (WHO 2016), and then we split the resistant populations into three groups, depending on the percentage of mortality observed. Discriminating dose assays provide an estimate of the prevalence of resistance in a population but do not indicate the strength of this resistance nor give any indication of the mechanism(s) underpinning the resistance. As PBO works primarily by inhibiting the metabolism of pyrethroids by cytochrome P450s, this synergist is likely to have had greatest impact in populations where resistance was primarily conferred by elevated P450 activity and further stratification according to resistance mechanisms might have proved informative. However, in reality, characterization of resistance in mosquitoes is still primarily performed by bioassays alone and the relevant contributions of different resistance mechanisms to the phenotype remain unknown. An exception to this is seen in *An funestus*, where pyrethroid resistance is almost entirely due to elevated P450 activity (Churcher 2016). Unfortunately, only one data set from experimental hut trials conducted where *An funestus* was the primary vector was made available to us at the time of this review.

Other examples of missing data that may have influenced study results include the absence of data on resistance status in some settings. Three experimental hut trials did not measure resistance at the time of the trial (Moore 2016; N'Guessan 2010; Pennetier 2013). For two of these trials, we used proxies for resistance; however, no proxy data were available for *An funestus* in Moore 2016, and hence we did not include this population in the stratified analysis. Three trials did not share their data with the review authors; these included trials on nets from two of the more recent manufacturers to produce pyrethroid-PBO nets (N'Guessan 2016; Tungu 2017), which precluded stratified analysis for these net types. For clinical trials, both species composition and resistance level may vary between clusters and/or over the duration of the trial (e.g. the Uganda trial - Staedke 2020 - involved 104 clusters across the country as part of the national LLIN campaign). The population was classified as highly pyrethroid resistant based on data provided by the study authors (WHO tube bioassay conducted in Banangaizi East: deltamethrin 0.05%, 20.7% mosquito mortality, n = 163), but the resistance phenotype of the vector population is likely to vary considerably between clusters.

One key finding of this trial was the decline in performance of pyrethroid-PBO nets after washing. However, as discussed above, it is not clear how the standardized washing protocol employed in experimental hut trials of LLINs reflects the actual chemical retention of active ingredients under operational use. It is encouraging to note that the impact of pyrethroid-PBO nets in reducing parasite prevalence was sustained over two years, hence the policy implications of the loss in bio efficacy after washing remain to be determined.

Agreements and disagreements with other studies or reviews

This is an update of the first Cochrane Review of pyrethroid-PBO nets (Gleave 2018). An earlier meta-analysis of experimental hut data indicated that pyrethroid-PBO nets would have the greatest impact against mosquito populations with intermediate levels of resistance (Churcher 2016). Using transmission models to convert entomological outputs into estimates of public health benefit, the authors noted that the impact of pyrethroid-PBO nets would vary depending on mosquito species, resistance levels, parasite prevalence, and LLIN usage. The importance of taking these key parameters into account when predicting the public health impact of a switch to pyrethroid-PBO nets has been somewhat lost in policy documents and operational guidelines, which seek to provide a simple decision rule to aid net selection. Hence, in the WHO report from the 2017 Evidence Review Group on 'Conditions for deployment of mosquito nets treated with pyrethroid and piperonyl butoxide', it is recommended that "National malaria control programmes and their partners should consider deployment of pyrethroid-PBO nets in areas where pyrethroid resistance has been confirmed in the main malaria vectors" (WHO 2017). In technical guidelines from one of the major net distributors, the PMI, the conditions for deployment of PBO nets include "moderate levels of pyrethroid resistance (defined as 35% to 80% mortality), evidence that PBO restores pyrethroid susceptibility, and moderate to high malaria prevalence" (PMI 2018). The PMI definition of moderate resistance overlaps with our definitions of moderate and low resistance. However in our review, the best evidence for superior efficacy of pyrethroid-PBO nets is derived from areas with high resistance (< 30% mortality), and very little evidence suggests improved performance in areas with moderate or low levels of resistance. The differences between these trials may have arisen from incorporation of a large data set of laboratory bioassays comparing mosquito mortality with or without pre-exposure to PBO in the modelling study. These laboratory bioassays rely on use of a single discriminating dose and identified multiple trials where highly resistant populations were not impacted by PBO. In the current review, the mosquito populations included were limited to sites in which experimental hut trials had been conducted, and this may not have fully captured the full diversity of resistance mechanisms in *Anopheles* mosquitoes. This again highlights the importance of further trials on the influence of resistance mechanisms on the impact of pyrethroid-PBO LLINs.

AUTHORS' CONCLUSIONS

Implications for practice

The findings of this review support the recent WHO policy recommendation that pyrethroid-piperonyl butoxide (PBO) nets should be considered for deployment in areas where pyrethroid resistance has been confirmed in the main malaria vectors (WHO-GMP 2017a). It is encouraging to note that both randomized controlled trials (RCTs) of pyrethroid-PBO nets found that the superior protective efficacy of Olyset Plus compared to that of standard Olyset nets was maintained at 21/25 months of use; the Tanzania trial has been extended further to establish whether this effect lasts the full duration of an LLIN's intended 36-month life span, but results are not yet publicly available. The WHO has declared Olyset Plus as first-in-class for pyrethroid-PBO nets; as a result, pyrethroid-PBO nets from other manufacturers will not

be required to generate epidemiological evidence showing their efficacy.

When evaluating these trials, it is important to remember that the PBO is an additive to the nets that is intended to increase their efficacy against pyrethroid-resistant mosquito populations. No evidence suggests that pyrethroid-PBO nets are less effective than standard LLINs for inducing mosquito mortality in any setting. For personal protection, blood-feeding rates are similarly decreased under all resistance scenarios when unwashed PBO nets are used, although this has not been shown for washed nets in low-resistance or susceptible areas (low-certainty evidence). Hence if pyrethroid-PBO nets perform as well as, or better than, standard LLINs, the decision on whether to switch to nets incorporating the synergist is largely a question of economics. With fixed budgets, there is a risk that the target of universal coverage of LLINs may be more difficult to reach if more expensive pyrethroid-PBO nets are deployed. Indeed, the WHO clearly states that countries should consider deploying pyrethroid-PBO nets only in situations where coverage with standard vector-control interventions is not reduced (WHO-GMP 2017c). Trials of the cost-effectiveness of pyrethroid-PBO nets have not yet been possible due to uncertainties over the price differential between pyrethroid-PBO nets and LLINs.

Implications for research

Experimental hut trials simultaneously comparing different pyrethroid-PBO nets in areas where mosquitoes have high levels of pyrethroid resistance are needed to demonstrate equivalency and to inform procurement decisions, particularly given the very different approaches used to incorporate PBO into LLINs employed by different manufacturers. The issue of durability of bioactive levels of the synergist on the nets also needs further study; current WHO protocols for measuring LLIN durability will need to be adjusted to utilize pyrethroid-resistant colonies of mosquitoes, so that the impact of PBO, and not just of the insecticide, can be measured over the net's intended life span. The issue of the value of entomological endpoints in estimating the public health value of new types of nets remains contentious (Killeen 2018; WHO-GMP 2017c). Performing experimental hut trials alongside future randomized controlled trials of nets containing synergists, or other novel active ingredients, would help resolve this issue.

In relation to reporting trial results, study authors need to record the level of resistance in the local mosquito population at the time of the trial and should include this when reporting the results. Data on resistance mechanisms would also be of value toward a improved understanding of how this influences the performance of pyrethroid-PBO nets.

Limitations of this review

One of the problems in this research field is that pyrethroid-PBO nets are commercial products. The pyrethroid-PBO nets currently undergoing RCTs have had additional alterations made to them, such as changing the concentration or rate at which the pyrethroid is released. However, these are the products for which policy decisions are needed that are based on evidence related to their relative effectiveness. Thus, in this Cochrane Review, we examined the evidence concerning the effectiveness of commercial products. During these comparisons, we considered other potential confounding factors.

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REFERENCES

References to studies included in this review

Awolola 2014 *{published data only}*

Awolola ST, Adeogun AO, Olojede JB, Oduola AO, Oyewole IO, Amajoh CN. Impact of PermaNet 3.0 on entomological indices in an area of pyrethroid resistant Anopheles gambiae in south-western Nigeria. *Parasites & Vectors* 2014;**7**:236. [DOI: 10.1186/1756-3305-7-236]

Bayili 2017 *{published data only (unpublished sought but not used)}*

Bayili K. Phase II field evaluation of long-lasting nets DawaPlus 3.0 (deltamethrin and PBO in roof panel; deltamethrin alone in the side panels) and DawaPlus 4.0 (deltamethrin and PBO) of Tana Netting against natural populations of Anopheles gambiae s.l in Burkina Faso. Report of the twentieth WHOPES working group meeting, WHO/HQ, Geneva, 20–24 March 2017. http://apps.who.int/iris/bitstream/handle/10665/258921/WHO-HTM-NTD-WHOPES-2017.04-eng.pdf;jsessionid=87560DBD471E349CBB7A99796C513DBC?sequence=1 (accessed 13 September 2017). [WHO/HTM/NTD/WHOPES/2017.04]

Cisse 2017 *{published and unpublished data}*

Cisse MB, Sangare D, Oxborough RM, Dicko A, Dengela D, Sadou A, et al. A village level cluster-randomized entomological evaluation of combination long-lasting insecticidal nets containing pyrethroid plus PBO synergist in Southern Mali. *Malaria Journal* 2017;**16**:477. [DOI: 10.1186/s12936-017-2124-1]

Corbel 2010 *{published data only}*

Corbel V, Chabi J, Dabiré RK, Etang J, Nwane P, Pigeon O, et al. Field efficacy of a new mosaic long-lasting mosquito net (PermaNet 3.0) against pyrethroid-resistant malaria vectors: a multi centre study in Western and Central Africa. *Malaria Journal* 2010;**9**:113. [DOI: 10.1186/1475-2875-9-113]

Koudou 2011 *{published data only}*

Koudou BG, Koffi AA, Malone D, Hemingway J. Efficacy of PermaNet® 2.0 and PermaNet® 3.0 against insecticide-resistant Anopheles gambiae in experimental huts in Côte d'Ivoire. *Malaria Journal* 2011;**10**(1):172. [DOI: 10.1186/1475-2875-10-172]

Menze 2020 *{published and unpublished data}*

Menze B, Kouamo MF, Wondji MJ, Tchapga W, Tchoupo M, Kusimo MO, et al. An experimental hut evaluation of PBO-based and pyrethroid-only nets against the malaria vector *Anopheles funestus* reveals a loss of bed nets efficacy associated with GSTe2 metabolic resistance. *Genes* 2020;**11**(2):143. [DOI: 10.3390/genes11020143]

Moore 2016 *{published and unpublished data}*

Moore S. Field evaluation of an alpha-cypermethrin+PBO long-lasting insecticidal net (Veeralin LN) against natural populations of Anopheles arabiensis in experimental huts, Tanzania [Unpublished report to the WHO Pesticide Evaluation Scheme (WHOPES)]. Report of the nineteenth WHOPES working group meeting: WHO/HQ, Geneva, 8–11 February 2016. http://apps.who.int/iris/bitstream/handle/10665/205588/9789241510400_eng.pdf?sequence=1 (accessed 24 August 2017). [WHO/HTM/NTD/WHOPES/2016.2]

Mzilahowa 2014 *{unpublished data only}*

Mzilahowa T, Luka M, Chiumia M, Gimnig J. Efficacy of the PermaNet 3.0 and the Olyset Plus against pyrethroid resistant An funestus and An arabiensis (as supplied 14 March 2015). Data on file (received 14 March 2015).

N'Guessan 2010 *{published data only}*

N'Guessan R, Asidi A, Boko P, Odjo A, Akogbeto M, Pigeon O, et al. An experimental hut evaluation of PermaNet(®) 3.0, a deltamethrin-piperonyl butoxide combination net, against pyrethroid-resistant Anopheles gambiae and Culex quinquefasciatus mosquitoes in southern Benin. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 2010;**104**(12):758-65. [DOI: 10.1016/j.trstmh.2010.08.008]

Oumbouke 2019 *{published data only}*

Oumbouke WA, Rowland M, Koffi AA, Alou LPA, Camara S, N'Guessan R. Evaluation of an alpha-cypermethrin + PBO mixture long-lasting insecticidal net VEERALIN® LN against pyrethroid resistant Anopheles gambiae s.s.: an experimental hut trial in M'bé, central Côte d'Ivoire. *Parasites & Vectors* 2019;**12**(1):544. [DOI: 10.1186/s13071-019-3796-x]

Pennetier 2013 *{published data only}*

Pennetier C, Bouraima A, Chandre F, Piameu M, Etang J, Rossignol M, et al. Efficacy of Olyset(R) Plus, a new long-lasting insecticidal net incorporating permethrin and piperonyl-butoxide against multi-resistant malaria vectors [corrected]. *PLoS One* 2013;**8**(10):e75134. [DOI: 10.1371/journal.pone.0075134]

Protopopoff 2018 *{published data only}*

Protopopoff N, Charlwood D, Mosha J, Wright A, Kisinza W, Mosha F, et al. Evaluation of a novel long lasting insecticidal net to indoor residual spray product, separately to together, against malaria transmitted by pyrethroid resistant mosquitoes in northwest Tanzania: a cluster randomized controlled trial. *Lancet* 2018;**391**(10130):1577-88. [DOI: 10.1016/S0140-6736(18)30427-6]

Staedke 2020 *{published and unpublished data}*

ISRCTN17516395. Impact of long-lasting insecticide treated bednets with and without piperonyl butoxide (PBO) on malaria indicators in Uganda. www.isrctn.com/ISRCTN17516395 (accessed 24 August 2018).

* Staedke SG, Gonahasa S, Dorsey G, Kamya MR, Maiteki-Sebuguzi C, Lynd A, et al. Effect of long-lasting insecticidal nets with and without piperonyl butoxide on malaria indicators in Uganda (LLINEUP): a pragmatic, cluster-randomised trial embedded in a national LLIN distribution campaign. *Lancet* 2020;**395**(10232):1292-303. [DOI: 10.1016/S0140-6736(20)30214-2]

Stiles-Ocran 2013 *{unpublished data only}*

Stiles-Ocran J. Field evaluation of PermaNet® 3.0 in controlling pyrethroid resistant Anopheles gambiae in the Chirano Area, Western Region, Ghana (as supplied 12 March 2015). Data on file 2013.

Toé 2018 *{published and unpublished data}*

Toé KH, Müller P, Badolo A, Traore A, Sagnon N, Dabiré RK, et al. Do bednets including piperonyl butoxide offer additional protection against populations of Anopheles gambiae s.l. that are highly resistant to pyrethroids? An experimental hut evaluation in Burkina Faso. *Medical and Veterinary Entomology* 2018;**32**(4):407-16. [DOI: 10.1111/mve.12316]

Tungu 2010 *{published data only}***10.1186/1475-2875-9-21**

Tungu P, Magesa S, Maxwell C, Malima R, Masue D, Sudi W, et al. Evaluation of PermaNet 3.0 a deltamethrin-PBO combination net against Anopheles gambiae and pyrethroid resistant Culex quinquefasciatus mosquitoes: an experimental hut trial in Tanzania. *Malaria Journal* 2010;**9**:21. [DOI: 10.1186/1475-2875-9-21]

References to studies excluded from this review

Darriet 2011 *{published data only}*

Darriet F, Chandre F. Combining piperonyl butoxide and dinotefuran restores the efficacy of deltamethrin mosquito nets against resistant Anopheles gambiae (Diptera: Culicidae). *Journal of Medical Entomology* 2011;**48**(4):952-5. [DOI: 10.1603/ME11022]

Darriet 2013 *{published data only}*

Darriet F, Chandre F. Efficacy of six neonicotinoid insecticides alone and in combination with deltamethrin and piperonyl butoxide against pyrethroid-resistant Aedes aegypti and Anopheles gambiae (Diptera: Culicidae). *Pest Management Science* 2013;**69**(8):905-10. [DOI: 10.1002/ps.3446]

References to studies awaiting assessment

Koudou 2012 *{unpublished data only}*

Koudou B, Malone D. Does PermaNet® 3.0 protect against pyrethroid resistant mosquitoes? Manuscript in preparation for Acta Tropica (received 14 December 2012).

Shono 2017 *{published data only}*

Shono Y, Ohashi K, Lucas JR. Biological performance of Olyset® Plus, a long-lasting mosquito net incorporating a mixture of a pyrethroid and synergist. *Acta Horticulturae* 2017;**1169**:77-81.

Tungu 2017 *{published data only (unpublished sought but not used)}*

Tungu P. Phase II study to evaluate the efficacy and wash resistance of DawaPlus 3.0 and DawaPlus 4.0 against natural populations of Anopheles funestus s.s. in experimental huts in Muheza, Tanzania. Report of the twentieth WHOPES working group meeting, WHO/HQ, Geneva, 20–24 March 2017. http://apps.who.int/iris/bitstream/handle/10665/258921/WHO-HTM-NTD-WHOPES-2017.04-eng.pdf;jsessionid=87560DBD471E349CBB7A99796C513DBC?sequence=1 (accessed 13 September 2017). [WHO/HTM/NTD/WHOPES/2017.04]

References to ongoing studies

ISRCTN99611164 *{unpublished data only}*

ISRCTN99611164. Comparative evaluation of standard and dual-treated insecticide bednets in Sud Ubangi, Democratic Republic of Congo. https://doi.org/10.1186/ISRCTN99611164 (accessed prior to 1 May 2021).

NCT03289663 *{unpublished data only}*

NCT03289663. Effectiveness study of bednets treated with synergistic combination of insecticides in an area with pyrethroid-resistant vectors in the Democratic Republic of the Congo [Effectiveness study of new generation bednets in the context of conventional insecticide resistance in the Democratic Republic of Congo]. clinicaltrials.gov/show/NCT03289663 (first received 24 August 2018).

NCT04182126 *{unpublished data only}*

NCT04182126. Environmental modifications in sub-Saharan Africa: changing epidemiology, transmission and pathogenesis of Plasmodium falciparum and P. vivax malaria [HS#2017-3512, adaptive interventions for optimizing malaria control: a cluster-randomized SMART trial]. clinicaltrials.gov/ct2/show/NCT04182126 (first received 2 December 2019).

UMIN000019971 *{unpublished data only}*

Minakawa N, Kongere JO, Sonye GO, Lutiali PA, Awuor B, Kawada H, et al. A preliminary study on designing a cluster randomized control trial of two new mosquito nets to prevent malaria parasite infection. *Tropical Medicine and Health* 2020;**48**(1):98. [DOI: 10.1186/s41182-020-00276-x]

UMIN000019971. Effects of long lasting insecticidal nets incorporating piperonyl butoxidae on malaria transmission among children: a cluster randomized controlled field trial. https://upload.umin.ac.jp/cgi-open-bin/ctr_e/ctr_view.cgi?recptno=R000023020 (first posted 25 December 2015).

Additional references

Abílio 2015

Abílio AP, Marrune P, de Deus N, Mbofana F, Muianga P, Kampango A. Bio-efficacy of new long-lasting insecticide-treated bed nets against Anopheles funestus and Anopheles gambiae from central and northern Mozambique. *Malaria Journal* 2015;**14**:352. [DOI: 10.1186/s12936-015-0885-y]

Adeogun 2012

Adeogun AO, Olojede JB, Oduola AO, Awolola TS. Efficacy of a combination long lasting insecticidal net (PermaNet® 3.0) against pyrethroid resistant Anopheles gambiae s.s. and Culex quinquefasciatus: an experimental hut trial in Nigeria. *Nigerian Journal of Clinical & Biomedical Research* 2012;**6**:37-50.

Aïzoun 2013

Aïzoun N, Ossè R, Azondekon R, Alia R, Oussou O, Gnanguenon V, et al. Comparison of the standard WHO

susceptibility tests and the CDC bottle bioassay for the determination of insecticide susceptibility in malaria vectors and their correlation with biochemical and molecular biology assays in Benin, West Africa. *Parasites & Vectors* 2013;**6**:147.

Bhatt 2015

Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U, et al. The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. *Nature* 2015;**526**(7572):207-11.

Bobanga 2013

Bobanga T, Ayieko W, Zanga M, Umesumbu S, Landela A, Fataki O, et al. Field efficacy and acceptability of PermaNet® 3.0 and OlysetNet® in Kinshasa, Democratic Republic of the Congo. *Journal of Vector Borne Diseases* 2013;**50**(3):206-14.

Churcher 2016

Churcher TS, Lissenden N, Griffin JT, Worrall E, Ranson H. The impact of pyrethroid resistance on the efficacy and effectiveness of bednets for malaria control in Africa. *eLife* 2016;**5**:e16090. [DOI: http://dx.doi.org/10.7554/eLife.16090]

Deeks 2017

Deeks JJ, Higgins JP, Altman DG (editors), on behalf of the Cochrane Statistical Methods Group. Chapter 9. Analysing data and undertaking meta-analyses. In: Higgins JP, Churchill R, Chandler J, Cumpston MS (editors). Cochrane Handbook for Systematic Reviews of Interventions version 5.2.0 (updated June 2017), Cochrane, 2017. Available from www.training.cochrane.org/handbook.

Djègbè 2011

Djègbè I, Boussari O, Sidick A, Martin T, Ranson H, Chandre F, et al. Dynamics of insecticide resistance in malaria vectors in Benin: first evidence of the presence of L1014S kdr mutation in Anopheles gambiae from West Africa. *Malaria Journal* 2011;**10**:261.

GRADEpro GDT 2015 [Computer program]

McMaster University (developed by Evidence Prime) GRADEpro GDT. Version accessed 5 May 2017. Hamilton (ON): McMaster University (developed by Evidence Prime), 2015. Available at gradepro.org.

Hawley 2003

Hawley WA, Phillips-Howard PA, ter Kuile FO, Terlouw DJ, Vulule JM, Ombok M, et al. Community-wide effects of permethrin-treated bed nets on child mortality and malaria morbidity in western Kenya. *American Journal of Tropical Medicine & Hygiene* 2003;**68**(4 Suppl):121-7. [DOI: 10.4269/ajtmh.2003.68.121]

Higgins 2003

Higgins JP, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. *BMJ* 2003;**327**:557-60.

Higgins 2011

Higgins JP, Deeks JJ, Altman DG, Cochrane Statistical Methods Group. Chapter 16. Special topics in statistics. In: Higgins JP, Green S, editor(s). Cochrane Handbook for Systematic Reviews of Interventions version 5.1.0 (updated March 2011), The Cochrane Collaboration, 2011. Available from handbook.cochrane.org.

Higgins 2017

Higgins JP, Altman DG, Sterne JA (editors). Chapter 8. Assessing risk of bias in included studies. In: Higgins JP, Churchill R, Chandler J, Cumpston MS (editors). Cochrane Handbook for Systematic Reviews of Interventions version 5.2.0 (updated June 2017), Cochrane, 2017. Available from www.training.cochrane.org/handbook.

Killeen 2018

Killeen G, Ranson H. Insecticide-resistant malaria vectors must be tackled. *Lancet* 2018;**391**(10130):1551-2.

Kleinschmidt 2018

Kleinschmidt I, Bradley J, Knox TB, Mnzava AP, Kafy HT, Mbogo C, et al. Implications of insecticide resistance for malaria vector control with long-lasting insecticidal nets: a WHO-coordinated, prospective, international, observational cohort study. *Lancet* 2018;**18**:20172-5. [DOI: 10.1016/S1473-3099(18)30172-5]

Lengeler 2004

Lengeler C. Insecticide-treated bed nets and curtains for preventing malaria. *Cochrane Database of Systematic Reviews* 2004, Issue 2. Art. No: CD000363. [DOI: 10.1002/14651858.CD000363.pub2]

Maxwell 2002

Maxwell CA, Msuya E, Sudi M, Njunwa KJ, Carneiro IA, Curtis CF. Effect of community-wide use of insecticide-treated nets for 3-4 years on malarial morbidity in Tanzania. *Tropical Medicine & International Health* 2002;**7**(12):1003-8. [DOI: 10.1046/j.1365-3156.2002.00966]

Mitchell 2012

Mitchell SN, Stevenson BJ, Müller P, Wilding CS, Egyir-Yawson A, Field SG, et al. Identification and validation of a gene causing cross-resistance between insecticide classes in Anopheles gambiae from Ghana. *Proceedings of the Nationall Academy of Sciences of the United States of America* 2012;**109**(16):6147-52. [DOI: 10.1073/pnas.1203452109]

MPAC 2016

Malaria Policy Advisory Committee. WHO Malaria Policy Advisory Committee (MPAC) meeting report (September 2016). www.who.int/malaria/publications/atoz/mpac-september2016-report.pdf?ua=1 (accessed 1 May 2017).

N'Guessan 2007

N'Guessan R, Corbel V, Akogbéto M, Rowland M. Reduced efficacy of insecticide-treated nets and indoor residual spraying for malaria control in pyrethroid resistance area, Benin. *Emerging Infectious Diseases* 2007;**13**(2):199-206. [DOI: 10.3201/eid1302.060631]

Parker 2015

Parker J, Angarita-Jaimes N, Abe M, Towers CE, Towers D, McCall P. Infrared video tracking of Anopheles gambiae at

insecticide-treated bed nets reveals rapid decisive impact after brief localised net contact. *Scientific Reports* 2015;**5**:e13392.

PMI 2018

PMI. U.S. President's Malaria Initiative technical guidance, 2018. www.pmi.gov/docs/default-source/default-document-library/tools-curricula/pmi-technical-guidance-(february-2017).pdf?sfvrsn=16.

Ranson 2011

Ranson H, N'Guessan R, Lines J, Moiroux N, Nkuni Z, Corbel V. Pyrethroid resistance in African Anopheline mosquitoes: what are the implications for malaria control? *Trends in Parasitology* 2011;**27**(2):91-8. [DOI: 10.1016/j.pt.2010.08.004]

Ranson 2016

Ranson H, Lissenden N. Insecticide resistance in African Anopheles mosquitoes: a worsening situation that needs urgent action to maintain malaria control. *Trends in Parasitology* 2016;**32**(3):187-96. [DOI: 10.1016/j.pt.2015.11.010]

Review Manager 2014 [Computer program]

Nordic Cochrane Centre, The Cochrane Collaboration Review Manager 5 (RevMan 5). Version 5.3. Copenhagen: Nordic Cochrane Centre, The Cochrane Collaboration, 2014.

Ridl 2008

Ridl FC, Bass C, Torrez M, Govender D, Ramdeen V, Yellot L, et al. A pre-intervention study of malaria vector abundance in Rio Muni, Equatorial Guinea: their role in malaria transmission and the incidence of insecticide resistance alleles. *Malaria Journal* 2008;**7**:194. [DOI: 10.1186/1475-2875-7-194]

Riveron 2015

Riveron JM, Chiumia M, Menze BD, Barnes KG, Irving H, Ibrahim SS, et al. Rise of multiple insecticide resistance in Anopheles funestus in Malawi: a major concern for malaria vector control. *Malaria Journal* 2015;**14**:344. [DOI: 10.1186/s12936-015-0877-y]

Schünemann 2013

Schünemann H, Brożek J, Guyatt G, Oxman A, editor(s). Handbook for grading the quality of evidence and the strength of recommendations using the GRADE approach (updated October 2013). GRADE Working Group, 2013. Available from gdt.guidelinedevelopment.org/app/handbook/handbook.html.

Stevenson 2011

Stevenson BJ, Bibby J, Pignatelli P, Muangnoicharoen S, O'Neill PM, Lian LY, et al. Cytochrome P450 6M2 from the malaria vector Anopheles gambiae metabolizes pyrethroids: sequential metabolism of deltamethrin revealed. *Insect Biochemistry and Molecular Biology* 2011;**41**(7):492-502. [DOI: 10.1016/j.ibmb.2011.02.003]

Strode 2014

Strode C, Donegan S, Garner P, Enayati AA, Hemingway J. The impact of pyrethroid resistance on the efficacy of insecticide-treated bed nets against African Anopheline mosquitoes: systematic review and meta-analysis. *PLoS Medicine* 2014;**11**(3):e1001619. [DOI: 10.1371/journal.pmed.1001619]

Sumitomo 2013

Sumitomo Chemical Co Ltd. Olyset Plus Technical Brochure 2013. sumivector.com/sites/default/files/site-content/pdf/Olyset_Plus_Technical_Brochure_Jan_2013_ENG.pdf (accessed 1 May 2017).

Vestergaard 2015

Vestergaard. Technical basis for deployment of PermaNet® 3.0 in areas with pyrethroid-resistant malaria vectors. PermaNet® 3.0 Technical Brochure. January 2015. www.vestergaard.com/images/pdf/PN3_Tech_Eng_2015.pdf (accessed 1 May 2017).

WHO 2013

World Health Organization Pesticide Evaluation Scheme. Guidelines for laboratory and field testing of long-lasting insecticidal nets. apps.who.int/iris/bitstream/10665/80270/1/9789241505277_eng.pdf (accessed 24 August 2018).

WHO 2016

World Health Organization. Test Procedures for Insecticide Resistance Monitoring in Malaria Vector Mosquitoes. Second edition. Geneva: WHO, 2016.

WHO 2017

World Health Organization/Department of Control of Neglected Tropical Diseases. Design of epidemiological trials for vector control products, Report of a WHO Expert Advisory Group; Château de Penthes, Geneva, 24–25 April 2017. www.who.int/neglected_diseases/vector_ecology/resources/WHO_HTM_NTD_VEM_2017.04/en/ (accessed 11 July 2017).

WHO 2019a

World Health Organization. World malaria report 2019. www.who.int/malaria/publications/world_malaria_report/en/.

WHO 2019b

World Health Organization. Guidelines for malaria vector control. https://apps.who.int/iris/bitstream/handle/10665/310862/9789241550499-eng.pdf?ua=1 (accessed prior to 13 May 2021).

WHO-GMP 2015

World Health Organization Global Malaria Programme. Conditions for use of long-lasting insecticidal nets treated with a pyrethroid and piperonyl butoxide. www.who.int/malaria/areas/vector_control/use-of-pbo-treated-llins-report-nov2015.pdf (accessed 24 August 2018).

WHO-GMP 2017a

World Health Organization. Conditions for deployment of mosquito nets treated with a pyrethroid and piperonyl butoxide. apps.who.int/iris/bitstream/handle/10665/258939/WHO-HTM-GMP-2017.17-eng.pdf?sequence=1 (accessed 24 August 2018).

WHO-GMP 2017b

World Health Organization. The evaluation process for vector control products. www.who.int/malaria/publications/atoz/evaluation-process-vector-control-products/en/ (accessed 24 August 2018).

WHO-GMP 2017c

World Health Organization Global Malaria Programme. Malaria vector control policy recommendations and their applicability to product evaluation. www.who.int/malaria/publications/atoz/vector-control-recommendations/en/ (accessed 24 August 2018).

WHOPES 2016

WHO/Department of Control of Neglected Tropical Diseases. Report of the nineteenth WHOPES working group meeting: WHO/HQ, Geneva, 8–11 February 2016. Review of Veeralin LN, VectoMax GR, Bactivec SC. apps.who.int/iris/bitstream/10665/205588/1/9789241510400_eng.pdf (accessed 1 May 2017).

Yewhalaw 2012

Yewhalaw D, Asale A, Tushune K, Getachew Y, Duchateau L, Speybroeck N. Bio-efficacy of selected long-lasting insecticidal nets against pyrethroid resistant Anopheles arabiensis from South-Western Ethiopia. *Parasites & Vectors* 2012;**5**:159. [DOI: 10.1186/1756-3305-5-159]

Zaim 2000

Zaim M, Aitio A, Nakashima N. Safety of pyrethroid-treated mosquito nets. *Medical and Veterinary Entomology* 2000;**14**(1):1-5.

References to other published versions of this review

Gleave 2017

Gleave K, Lissenden N, Richardson M, Ranson H. Piperonyl butoxide (PBO) combined with pyrethroids in long-lasting insecticidal nets (LLINs) to prevent malaria in Africa. *Cochrane Database of Systematic Reviews* 2017, Issue 8. Art. No: CD012776. [DOI: 10.1002/14651858.CD012776]

Gleave 2018

Gleave K, Lissenden N, Richardson M, Choi L, Ranson H. Piperonyl butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa. *Cochrane Database of Systematic Reviews* 2018, Issue 11. Art. No: CD012776. [DOI: 10.1002/14651858.CD012776.pub2]

* Indicates the major publication for the study

CHARACTERISTICS OF STUDIES

Characteristics of included studies [author-defined order]

Staedke 2020

Study characteristics	
Methods	Cluster-randomized controlled village trial
Participants	Households with at least 1 adult resident and 1 child aged 2 to 10 years, *Anopheles* species
Interventions	Control: LLIN, PermaNet 2.0 Intervention: LLIN, PermaNet 3.0 Control: LLIN, Olyset Net Intervention: LLIN, Olyset Plus
Outcomes	Primary outcomes; parasite prevalence (proportion of thick blood smears that are positive for asexual parasites) in children ages 2 to 10 years, assessed before net distribution and 3 times after nets are distributed Secondary outcomes: prevalence of anaemia; mean haemoglobin in children ages 2 to 10 years; vector density; measures of LLIN ownership; coverage, use, and integrity
Mosquito resistance status	Resistance - high
Net treatment	Nets unholed and unwashed
Location(s)	Uganda - East and West, 104 sub-districts
Notes	
Risk of bias	

Staedke 2020 *(Continued)*

Bias	Authors' judgement	Support for judgement
Recruitment bias	Low risk	No participants were recruited after clusters had been randomized
Were the mosquitoes in LLIN and LLIN + PBO groups comparable	Unclear risk	Resistance monitoring was not conducted at all study sites due to the size of the RCT
Collectors blinded	High risk	LLIN allocation was not masked; therefore risk of detection bias was high for entomological outcomes
Household blinded	Low risk	LLIN allocation was not masked, but this is unlikely to affect the primary outcome (parasite prevalence)
Treatment allocation (sequence randomly/adequately generated)	Low risk	Randomization was used to allocate clusters to study groups
Allocation concealment (selection bias)	Low risk	Randomization was carried out to allocate treatments to clusters
Were incomplete outcome data adequately addressed	Low risk	No outcome data were incomplete; intention-to-treat analysis was conducted
Were the raw data reported for LLIN and LLIN + PBO groups	Low risk	No outcome data were missing
Clusters lost to follow-up	Unclear risk	14 clusters were lost to follow-up in the final time point (25 months) due to the COVID-19 pandemic
Selective reporting (reporting bias)	Low risk	All intended outcomes stated in the pre-published protocol were reported in the final publication
Correct statistical methods; adjusted for clustering	Low risk	Clustering was not taken into account and adjusted for during statistical analysis. Trial authors did however provide us with an ICC, so we could adjust for clustering
Trial authors' conflicting interest	Low risk	Trial authors declared no conflicting interests

Awolola 2014

Study characteristics	
Methods	Village trial
Participants	Ilara - *An gambiae* (100% S-form) Irolu - 95% *An gambiae* (100% S-form), 4.5% *An arabiensis* Ijesa - 98.1% *An gambiae* (80% S-form, 19% M-form), 1.6% *An arabiensis*
Interventions	Control: LLIN, PermaNet 2.0 Intervention: LLIN, PermaNet 3.0

Awolola 2014 *(Continued)*

Outcomes	Mosquito mortality, blood feeding, sporozoite rate, mosquito density, parity rate
Mosquito resistance status	Ilara - resistant - low (deltamethrin, 72.5% mortality, N = 120) Irolu - resistant - low (deltamethrin, 62.5% mortality, N = 120) Ijesa - resistant - low (deltamethrin, 66.7% mortality, N = 120)
Net treatment	Nets unholed and unwashed
Location(s)	Ilara, Nigeria - untreated net Irolu, Nigeria - PermaNet 2.0 Ijesa, Nigera - PermaNet 3.0
Notes	Trial conducted: March 2012 to March 2013

Risk of bias

Bias	Authors' judgement	Support for judgement
Recruitment bias	Low risk	Recruiment bias is related to human participants and so is not applicable to this study
Were the mosquitoes in LLIN and LLIN + PBO groups comparable	Unclear risk	Mosquito species composition varied slightly pre-trial and post-trial between treatment villages. However, resistance level was the same
Collectors blinded	High risk	Not stated whether collectors where blinded; therefore judged as high risk, as this is likely to impact searching efforts
Household blinded	Low risk	Unclear whether households were blinded – not stated in the publication. We judged this as low risk, as it is unlikely to affect the outcome
Treatment allocation (sequence randomly/adequately generated)	Low risk	Villages were randomly assigned to treatment arms
Allocation concealment (selection bias)	Low risk	Allocation concealment procedures were not adhered to; however this is unlikely to affect the results
Were incomplete outcome data adequately addressed	Low risk	There were no incomplete data
Were the raw data reported for LLIN and LLIN + PBO groups	Low risk	All necessary data were reported
Clusters lost to follow-up	Low risk	No clusters were lost to follow-up
Selective reporting (reporting bias)	Low risk	It appears that all measured outcomes were reported
Correct statistical methods; adjusted for clustering	High risk	Study did not take clustering into account for statistical methods

Awolola 2014 *(Continued)*

Trial authors' conflicting interest	Low risk	Trial authors declared no conflicting interests; however the study was funded by Vestergaard (net manufacturers). Views and findings in the publication are stated to be those of the trial authors

Bayili 2017

Study characteristics		
Methods	Experimental hut trial	
Participants	*An coluzzii*	
Interventions	Control: LLIN, DawaPlus 2.0 Intervention: LLIN, DawaPlus 3.0, DawaPlus 4.0	
Outcomes	Mosquito mortality, blood feeding, deterrence, exophily	
Mosquito resistance status	Resistant - high (6% mortality, N = 98)	
Net treatment	Nets holed, nets unwashed and washed (x 20)	
Location(s)	Vallée du Kou, Burkina Faso	
Notes	Trial conducted: August 2016 to October 2016	
Risk of bias		
Bias	**Authors' judgement**	**Support for judgement**
Were the mosquitoes in LLIN and LLIN + PBO groups comparable	Low risk	The hut trial was conducted in the same area; therefore characteristics are similar
Collectors blinded	Unclear risk	Paper does not state whether collectors were blinded
Sleepers blinded	Unclear risk	Paper does not state whether sleepers were blinded
Sleeper bias	Low risk	Sleepers were rotated between huts according to a Latin square design
Treatment allocation (sequence randomly/adequately generated)	Low risk	Treatments were not randomly allocated to huts; however the trial completed a full rotation through the huts
Treatment rotation	Low risk	Treatments were rotated between huts according to a Latin square design + 2 weeks
Standardized hut design	Low risk	Huts were built previously according to standard West African design
Hut cleaning between treatments	Unclear risk	Trial authors do not state whether huts were cleaned between treatments
Were incomplete outcome data adequately addressed	Low risk	No data were incomplete

Bayili 2017 *(Continued)*

Were the raw data reported for LLIN and LLIN + PBO groups	Low risk	All necessary data were reported
Trial authors' conflicting interest	Low risk	Trial authors declare no conflicting interest in the WHOPES report

Cisse 2017

Study characteristics	
Methods	Village trial
Participants	*An gambiae* s.s.
Interventions	Control: LLIN, Olyset Net, PermaNet 2.0 Intervention: LLIN, Olyset Plus, PermaNet 3.0
Outcomes	Sporozoite rate, mosquito density, parity rate
Mosquito resistance status	Olyset Net villages - resistance - high (1% mortality, N = 305) Olyset Plus villages - resistance - high (2% mortality, N = 411) PermaNet 2.0 villages - resistance - high (29% mortality, N = 410) PermaNet 3.0 villages - resistance - moderate (38% mortality, N = 408)
Net treatment	Nets unholed and unwashed
Location(s)	Sikasso region, Mali PermaNet 2.0 villages - Beko East, Dalabani, Berila, Dierila PermaNet 3.0 villages - Beko West, Farabacoura East, Kola Djokada, Tieblembougou Olyset Net villages - Karako, Geleba 2, Toula East, Toula West Olyset Plus villages - Dialake, Farabacoura West, Deneklin, Faradjele
Notes	Trial conducted: January 2014 to January 2015
Risk of bias	

Bias	**Authors' judgement**	**Support for judgement**
Recruitment bias	Low risk	Recruiment bias is related to human participants and so is not applicable to this study
Were the mosquitoes in LLIN and LLIN + PBO groups comparable	Unclear risk	Mosquito species composition is constant between villages; however resistance level varies slightly
Collectors blinded	High risk	Not stated whether collectors where blinded; therefore judged as high risk, as this is likely to affect searching efforts
Household blinded	Low risk	Unclear whether households were blinded – not stated in the publication. We judged this as low risk, as this is unlikely to affect the outcome

Cisse 2017 *(Continued)*

Treatment allocation (sequence randomly/adequately generated)	Low risk	Villages were randomly assigned to treatment arms
Allocation concealment (selection bias)	Low risk	Allocation concealment procedures were not adhered to; however this is unlikely to affect study results
Were incomplete outcome data adequately addressed	Low risk	No data were incomplete
Were the raw data reported for LLIN and LLIN + PBO groups	Low risk	All necessary data were reported
Clusters lost to follow-up	Low risk	No clusters were lost to follow-up
Selective reporting (reporting bias)	Low risk	It appears that all measured outcomes were reported
Correct statistical methods; adjusted for clustering	High risk	Study did not take clustering into account for statistical methods
Trial authors' conflicting interest	Low risk	Trial authors have no competing interests

Corbel 2010

Study characteristics	
Methods	Experimental hut trial
Participants	Vallée du Kou, Burkina Faso - 100% *An gambiae*: M-form (15%), S-form (85%) Malanville, Benin - 95% *An gambiae*: M-form (100%), 5% *An arabiensis* Pitoa, Cameroon - 5% *An gambiae*: S-form (100%), 95% *An arabiensis*
Interventions	Control: LLIN, PermaNet 2.0 Intervention: LLIN, PermaNet 3.0
Outcomes	Mosquito mortality, blood feeding, deterrence, exophily
Mosquito resistance status	Vallée du Kou, Burkina Faso - resistant – high (deltamethrin, 23% mortality, N = 100) Malanville, Benin - resistant – low (deltamethrin, 85% mortality, N = 100) Pitoa, Cameroon - resistant – low (deltamethrin, 70% mortality, N = 100)
Net treatment	Nets holed, nets unwashed and washed (x 20)
Location(s)	Vallée du Kou, Burkina Faso Malanville, Benin

Corbel 2010 *(Continued)*

	Pitoa, Cameroon
Notes	Trial conducted: Vallée du Kou, Burkina Faso - September 2007 to November 2007 Malanville, Benin - July 2008 to September 2008 Pitoa, Cameroon - July 2008 to September 2008

Risk of bias

Bias	Authors' judgement	Support for judgement
Were the mosquitoes in LLIN and LLIN + PBO groups comparable	Low risk	Huts situated in the same area: mosquito characteristics will be the same
Collectors blinded	Unclear risk	Unclear whether collectors were blinded – not stated in the publication
Sleepers blinded	Unclear risk	Unclear whether sleeper was blinded – not stated in the publication
Sleeper bias	Low risk	Sleepers were rotated between huts according to a Latin square design
Treatment allocation (sequence randomly/adequately generated)	Low risk	Treatments were randomly allocated to huts
Treatment rotation	Low risk	Treatments were rotated between huts according to a Latin square design
Standardized hut design	Low risk	Huts were built according to a standard West African design
Hut cleaning between treatments	Unclear risk	Unclear whether huts were cleaned between treatments – not stated in the publication
Were incomplete outcome data adequately addressed	Low risk	No outcome data were incomplete
Were the raw data reported for LLIN and LLIN + PBO groups	Low risk	All necessary data were reported
Trial authors' conflicting interest	Low risk	Trial authors have no competing interests

Koudou 2011

Study characteristics

Methods	Experimental hut trial
Participants	*An gambiae* s.s.
Interventions	Control: LLIN, PermaNet 2.0

Koudou 2011 *(Continued)*

	Intervention: LLIN, PermaNet 3.0
Outcomes	Mosquito mortality, deterrence, exophily
Mosquito resistance status	Resistant - high (deltamethrin, 10.6% mortality, N = 80 min)
Net treatment	Nets not holed, nets unwashed and washed (x 20)
Location(s)	Yaokoffikro, Côte d'Ivoire
Notes	Trial conducted: April 2009 to July 2009

Risk of bias

Bias	Authors' judgement	Support for judgement
Were the mosquitoes in LLIN and LLIN + PBO groups comparable	Low risk	Huts situated in the same area – mosquito characteristics will be the same
Collectors blinded	Unclear risk	Unclear whether collectors were blinded – not stated in the publication
Sleepers blinded	Unclear risk	Unclear whether sleeper was blinded – not stated in the publication
Sleeper bias	Low risk	Sleepers were rotated between huts according to a Latin square design
Treatment allocation (sequence randomly/adequately generated)	Low risk	Treatments were not randomly allocated to the huts However, results from trials performed before this trial show no significant differences in attractiveness of the different huts
Treatment rotation	Low risk	Treatments were rotated between huts according to a Latin square design
Standardized hut design	Low risk	Huts were built according to a standard West African design
Hut cleaning between treatments	Low risk	All huts were cleaned between treatments
Were incomplete outcome data adequately addressed	Low risk	No outcome data were incomplete
Were the raw data reported for LLIN and LLIN + PBO groups	Low risk	All necessary data were reported
Trial authors' conflicting interest	Low risk	Trial authors declared they had no conflicting interests

Moore 2016

Study characteristics	
Methods	Experimental hut trial

Moore 2016 *(Continued)*

Participants	*An arabiensis* (100%), *An funestus* group (95% s.s.)
Interventions	Control: LLIN, MAGNet LN Intervention: LLIN, Veeralin LN
Outcomes	Mosquito mortality, blood feeding, deterrence, exophily
Mosquito resistance status	*An arabiensis* - susceptible (alpha-cypermethrin, 100% mortality, N = 97) *An funestus* - unclassified
Net treatment	Nets holed, nets unwashed and washed (x 20)
Location(s)	Ifakara, Tanzania
Notes	Although additional data provided, they show resistance to deltamethrin and permethrin in *An gambiae s.l.*

Risk of bias

Bias	Authors' judgement	Support for judgement
Were the mosquitoes in LLIN and LLIN + PBO groups comparable	Low risk	The hut trial was conducted in the same area; therefore characteristics are similar
Collectors blinded	Unclear risk	Paper does not state whether collectors were blinded
Sleepers blinded	Unclear risk	Paper does not state whether sleepers were blinded
Sleeper bias	Low risk	Sleepers were rotated between huts according to a Latin square design
Treatment allocation (sequence randomly/adequately generated)	Low risk	Treatments were not randomly allocated to huts; however the trial completed a full rotation through the huts
Treatment rotation	Low risk	Treatments were rotated between huts according to a Latin square design
Standardized hut design	Low risk	Study used the standard design of the Ifakara experimental huts
Hut cleaning between treatments	Unclear risk	The paper does not state whether huts were cleared between treatments
Were incomplete outcome data adequately addressed	Low risk	No outcome data were incomplete
Were the raw data reported for LLIN and LLIN + PBO groups	Low risk	No outcome data were missing
Trial authors' conflicting interest	Low risk	Trial authors declared they received prescribed standard fees from Vestergaard Frandsen for evaluating its pesticide products; however this is standard practice

Mzilahowa 2014

Study characteristics	
Methods	Village trial
Participants	*An gambiae* s. l., *An funestus* group
Interventions	Control: LLIN, Olyset Net, PermaNet 2.0 Intervention: LLIN, Olyset Plus, PermaNet 3.0
Outcomes	Mosquito density, parity rate
Mosquito resistance status	*An funestus* (Balaka district) Permethrin - resistant - moderate (55.5% mortality, N = unknown) Deltamethrin - resistant - high (14.9% mortality, N = unknown) *An gambiae* (Balaka district) Permethrin - resistant - low (84.4% mortality, N = unknown) (Machinga district) Deltamethrin - resistant - moderate (54.5% mortality, N = unknown)
Net treatment	Nets unholed and unwashed
Location(s)	Balaka district, Malawi (12 villages)
Notes	Trial conducted: December 2012 to June 2014

Risk of bias		
Bias	**Authors' judgement**	**Support for judgement**
Recruitment bias	Low risk	Recruiment bias is related to human participants and so is not applicable to this study
Were the mosquitoes in LLIN and LLIN + PBO groups comparable	Unclear risk	Mosquito species composition and resistance status are not recorded per village. Village names are not provided in the study; instead villages are grouped by treatment type
Collectors blinded	High risk	Not stated whether collectors were blinded; therefore judged as high risk, as this is likely to affect searching effort
Household blinded	Low risk	Unclear whether households were blinded – not stated in the publication. We judged this as low risk, as this is unlikely to affect the outcome
Treatment allocation (sequence randomly/adequately generated)	Low risk	Villages were randomly assigned to treatment arms
Allocation concealment (selection bias)	Low risk	Allocation concealment procedures were not adhered to; however this is unlikely to affect the results

Mzilahowa 2014 *(Continued)*

Were incomplete outcome data adequately addressed	Low risk	No outcome data were incomplete
Were the raw data reported for LLIN and LLIN + PBO groups	Low risk	All necessary data were reported
Clusters lost to follow-up	Low risk	No clusters were lost to follow-up
Selective reporting (reporting bias)	Low risk	It appears that all measured outcomes were reported
Correct statistical methods; adjusted for clustering	High risk	Study did not take clustering into account when statistical methods were performed
Trial authors' conflicting interest	Unclear risk	No information on trial authors' possible conflicting interests is provided

N'Guessan 2010

Study characteristics		
Methods	Experimental hut trial	
Participants	*An gambiae*	
Interventions	Control: LLIN, PermaNet 2.0 Intervention: LLIN, PermaNet 3.0	
Outcomes	Mosquito mortality, blood feeding, deterrence, exophily	
Mosquito resistance status	Proxy data. Adjara, Benin: resistant - moderate (deltamethrin, 50% mortality, N = 56) (Aïzoun 2013)	
Net treatment	Nets holed, nets unwashed and washed (x 20)	
Location(s)	Akron, Benin	
Notes	Trial conducted: October 2008 to January 2009	
Risk of bias		
Bias	**Authors' judgement**	**Support for judgement**
Were the mosquitoes in LLIN and LLIN + PBO groups comparable	Low risk	Huts were situated in the same area – mosquito characteristics will be the same
Collectors blinded	Unclear risk	Unclear whether collectors were blinded – not stated in the publication
Sleepers blinded	Unclear risk	Unclear whether sleeper was blinded – not stated in the publication
Sleeper bias	Low risk	Sleepers were rotated between huts according to a Latin square design

N'Guessan 2010 *(Continued)*

Treatment allocation (sequence randomly/adequately generated)	Low risk	Treatments were randomly allocated to huts
Treatment rotation	Low risk	Treatments were rotated between huts according to a Latin square design
Standardized hut design	Low risk	Huts were built according to a standard West African design
Hut cleaning between treatments	Low risk	All huts were cleaned between treatments
Were incomplete outcome data adequately addressed	Low risk	No outcome data were incomplete
Were the raw data reported for LLIN and LLIN + PBO groups	Low risk	All necessary data were reported
Trial authors' conflicting interest	Unclear risk	The trial was sponsored by Vestergaard (net manufacturers), which also commented on the manuscript

Pennetier 2013

Study characteristics	
Methods	Experimental hut trial
Participants	95% *An gambiae*: M-form (100%), 5% *An arabiensis* (Corbel 2010)
Interventions	Control: LLIN, Olyset Net Intervention: LLIN, Olyset Plus
Outcomes	Mosquito mortality, blood feeding, deterrence, exophily
Mosquito resistance status	Proxy data. Resistant - high (permethrin, 22% mortality, N = 100) (Djègbè 2011)
Net treatment	Nets holed, nets unwashed and washed (x 20)
Location(s)	Malanville, Benin
Notes	Trial conducted: September 2011 to December 2011

Risk of bias

Bias	Authors' judgement	Support for judgement
Were the mosquitoes in LLIN and LLIN + PBO groups comparable	Low risk	Huts situated in the same area – mosquito characteristics will be the same
Collectors blinded	Unclear risk	Unclear whether collectors were blinded – not stated in the publication
Sleepers blinded	Unclear risk	Unclear whether sleeper was blinded – not stated in the publication

Pennetier 2013 *(Continued)*

Sleeper bias	Low risk	Sleepers were rotated between huts according to a Latin square design
Treatment allocation (sequence randomly/adequately generated)	Low risk	Treatments were not randomized to huts but instead were rotated fully between all huts according to a Latin square design
Treatment rotation	Low risk	Treatments were rotated between huts according to a Latin square design
Standardized hut design	Low risk	Huts were built according to a standard West African design
Hut cleaning between treatments	Low risk	All huts were cleaned between treatments
Were incomplete outcome data adequately addressed	Low risk	No outcome data were incomplete
Were the raw data reported for LLIN and LLIN + PBO groups	Low risk	All necessary data were reported
Trial authors' conflicting interest	Low risk	Funders of the trial stated that they had no part in data collection, data analysis, or manuscript preparation

Protopopoff 2018

Study characteristics		
Methods	Cluster-randomized controlled village trial	
Participants	3966 children analysed (21 months after intervention) aged 6 months to 14 years (excluding the severely ill), *Anopheles* species (pooled). Total core cluster population ranged from 14,845 to 16,358	
Interventions	Control: LLIN, Olyset Net Intervention: LLIN, Olyset Plus	
Outcomes	Malaria parasite prevalence, sporozoite rate, mosquito density	
Mosquito resistance status	Resistance - high (17.8% mortality, N = 107)	
Net treatment	Nets unholed and unwashed	
Location(s)	Muleba District, Tanzania	
Notes	Trial conducted: March 2014 to December 2016	
Risk of bias		
Bias	**Authors' judgement**	**Support for judgement**
Recruitment bias	Low risk	No participants were recruited after clusters had been randomized

Protopopoff 2018 *(Continued)*

Were the mosquitoes in LLIN and LLIN + PBO groups comparable	Unclear risk	Resistance level was available only for the whole district - not at the village level
Collectors blinded	Low risk	Field workers were masked to net treatment
Household blinded	Low risk	Inhabitants were masked to net treatment
Treatment allocation (sequence randomly/adequately generated)	Low risk	Restricted randomization was used to allocate clusters to study groups
Allocation concealment (selection bias)	Low risk	Restricted randomization was used to allocate treatments to clusters
Were incomplete outcome data adequately addressed	Low risk	No outcome data were incomplete
Were the raw data reported for LLIN and LLIN + PBO groups	Low risk	No outcome data were missing
Clusters lost to follow-up	Low risk	No clusters were lost to follow-up
Selective reporting (reporting bias)	Low risk	It appears that all measured outcomes were reported
Correct statistical methods; adjusted for clustering	Low risk	Clustering was taken into account and was adjusted for during statistical analysis
Trial authors' conflicting interest	Low risk	Trial authors declared no conflicting interests

Stiles-Ocran 2013

Study characteristics	
Methods	Village trial
Participants	*An gambiae*
Interventions	Control: LLIN, PermaNet 2.0 Intervention: LLIN, PermaNet 3.0
Outcomes	Sporozoite rate, mosquito density, parity rate
Mosquito resistance status	Futa - resistant - moderate (33.3% mortality, N = 96) Abrabra- resistant - moderate (43.7% mortality, N = 126) Kunkumso - resistant - high (28.4% mortality, N = 109) Anyinabrim - resistant - moderate (53.2% mortality, N = 109) Wenchi - resistant - low (61.9% mortality, N =126)

Stiles-Ocran 2013 *(Continued)*

Net treatment	Nets unholed and unwashed
Location(s)	Futa, Ghana - no net control Abrabra, Ghana - PermaNet 2.0 Kunkumso, Ghana - PermaNet 2.0 Anyinabrim, Ghana - PermaNet 3.0 Wench, Ghana - PermaNet 3.0
Notes	Trial conducted: November 2010 to August 2011

Risk of bias

Bias	Authors' judgement	Support for judgement
Recruitment bias	Low risk	Recruiment bias is related to human participants and so is not applicable to this study
Were the mosquitoes in LLIN and LLIN + PBO groups comparable	Unclear risk	Mosquito species composition varied slightly. Resistance level varies between villages. However, pre-trial and post-trial data are provided
Collectors blinded	High risk	Not stated whether collectors were blinded; therefore judged as high risk, as this is likely to affect searching efforts
Household blinded	Low risk	Unclear whether households were blinded – not stated in the publication. We judged this as low risk, as this is unlikely to impact the outcome
Treatment allocation (sequence randomly/adequately generated)	Low risk	Villages were randomly assigned to treatment arms
Allocation concealment (selection bias)	Low risk	Allocation concealment procedures were not adhered to; however this is unlikely to affect the results
Were incomplete outcome data adequately addressed	Low risk	No outcome data were incomplete
Were the raw data reported for LLIN and LLIN + PBO groups	Low risk	All necessary data were reported
Clusters lost to follow-up	Low risk	No clusters were lost to follow-up
Selective reporting (reporting bias)	Low risk	It appears that all measured outcomes were reported
Correct statistical methods; adjusted for clustering	High risk	Study did not take clustering into account for statistical methods
Trial authors' conflicting interest	Unclear risk	Study data were collected for use in Vestergaard PermaNet 3.0 product dossier

Toé 2018

Study characteristics	
Methods	Experimental hut trial
Participants	*An coluzzii*
Interventions	Control: LLIN, PermaNet 2.0, Olyset Net Intervention: LLIN, PermaNet 3.0, Olyset Plus
Outcomes	Mosquito mortality, blood feeding, deterrence, exophily
Mosquito resistance status	Vallée du Kou 5 - resistant – high (deltamethrin, 2.5% mortality, N = 163; permethrin, 5% mortality, N = 153) Tengrela - resistant – high (deltamethrin, 34% mortality, N = 85; permethrin, 14% mortality, N = 101)
Net treatment	Nets holed, nets unwashed
Location(s)	Vallée du Kou 5, Burkina Faso Tengrela, Burkina Faso
Notes	Trial conducted: September 2014 to October 2014

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Were the mosquitoes in LLIN and LLIN + PBO groups comparable	Low risk	Huts situated in the same area – mosquito characteristics will be the same
Collectors blinded	Unclear risk	Unclear whether collectors were blinded – not stated in the publication
Sleepers blinded	Unclear risk	Unclear whether sleeper was blinded – not stated in the publication
Sleeper bias	Low risk	Sleepers were rotated between huts according to a Latin square design
Treatment allocation (sequence randomly/adequately generated)	Low risk	Treatments were not randomized to huts but instead were rotated fully between all huts according to a Latin square design
Treatment rotation	Low risk	Treatments were rotated between huts according to a Latin square design
Standardized hut design	Low risk	Huts were built according to a standard West African design
Hut cleaning between treatments	Unclear risk	Unclear whether huts were cleaned between treatments – not stated in the publication
Were incomplete outcome data adequately addressed	Low risk	No outcome data were incomplete
Were the raw data reported for LLIN and LLIN + PBO groups	Low risk	All necessary data were reported

Toé 2018 *(Continued)*

Trial authors' conflicting interest	Low risk	Trial authors had no competing interests

Tungu 2010

Study characteristics		
Methods	Experimental hut trial	
Participants	*An gambiae*	
Interventions	Control: LLIN, PermaNet 2.0 Intervention: LLIN, PermaNet 3.0	
Outcomes	Mosquito mortality, blood feeding, deterrence, exophily	
Mosquito resistance status	Susceptible (deltamethrin, 100% mortality, N = not stated)	
Net treatment	Nets holed, nets unwashed and washed (x 20)	
Location(s)	Zeneti, Muheza, Tanzania	
Notes	Trial conducted: July 2008 to October 2008	
Risk of bias		
Bias	**Authors' judgement**	**Support for judgement**
Were the mosquitoes in LLIN and LLIN + PBO groups comparable	Low risk	Huts situated in the same area – mosquito characteristics will be the same
Collectors blinded	Unclear risk	Unclear whether collectors were blinded – not stated in the publication
Sleepers blinded	Unclear risk	Unclear whether sleeper was blinded – not stated in the publication
Sleeper bias	Low risk	Sleepers were rotated between huts according to a Latin square design
Treatment allocation (sequence randomly/adequately generated)	Low risk	Treatments were randomly allocated to huts
Treatment rotation	Low risk	Treatments were rotated between huts according to a Latin square design
Standardized hut design	Low risk	Huts were built according to a standard West African design
Hut cleaning between treatments	Low risk	All huts were cleaned between treatments
Were incomplete outcome data adequately addressed	Low risk	No outcome data were incomplete

Tungu 2010 *(Continued)*

Were the raw data reported for LLIN and LLIN + PBO groups	Low risk	All necessary data were reported
Trial authors' conflicting interest	Low risk	Trial authors had no competing interests

Menze 2020

Study characteristics		
Methods	Experimental hut trial	
Participants	*An funestus*	
Interventions	Control: LLIN, PermaNet 2.0, Olyset Net Intervention: LLIN, PermaNet 3.0, Olyset Plus	
Outcomes	Mosquito mortality, blood feeding, exophily	
Mosquito resistance status	Moderate	
Net treatment	Nets unwashed and holed	
Location(s)	Mibellon, Cameroon	
Notes		
Risk of bias		
Bias	**Authors' judgement**	**Support for judgement**
Were the mosquitoes in LLIN and LLIN + PBO groups comparable	Low risk	Huts situated in the same area – mosquito characteristics will be the same
Collectors blinded	Unclear risk	Unclear whether collectors were blinded – not stated in the publication
Sleepers blinded	Unclear risk	Unclear whether collectors were blinded – not stated in the publication
Sleeper bias	Low risk	Sleepers were rotated between huts according to a Latin square design
Treatment allocation (sequence randomly/adequately generated)	Low risk	Treatments were not randomized to huts but instead were rotated fully between all huts according to a Latin square design
Treatment rotation	Low risk	Treatments were rotated between huts according to a Latin square design
Standardized hut design	Low risk	Huts were built according to a standard West African design
Hut cleaning between treatments	Low risk	All huts were cleaned between treatments

Menze 2020 *(Continued)*

Were incomplete outcome data adequately addressed	Low risk	No outcome data were incomplete
Were the raw data reported for LLIN and LLIN + PBO groups	Low risk	No outcome data were missing
Correct statistical methods; adjusted for clustering	Low risk	Clustering was not taken into account and adjusted for during statistical analysis. We adjusted for clustering by using an ICC value of 0.1
Trial authors' conflicting interest	Low risk	Trial authors state that they have no competing interests

Oumbouke 2019

Study characteristics		
Methods	Experimental hut trial	
Participants	*An gambiae*	
Interventions	Control: LLIN, MAGNet LN Intervention: LLIN, Veeralin LN	
Outcomes	Mosquito mortality, blood feeding, deterrence, exophily	
Mosquito resistance status	Low resistance	
Net treatment	Nets holed, nets unwashed and washed (x 20)	
Location(s)	M'be Côte d'Ivoire	
Notes		
Risk of bias		
Bias	**Authors' judgement**	**Support for judgement**
Were the mosquitoes in LLIN and LLIN + PBO groups comparable	Low risk	Huts situated in the same area – mosquito characteristics will be the same
Collectors blinded	Unclear risk	Unclear whether collectors were blinded – not stated in the publication
Sleepers blinded	Unclear risk	Unclear whether collectors were blinded – not stated in the publication
Sleeper bias	Low risk	Sleepers rotated between huts according to a Latin square design
Treatment allocation (sequence randomly/adequately generated)	Low risk	Treatments were randomly allocated to huts

Oumbouke 2019 *(Continued)*

Treatment rotation	Low risk	Treatment were rotated between huts according to a Latin Square design
Standardized hut design	Low risk	Huts were built previously according to standard West African hut design
Hut cleaning between treatments	Low risk	Huts were thoroughly cleaned and aired for a day at the end of each rotation
Were incomplete outcome data adequately addressed	Low risk	No outcome data were incomplete
Were the raw data reported for LLIN and LLIN + PBO groups	Low risk	No outcome data were missing
Correct statistical methods; adjusted for clustering	Low risk	Clustering was not taken into account and adjusted for during statistical analysis. We adjusted for clustering using an ICC value of 0.1
Trial authors' conflicting interest	Low risk	Trial authors state that they have no conflicting interests

An arabiensis: *Anopheles arabiensis*; *An coluzzii*: *Anopheles coluzzii*; *An funestus: Anopheles funestus*; *An gambiae*: *Anopheles gambiae*; ITN: insecticide-treated net; LLIN: long-lasting insecticidal net; PBO: piperonyl butoxide.

Characteristics of excluded studies *[ordered by study ID]*

Study	Reason for exclusion
Darriet 2011	Study included laboratory data only
Darriet 2013	Study included laboratory data only

Characteristics of studies awaiting classification *[ordered by study ID]*

Koudou 2012

Methods	Village trial
Participants	Bouaké - 100% *An gambiae*: (70% S-form, 30% M-form) Tiassalé - 100% *An gambiae*: (70% S-form, 30% M-form)
Interventions	Control: LLIN, PermaNet 2.0 Extra Intervention: LLIN, PermaNet 3.0
Outcomes	Blood feeding, mosquito density
Mosquito Resistance Status	Bouaké - resistant - moderate (43.9% mortality, N = 114) Tiassalé - resistant - moderate (7.5% mortality, N = 106)
Net Treatment	Nets unholed and unwashed
Location(s)	Bouaké, Côte d'Ivoire

Koudou 2012 *(Continued)*

	Tiassalé, Côte d'Ivoire
Notes	Trial conducted: November 2009 to January 2012

Shono 2017

Methods	Not available
Participants	*An funestus: Anopheles funestus*; *An gambiae*: *Anopheles gambiae*
Interventions	
Outcomes	Not available
Mosquito Resistance Status	Not available
Net Treatment	Control: LLIN, Olyset Net Intervention: LLIN, Olyset Plus
Location(s)	Not available
Notes	

Tungu 2017

Methods	Experimental hut trial
Participants	*An funestus*
Interventions	Control: LLIN, DawaPlus 2.0 Intervention: LLIN, DawaPlus 3.0, DawaPlus 4.0
Outcomes	Mosquito mortality, blood feeding, deterrence, exophily
Mosquito Resistance Status	
Net Treatment	Nets holed, nets unwashed and washed (x 20)
Location(s)	Muheza, Tanzania
Notes	

Characteristics of ongoing studies *[ordered by study ID]*

ISRCTN99611164

Study name	Comparative evaluation of standard insecticide-treated bed nets and co-treated bed nets on malaria prevalence in Sud Ubangi, Democratic Republic of Congo: a cluster-randomised trial

ISRCTN99611164 *(Continued)*

Methods	Cluster-randomized trial
Participants	Women (> 15 years) attending first ANC appointment at a clinic that is taking part in the study, who consent to be enrolled in the study 20 visitors per month at each of 7 antenatal clinics (held monthly) in each of 17 study clusters, which gives a total of approximately 2400 participants per month, 28,500 per year, and 86,000 in total
Interventions	Control: bed net treated with pyrethroid only Intervention: bed net treated with both pyrethroid and PBO
Outcomes	1. Determination of parasite prevalence in women visiting monthly antenatal clinics 2. Entomological collections for surveillance of insecticide resistance and mosquito abundance and parasite infection 3. Assessment of bed net durability (physical and chemical analysis) and bio-efficacy (against mosquitoes) over time
Starting date	November 2019 (recruitment start date 01/06/2020)
Contact information	Dr David Weetman
Notes	

NCT03289663

Study name	Effectiveness study of new-generation bed nets in the context of conventional insecticide resistance in the Democratic Republic of the Congo (Net-PBO)
Methods	Cluster-randomized trial
Participants	1680 participants; 0 to 10-year-old subjects in 30 villages
Interventions	Control: bed net treated with pyrethroid only Intervention: bed net treated with both pyrethroid and PBO (IRS and LSM included in trial)
Outcomes	Incidence rate of laboratory-confirmed clinical cases of malaria (time frame: participants will be actively followed up for 12 months, and any suspected case of clinical malaria will immediately lead to microscopy and RDT for confirmation). Microscopy to confirm the diagnosis of malaria sporozoite rate (time frame: *Anopheles* mosquitoes will be captured every 3 months during 1 year), sporozoite detection by ELISA to determine infectivity of *Anopheles*
Starting date	2 October 2017
Contact information	
Notes	

NCT04182126

Study name	HS#2017-3512. Adaptive interventions for optimizing malaria control: a cluster-randomized SMART trial
Methods	Cluster-randomized trial
Participants	122,872 participants (6 months and older, all sexes)
Interventions	Other: regular long-lasting insecticidal nets (Olyset) Other: LLIN plus piperonyl butoxide-treated LLIN (Olyset Plus)
Outcomes	Annual clinical malaria incidence rate Malaria parasite prevalence Malaria vector density Malaria transmission intensity
Starting date	01/12/2019
Contact information	Dr Guiyun Yan
Notes	

UMIN000019971

Study name	A preliminary study on designing a cluster randomized control trial of two mosquito nets to prevent malaria parasite infection
Methods	Cluster-randomized trial
Participants	1360 target participants Children targeted for malaria transmission survey are aged between 7 and 131 months Children between 60 and 131 months old are schoolchildren; 170 children are randomly selected from each cluster for survey
Interventions	Control: bed net treated with pyrethroid only Intervention: bed net treated with both pyrethroid and PBO
Outcomes	*Plasmodium falciparum* parasite infection after distribution of bed nets with PBO: PCR-based infection Slide-based infection Haemoglobin amount
Starting date	
Contact information	Dr Noboru Minakawa
Notes	

ELISA: enzyme-linked immunosorbent assay; PBO: piperonyl butoxide.

DATA AND ANALYSES

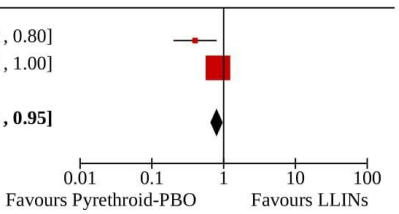
Comparison 1. Commercial pyrethroid-PBO nets versus commercial LLINs: village trials

Outcome or subgroup title	No. of studies	No. of participants	Statistical method	Effect size
1.1 Parasite prevalence (pyrethroid-PBO nets vs non-PBO LLINs, latest end points in RCT)	2		Odds Ratio (IV, Fixed, 95% CI)	0.79 [0.67, 0.95]
1.2 Parasite prevalence (pyrethroid-PBO nets vs non-PBO LLINs, shown at 4 different time points)	2		Odds Ratio (IV, Fixed, 95% CI)	Subtotals only
1.2.1 4 to 6 months	2		Odds Ratio (IV, Fixed, 95% CI)	0.74 [0.62, 0.89]
1.2.2 9 to 12 months	2		Odds Ratio (IV, Fixed, 95% CI)	0.72 [0.61, 0.86]
1.2.3 16 to 18 months	2		Odds Ratio (IV, Fixed, 95% CI)	0.88 [0.74, 1.04]
1.2.4 21 to 25 months	2		Odds Ratio (IV, Fixed, 95% CI)	0.79 [0.67, 0.95]
1.3 Mosquito sporozoite-positive (adjusted ICC 0.1)	4	424	Risk Ratio (M-H, Random, 95% CI)	0.82 [0.24, 2.75]
1.4 Mosquito parous (adjusted ICC 0.1)	3	220	Risk Ratio (M-H, Random, 95% CI)	0.97 [0.82, 1.13]

Analysis 1.1. Comparison 1: Commercial pyrethroid-PBO nets versus commercial LLINs: village trials, Outcome 1: Parasite prevalence (pyrethroid-PBO nets vs non-PBO LLINs, latest end points in RCT)

Study or Subgroup	log[OR]	SE	Weight	Odds Ratio IV, Fixed, 95% CI
Protopopoff 2018 (1)	-0.9163	0.3537	6.4%	0.40 [0.20 , 0.80]
Staedke 2020 (2)	-0.1827	0.0923	93.6%	0.83 [0.70 , 1.00]
Total (95% CI)			**100.0%**	**0.79 [0.67 , 0.95]**

Heterogeneity: Chi² = 4.03, df = 1 (P = 0.04); I² = 75%
Test for overall effect: Z = 2.57 (P = 0.01)
Test for subgroup differences: Not applicable

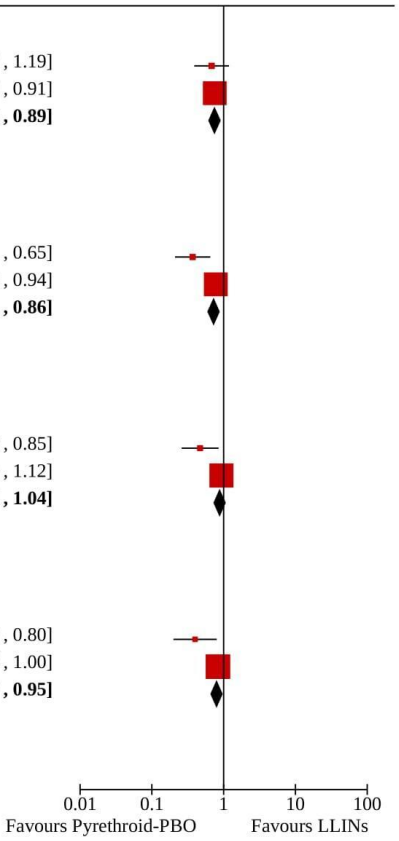


Footnotes

(1) 21 months after intervention
(2) 25 months after intervention

Analysis 1.2. Comparison 1: Commercial pyrethroid-PBO nets versus commercial LLINs: village trials, Outcome 2: Parasite prevalence (pyrethroid-PBO nets vs non-PBO LLINs, shown at 4 different time points)

Study or Subgroup	log[OR]	SE	Weight	Odds Ratio IV, Fixed, 95% CI
1.2.1 4 to 6 months				
Protopopoff 2018 (1)	-0.3857	0.2837	10.1%	0.68 [0.39 , 1.19]
Staedke 2020 (2)	-0.2845	0.095	89.9%	0.75 [0.62 , 0.91]
Subtotal (95% CI)			**100.0%**	**0.74 [0.62 , 0.89]**
Heterogeneity: Chi² = 0.11, df = 1 (P = 0.74); I² = 0%				
Test for overall effect: Z = 3.27 (P = 0.001)				
1.2.2 9 to 12 months				
Protopopoff 2018 (3)	-0.9943	0.289	9.8%	0.37 [0.21 , 0.65]
Staedke 2020 (4)	-0.2522	0.095	90.2%	0.78 [0.65 , 0.94]
Subtotal (95% CI)			**100.0%**	**0.72 [0.61 , 0.86]**
Heterogeneity: Chi² = 5.95, df = 1 (P = 0.01); I² = 83%				
Test for overall effect: Z = 3.60 (P = 0.0003)				
1.2.3 16 to 18 months				
Protopopoff 2018 (5)	-0.755	0.3021	8.7%	0.47 [0.26 , 0.85]
Staedke 2020 (6)	-0.0713	0.0934	91.3%	0.93 [0.78 , 1.12]
Subtotal (95% CI)			**100.0%**	**0.88 [0.74 , 1.04]**
Heterogeneity: Chi² = 4.68, df = 1 (P = 0.03); I² = 79%				
Test for overall effect: Z = 1.47 (P = 0.14)				
1.2.4 21 to 25 months				
Protopopoff 2018 (7)	-0.9163	0.3537	6.4%	0.40 [0.20 , 0.80]
Staedke 2020 (8)	-0.1827	0.0923	93.6%	0.83 [0.70 , 1.00]
Subtotal (95% CI)			**100.0%**	**0.79 [0.67 , 0.95]**
Heterogeneity: Chi² = 4.03, df = 1 (P = 0.04); I² = 75%				
Test for overall effect: Z = 2.57 (P = 0.01)				



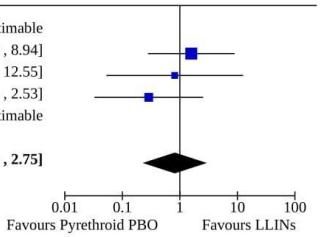
Footnotes

(1) 4 months after intervention
(2) 6 months after intervention
(3) 9 months after intervention
(4) 12 months after intervention
(5) 16 months after intervention
(6) 18 months after intervention
(7) 21 months after intervention
(8) 25 months after intervention

Analysis 1.3. Comparison 1: Commercial pyrethroid-PBO nets versus commercial LLINs: village trials, Outcome 3: Mosquito sporozoite-positive (adjusted ICC 0.1)

Study or Subgroup	Pyrethroid-PBO nets Events	Total	LLINs Events	Total	Weight	Risk Ratio M-H, Random, 95% CI
Awolola 2014 (1)	0	4	0	16		Not estimable
Cisse 2017 (2)	3	38	2	40	49.1%	1.58 [0.28 , 8.94]
Cisse 2017 (3)	1	43	1	35	19.7%	0.81 [0.05 , 12.55]
Protopopoff 2018 (2)	1	106	4	122	31.2%	0.29 [0.03 , 2.53]
Stiles-Ocran 2013 (3)	0	9	0	11		Not estimable
Total (95% CI)		**200**		**224**	**100.0%**	**0.82 [0.24 , 2.75]**
Total events:	5		7			

Heterogeneity: Tau² = 0.00; Chi² = 1.46, df = 2 (P = 0.48); I² = 0%
Test for overall effect: Z = 0.33 (P = 0.74)
Test for subgroup differences: Not applicable

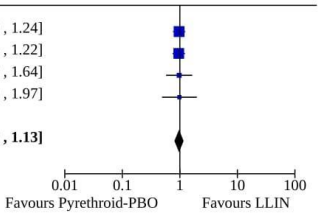


Footnotes
(1) Permanet 3.0, Low resistance
(2) Olyset Plus, High resistance
(3) Permanet 3.0, Moderate resistance

Analysis 1.4. Comparison 1: Commercial pyrethroid-PBO nets versus commercial LLINs: village trials, Outcome 4: Mosquito parous (adjusted ICC 0.1)

Study or Subgroup	Pyrethroid-PBO nets Events	Total	LLINs Events	Total	Weight	Risk Ratio M-H, Random, 95% CI
Cisse 2017 (1)	28	37	32	41	43.0%	0.97 [0.76 , 1.24]
Cisse 2017 (2)	30	40	29	37	42.3%	0.96 [0.75 , 1.22]
Mzilahowa 2014 (3)	10	18	16	28	9.4%	0.97 [0.58 , 1.64]
Stiles-Ocran 2013 (2)	5	8	7	11	5.3%	0.98 [0.49 , 1.97]
Total (95% CI)		**103**		**117**	**100.0%**	**0.97 [0.82 , 1.13]**
Total events:	73		84			

Heterogeneity: Tau² = 0.00; Chi² = 0.01, df = 3 (P = 1.00); I² = 0%
Test for overall effect: Z = 0.43 (P = 0.66)
Test for subgroup differences: Not applicable



Footnotes
(1) Olyset Plus, High resistance
(2) Permanet 3.0, Moderate resistance
(3) Permanet 3.0, *Anopheles funestus*

Comparison 2. Commercial pyrethroid-PBO nets versus commercial LLINs: hut trials

Outcome or subgroup title	No. of studies	No. of participants	Statistical method	Effect size
2.1 Mosquito mortality (pooled) hut/night (adjusted ICC 0.1)	10	15614	Risk Ratio (M-H, Random, 95% CI)	1.43 [1.26, 1.62]
2.1.1 Unwashed	10	8647	Risk Ratio (M-H, Random, 95% CI)	1.63 [1.29, 2.05]

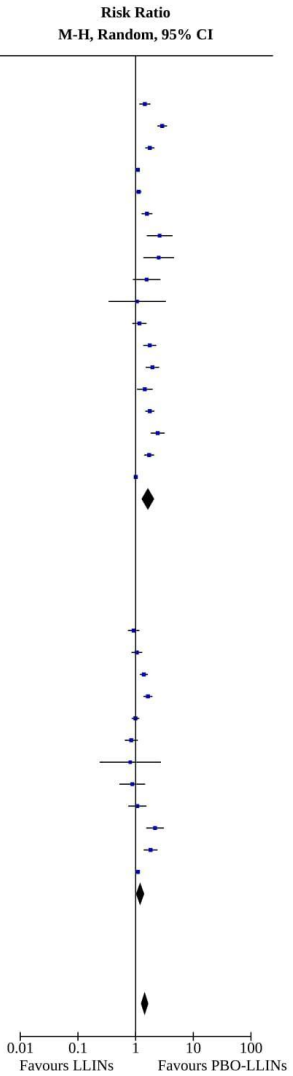
Outcome or subgroup title	No. of studies	No. of participants	Statistical method	Effect size
2.1.2 Washed	8	6967	Risk Ratio (M-H, Random, 95% CI)	1.19 [1.04, 1.38]
2.2 Mosquito blood-feeding success (pooled) hut/night (adjusted ICC 0.1)	9	12351	Risk Ratio (M-H, Random, 95% CI)	0.75 [0.66, 0.85]
2.2.1 Unwashed	9	7261	Risk Ratio (M-H, Random, 95% CI)	0.68 [0.57, 0.80]
2.2.2 Washed	7	5090	Risk Ratio (M-H, Random, 95% CI)	0.87 [0.74, 1.02]
2.3 Mosquito exophily (pooled) hut/night (adjusted ICC 0.1)	10	13214	Risk Ratio (M-H, Random, 95% CI)	1.00 [0.94, 1.06]
2.3.1 Unwashed	10	7699	Risk Ratio (M-H, Random, 95% CI)	1.00 [0.91, 1.10]
2.3.2 Washed	8	5515	Risk Ratio (M-H, Random, 95% CI)	1.00 [0.93, 1.07]
2.4 Mosquito mortality (high resistance) hut/night (adjusted ICC 0.1)	5	7997	Risk Ratio (M-H, Random, 95% CI)	1.58 [1.34, 1.86]
2.4.1 Unwashed	5	4896	Risk Ratio (M-H, Random, 95% CI)	1.84 [1.60, 2.11]
2.4.2 Washed	4	3101	Risk Ratio (M-H, Random, 95% CI)	1.20 [0.88, 1.63]
2.5 Mosquito blood-feeding success (high resistance) hut/night (adjusted ICC 0.1)	4	7134	Risk Ratio (M-H, Random, 95% CI)	0.66 [0.57, 0.76]
2.5.1 Unwashed	4	4458	Risk Ratio (M-H, Random, 95% CI)	0.60 [0.50, 0.71]
2.5.2 Washed	3	2676	Risk Ratio (M-H, Random, 95% CI)	0.81 [0.72, 0.92]
2.6 Mosquito mortality (moderate resistance) hut/night (adjusted ICC 0.1)	2	1027	Risk Ratio (M-H, Fixed, 95% CI)	1.47 [1.21, 1.78]
2.6.1 Unwashed	2	751	Risk Ratio (M-H, Fixed, 95% CI)	1.68 [1.33, 2.11]
2.6.2 Washed	1	276	Risk Ratio (M-H, Fixed, 95% CI)	1.07 [0.74, 1.54]
2.7 Mosquito blood-feeding success (moderate resistance) hut/night (adjusted ICC 0.1)	2	1034	Risk Ratio (M-H, Random, 95% CI)	0.91 [0.78, 1.05]

Outcome or subgroup title	No. of studies	No. of participants	Statistical method	Effect size
2.7.1 Unwashed	2	752	Risk Ratio (M-H, Random, 95% CI)	0.90 [0.72, 1.11]
2.7.2 Washed	1	282	Risk Ratio (M-H, Random, 95% CI)	0.91 [0.74, 1.13]
2.8 Mosquito mortality (low resistance) hut/night (adjusted ICC 0.1)	2	1970	Risk Ratio (M-H, Random, 95% CI)	1.30 [1.09, 1.56]
2.8.1 Unwashed	2	948	Risk Ratio (M-H, Random, 95% CI)	1.25 [0.99, 1.57]
2.8.2 Washed	2	1022	Risk Ratio (M-H, Random, 95% CI)	1.39 [0.95, 2.04]
2.9 Mosquito blood-feeding success (low resistance) hut/night (adjusted ICC 0.1)	2	1970	Risk Ratio (M-H, Random, 95% CI)	0.94 [0.56, 1.57]
2.9.1 Unwashed	2	948	Risk Ratio (M-H, Random, 95% CI)	0.75 [0.27, 2.11]
2.9.2 Washed	2	1022	Risk Ratio (M-H, Random, 95% CI)	1.07 [0.49, 2.33]
2.10 Mosquito mortality (susceptible) hut/night (adjusted ICC 0.1)	2	1916	Risk Ratio (M-H, Random, 95% CI)	1.05 [0.96, 1.15]
2.10.1 Unwashed	2	948	Risk Ratio (M-H, Random, 95% CI)	1.20 [0.64, 2.26]
2.10.2 Washed	2	968	Risk Ratio (M-H, Random, 95% CI)	1.07 [0.92, 1.25]
2.11 Mosquito blood-feeding success (susceptible) hut/night (adjusted ICC 0.1)	2	1916	Risk Ratio (M-H, Random, 95% CI)	0.87 [0.40, 1.89]
2.11.1 Unwashed	2	948	Risk Ratio (M-H, Random, 95% CI)	0.52 [0.12, 2.22]
2.11.2 Washed	2	968	Risk Ratio (M-H, Random, 95% CI)	1.25 [0.82, 1.91]
2.12 Mosquito mortality (high resistance/Permanet) hut/night (adjusted ICC 0.1)	3	2806	Risk Ratio (M-H, Random, 95% CI)	1.59 [1.26, 2.01]
2.12.1 Not Washed	3	1877	Risk Ratio (M-H, Random, 95% CI)	1.81 [1.56, 2.10]
2.12.2 Washed	2	929	Risk Ratio (M-H, Random, 95% CI)	1.18 [0.61, 2.28]

Outcome or subgroup title	No. of studies	No. of participants	Statistical method	Effect size
2.13 Mosquito blood-feeding success (high resistance/Permanet) hut/night (adjusted ICC 0.1)	2	1943	Risk Ratio (M-H, Random, 95% CI)	0.58 [0.45, 0.76]
2.13.1 Unwashed	2	1439	Risk Ratio (M-H, Random, 95% CI)	0.53 [0.40, 0.69]
2.13.2 Washed	1	504	Risk Ratio (M-H, Random, 95% CI)	0.76 [0.61, 0.93]
2.14 Mosquito mortality (high resistance/Olyset) hut/night (adjusted ICC 0.1)	2	1410	Risk Ratio (M-H, Random, 95% CI)	1.73 [1.51, 1.97]
2.14.1 Unwashed	2	1257	Risk Ratio (M-H, Random, 95% CI)	1.72 [1.48, 1.99]
2.14.2 Washed	1	153	Risk Ratio (M-H, Random, 95% CI)	1.81 [1.25, 2.61]
2.15 Mosquito blood-feeding success (high resistance/Olyset) hut/night (adjusted ICC 0.1)	2	1470	Risk Ratio (M-H, Random, 95% CI)	0.63 [0.40, 0.98]
2.15.1 Unwashed	2	1257	Risk Ratio (M-H, Random, 95% CI)	0.67 [0.38, 1.18]
2.15.2 Washed	1	213	Risk Ratio (M-H, Random, 95% CI)	0.50 [0.27, 0.93]

Analysis 2.1. Comparison 2: Commercial pyrethroid-PBO nets versus commercial LLINs: hut trials, Outcome 1: Mosquito mortality (pooled) hut/night (adjusted ICC 0.1)

Study or Subgroup	PBO-LLINs Events	PBO-LLINs Total	LLINs Events	LLINs Total	Weight	Risk Ratio M-H, Random, 95% CI
2.1.1 Unwashed						
Bayili 2017 (1)	144	664	113	753	3.7%	1.45 [1.16 , 1.81]
Bayili 2017 (2)	197	457	125	837	3.8%	2.89 [2.38 , 3.50]
Corbel 2010 (3)	181	232	73	165	3.8%	1.76 [1.47 , 2.12]
Corbel 2010 (4)	170	176	177	200	4.1%	1.09 [1.03 , 1.16]
Corbel 2010 (5)	110	117	70	84	4.1%	1.13 [1.01 , 1.25]
Koudou 2011 (6)	117	214	78	224	3.7%	1.57 [1.26 , 1.95]
Menze 2020 (7)	32	106	19	164	2.5%	2.61 [1.56 , 4.35]
Menze 2020 (8)	34	140	12	124	2.1%	2.51 [1.36 , 4.63]
Moore 2016 (9)	23	161	22	239	2.3%	1.55 [0.90 , 2.69]
Moore 2016 (10)	5	68	6	87	0.9%	1.07 [0.34 , 3.35]
N'Guessan 2010 (11)	59	115	45	102	3.5%	1.16 [0.88 , 1.54]
Oumbouke 2019 (12)	79	156	62	215	3.5%	1.76 [1.35 , 2.28]
Pennetier 2013 (13)	53	66	39	95	3.5%	1.96 [1.49 , 2.56]
Toé 2018 (14)	62	221	57	293	3.3%	1.44 [1.05 , 1.97]
Toé 2018 (15)	125	199	116	325	3.8%	1.76 [1.47 , 2.11]
Toé 2018 (16)	116	249	52	269	3.5%	2.41 [1.83 , 3.18]
Toé 2018 (17)	146	272	97	310	3.8%	1.72 [1.41 , 2.09]
Tungu 2010 (18)	223	233	300	315	4.2%	1.00 [0.97 , 1.04]
Subtotal (95% CI)		**3846**		**4801**	**60.2%**	**1.63 [1.29 , 2.05]**
Total events:	1876		1463			
Heterogeneity: Tau² = 0.22; Chi² = 750.61, df = 17 (P < 0.00001); I² = 98%						
Test for overall effect: Z = 4.17 (P < 0.0001)						
2.1.2 Washed						
Bayili 2017 (2)	105	683	149	895	3.7%	0.92 [0.73 , 1.16]
Bayili 2017 (1)	136	780	141	848	3.7%	1.05 [0.85 , 1.30]
Corbel 2010 (5)	82	105	112	199	3.9%	1.39 [1.18 , 1.63]
Corbel 2010 (3)	183	371	122	404	3.8%	1.63 [1.36 , 1.96]
Corbel 2010 (4)	101	144	94	133	3.9%	0.99 [0.85 , 1.16]
Koudou 2011 (6)	72	224	77	201	3.6%	0.84 [0.65 , 1.09]
Moore 2016 (10)	4	67	6	81	0.8%	0.81 [0.24 , 2.74]
Moore 2016 (19)	23	186	28	198	2.5%	0.87 [0.52 , 1.46]
N'Guessan 2010 (11)	40	130	42	146	3.1%	1.07 [0.74 , 1.54]
Oumbouke 2019 (12)	60	158	38	217	3.2%	2.17 [1.53 , 3.08]
Pennetier 2013 (13)	64	96	43	117	3.5%	1.81 [1.38 , 2.39]
Tungu 2010 (18)	271	285	260	299	4.1%	1.09 [1.04 , 1.15]
Subtotal (95% CI)		**3229**		**3738**	**39.8%**	**1.19 [1.04 , 1.38]**
Total events:	1141		1112			
Heterogeneity: Tau² = 0.04; Chi² = 63.76, df = 11 (P < 0.00001); I² = 83%						
Test for overall effect: Z = 2.46 (P = 0.01)						
Total (95% CI)		**7075**		**8539**	**100.0%**	**1.43 [1.26 , 1.62]**
Total events:	3017		2575			
Heterogeneity: Tau² = 0.10; Chi² = 615.89, df = 29 (P < 0.00001); I² = 95%						
Test for overall effect: Z = 5.56 (P < 0.00001)						
Test for subgroup differences: Chi² = 5.08, df = 1 (P = 0.02), I² = 80.3%						



Footnotes

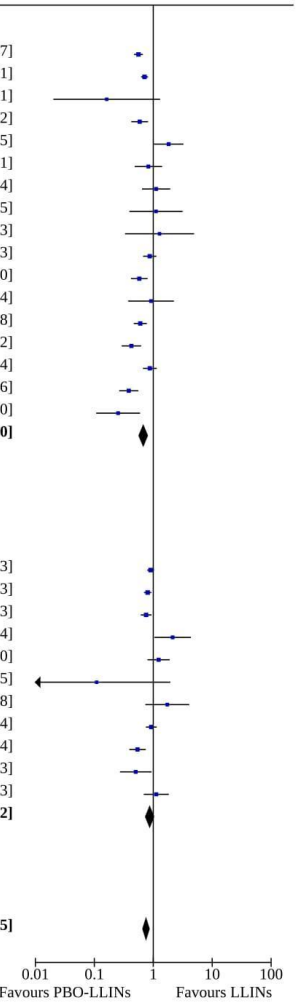
(1) Valle du Kou, DawaPlus 3.0, High resistance
(2) Valle du Kou, DawaPlus 4.0, High resistance
(3) Vallée du Kou, Permanet 3.0, High resistance
(4) Malanville, Permanet 3.0, Low resistance
(5) Pitoa, Permanet 3.0, Low resistance

Analysis 2.1. (Continued)

(4) Malanville, Permanet 3.0, Low resistance
(5) Pitoa, Permanet 3.0, Low resistance
(6) Yaokoffikro, Permanet 3.0, High resistance
(7) Mibellon, PermaNet 3.0, moderate resistance, *An funestus*
(8) Mibellon, Olyset Plus, Moderate resistance, *An funestus*
(9) Ifakara, Veeralin, Susceptible, *An arabiensis*
(10) Ifakara, Veeralin, Unclassified, *An funestus*
(11) Akron, Permanet 3.0, Moderate resistance
(12) Cote d'Ivoire, VEERALIN, Low resistance
(13) Malanaville, Olyset Plus, High resistance
(14) Tengrela, Olyset Plus, High resistance
(15) Vallee du Kou 5, Permanet 3.0, High resistance
(16) Tengrela, Permanet 3.0, High resistance
(17) Vallee du Kou 5, Olyset Plus, High resistance
(18) Zeneti, Permanet 3.0, Susceptible
(19) Ifakara, Veeralin, Susceptible, *An arabiensis*

Analysis 2.2. Comparison 2: Commercial pyrethroid-PBO nets versus commercial LLINs: hut trials, Outcome 2: Mosquito blood-feeding success (pooled) hut/night (adjusted ICC 0.1)

Study or Subgroup	PBO-LLINs Events	PBO-LLINs Total	LLINs Events	LLINs Total	Weight	Risk Ratio M-H, Random, 95% CI
2.2.1 Unwashed						
Bayili 2017 (1)	102	302	333	553	5.9%	0.56 [0.47 , 0.67]
Bayili 2017 (2)	182	425	290	482	6.2%	0.71 [0.62 , 0.81]
Corbel 2010 (3)	1	176	7	200	0.3%	0.16 [0.02 , 1.31]
Corbel 2010 (4)	48	232	58	165	4.5%	0.59 [0.42 , 0.82]
Corbel 2010 (5)	33	117	13	84	2.7%	1.82 [1.02 , 3.25]
Menze 2020 (6)	17	106	32	165	3.0%	0.83 [0.48 , 1.41]
Menze 2020 (7)	24	140	19	124	2.9%	1.12 [0.64 , 1.94]
Moore 2016 (8)	6	161	8	239	1.2%	1.11 [0.39 , 3.15]
Moore 2016 (9)	4	68	4	87	0.8%	1.28 [0.33 , 4.93]
N'Guessan 2010 (10)	55	115	56	102	5.1%	0.87 [0.67 , 1.13]
Oumbouke 2019 (11)	37	156	88	215	4.6%	0.58 [0.42 , 0.80]
Pennetier 2013 (10)	7	66	11	95	1.5%	0.92 [0.37 , 2.24]
Toé 2018 (12)	63	249	113	269	5.2%	0.60 [0.47 , 0.78]
Toé 2018 (13)	31	272	83	310	4.1%	0.43 [0.29 , 0.62]
Toé 2018 (14)	62	221	94	293	5.1%	0.87 [0.67 , 1.14]
Toé 2018 (15)	28	199	119	325	4.2%	0.38 [0.26 , 0.56]
Tungu 2010 (16)	6	233	32	315	1.6%	0.25 [0.11 , 0.60]
Subtotal (95% CI)		**3238**		**4023**	**58.7%**	**0.68 [0.57 , 0.80]**
Total events:	706		1360			
Heterogeneity: Tau² = 0.06; Chi² = 53.70, df = 16 (P < 0.00001); I² = 70%						
Test for overall effect: Z = 4.66 (P < 0.00001)						
2.2.2 Washed						
Bayili 2017 (2)	215	474	259	515	6.2%	0.90 [0.79 , 1.03]
Bayili 2017 (1)	170	420	277	550	6.1%	0.80 [0.70 , 0.93]
Corbel 2010 (4)	88	241	127	263	5.6%	0.76 [0.61 , 0.93]
Corbel 2010 (3)	23	178	10	165	2.1%	2.13 [1.05 , 4.34]
Corbel 2010 (5)	26	105	40	199	3.7%	1.23 [0.80 , 1.90]
Moore 2016 (9)	0	67	5	81	0.2%	0.11 [0.01 , 1.95]
Moore 2016 (8)	13	186	8	198	1.6%	1.73 [0.73 , 4.08]
N'Guessan 2010 (10)	70	130	85	146	5.6%	0.92 [0.75 , 1.14]
Oumbouke 2019 (11)	37	158	94	217	4.6%	0.54 [0.39 , 0.74]
Pennetier 2013 (17)	12	96	29	117	2.5%	0.50 [0.27 , 0.93]
Tungu 2010 (16)	30	285	28	299	3.3%	1.12 [0.69 , 1.83]
Subtotal (95% CI)		**2340**		**2750**	**41.3%**	**0.87 [0.74 , 1.02]**
Total events:	684		962			
Heterogeneity: Tau² = 0.03; Chi² = 28.71, df = 10 (P = 0.001); I² = 65%						
Test for overall effect: Z = 1.73 (P = 0.08)						
Total (95% CI)		**5578**		**6773**	**100.0%**	**0.75 [0.66 , 0.85]**
Total events:	1390		2322			
Heterogeneity: Tau² = 0.06; Chi² = 103.45, df = 27 (P < 0.00001); I² = 74%						
Test for overall effect: Z = 4.48 (P < 0.00001)						
Test for subgroup differences: Chi² = 4.60, df = 1 (P = 0.03), I² = 78.3%						



Footnotes

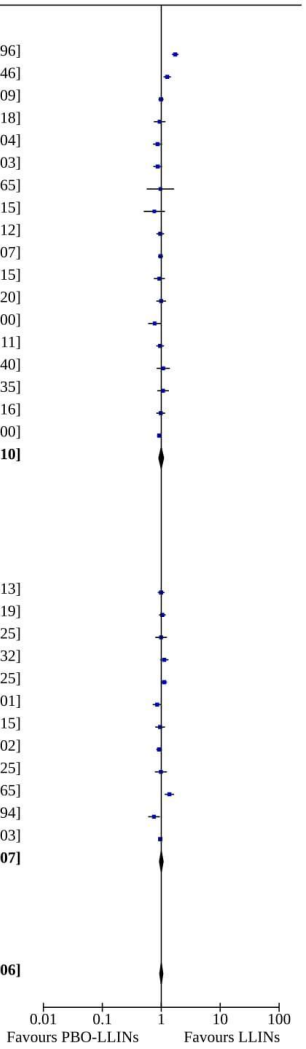
(1) Vallee du Kou, DawaPlus 4.0, High resistance
(2) Vallee du Kou, DawaPlus 3.0, High resistance
(3) Malanville, Permanet 3.0, Low resistance
(4) Vallée du Kou, Permanet 3.0, High resistance
(5) Pitoa, Permanet 3.0, Low resistance
(6) Mibellon, PermaNet 3.0, moderate resistance. *An funestus*

Analysis 2.2. (Continued)

(5) Pitoa, Permanet 3.0, Low resistance
(6) Mibellon, PermaNet 3.0, moderate resistance, *An funestus*
(7) Mibellon, Olyset Plus, Moderate resistance, *An funestus*
(8) Ifakara, Veeralin, Susceptible, *An arabiensis*
(9) Ifakara, Veeralin, Unclassified, *An funestus*
(10) Akron, Permanet 3.0, Moderate resistance
(11) Cote d'Ivoire, VEERALIN, Low resistance
(12) Tengrela, Permanet 3.0, High resistance
(13) Vallee du Kou 5, Olyset Plus, High resistance
(14) Tengrela, Olyset Plus, High resistance
(15) Vallee du Kou 5, Permanet 3.0, High resistance
(16) Zeneti, Permanet 3.0, Susceptible
(17) Malanville, Olyset Plus, High resistance

Analysis 2.3. Comparison 2: Commercial pyrethroid-PBO nets versus commercial LLINs: hut trials, Outcome 3: Mosquito exophily (pooled) hut/night (adjusted ICC 0.1)

Study or Subgroup	PBO-LLIN Events	PBO-LLIN Total	LLIN Events	LLIN Total	Weight	Risk Ratio M-H, Random, 95% CI
2.3.1 Unwashed						
Bayili 2017 (1)	210	302	223	553	3.9%	1.72 [1.52 , 1.96]
Bayili 2017 (2)	216	425	194	482	3.7%	1.26 [1.09 , 1.46]
Corbel 2010 (3)	185	232	133	165	4.2%	0.99 [0.90 , 1.09]
Corbel 2010 (4)	68	117	52	84	2.8%	0.94 [0.75 , 1.18]
Corbel 2010 (5)	93	176	122	200	3.3%	0.87 [0.72 , 1.04]
Koudou 2011 (6)	109	214	131	224	3.4%	0.87 [0.73 , 1.03]
Menze 2020 (7)	18	106	29	165	1.0%	0.97 [0.57 , 1.65]
Menze 2020 (8)	31	140	36	124	1.4%	0.76 [0.50 , 1.15]
Moore 2016 (9)	55	68	73	87	3.7%	0.96 [0.83 , 1.12]
Moore 2016 (10)	129	161	197	239	4.3%	0.97 [0.88 , 1.07]
N'Guessan 2010 (11)	67	115	64	102	2.9%	0.93 [0.75 , 1.15]
Oumbouke 2019	86	156	119	215	3.3%	1.00 [0.83 , 1.20]
Pennetier 2013 (12)	36	66	67	95	2.5%	0.77 [0.60 , 1.00]
Toé 2018 (13)	146	272	174	310	3.7%	0.96 [0.82 , 1.11]
Toé 2018 (14)	80	249	80	269	2.5%	1.08 [0.84 , 1.40]
Toé 2018 (15)	85	221	105	293	2.8%	1.07 [0.86 , 1.35]
Toé 2018 (16)	102	199	170	325	3.4%	0.98 [0.83 , 1.16]
Tungu 2010 (17)	184	233	269	315	4.4%	0.92 [0.85 , 1.00]
Subtotal (95% CI)		**3452**		**4247**	**57.5%**	**1.00 [0.91 , 1.10]**
Total events:	1900		2238			
Heterogeneity: Tau² = 0.03; Chi² = 100.21, df = 17 (P < 0.00001); I² = 83%						
Test for overall effect: Z = 0.04 (P = 0.97)						
2.3.2 Washed						
Bayili 2017 (2)	227	474	248	515	3.9%	0.99 [0.87 , 1.13]
Bayili 2017 (1)	211	420	265	550	3.9%	1.04 [0.92 , 1.19]
Corbel 2010 (4)	54	105	103	199	2.8%	0.99 [0.79 , 1.25]
Corbel 2010 (5)	120	178	99	165	3.5%	1.12 [0.96 , 1.32]
Corbel 2010 (3)	183	241	178	263	4.1%	1.12 [1.01 , 1.25]
Koudou 2011 (6)	113	224	119	201	3.4%	0.85 [0.72 , 1.01]
Moore 2016 (9)	49	67	62	81	3.2%	0.96 [0.79 , 1.15]
Moore 2016 (10)	140	186	162	198	4.2%	0.92 [0.83 , 1.02]
N'Guessan 2010 (11)	65	130	74	146	2.7%	0.99 [0.78 , 1.25]
Oumbouke 2019	102	158	102	217	3.3%	1.37 [1.14 , 1.65]
Pennetier 2013 (12)	50	96	81	117	2.8%	0.75 [0.60 , 0.94]
Tungu 2010 (17)	242	285	264	299	4.6%	0.96 [0.90 , 1.03]
Subtotal (95% CI)		**2564**		**2951**	**42.5%**	**1.00 [0.93 , 1.07]**
Total events:	1556		1757			
Heterogeneity: Tau² = 0.01; Chi² = 32.00, df = 11 (P = 0.0008); I² = 66%						
Test for overall effect: Z = 0.04 (P = 0.97)						
Total (95% CI)		**6016**		**7198**	**100.0%**	**1.00 [0.94 , 1.06]**
Total events:	3456		3995			
Heterogeneity: Tau² = 0.02; Chi² = 132.61, df = 29 (P < 0.00001); I² = 78%						
Test for overall effect: Z = 0.03 (P = 0.97)						
Test for subgroup differences: Chi² = 0.00, df = 1 (P = 0.96), I² = 0%						



Footnotes

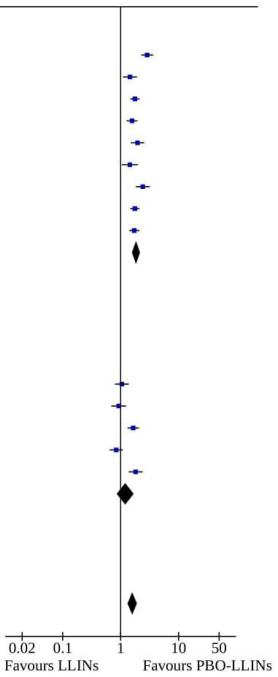
(1) Vallee du Kou. DawaPlus 4.0, High resistance
(2) Vallee du Kou. DawaPlus 3.0, High resistance
(3) Vallée du Kou, Permanet 3.0, HIgh resistance
(4) Pitoa, Permanet 3.0, Low resistance

Analysis 2.3. (Continued)

(3) Vallée du Kou, Permanet 3.0, HIgh resistance
(4) Pitoa, Permanet 3.0, Low resistance
(5) Malanville, Permanet 3.0, Low resistance
(6) Yaokoffikro, Permanet 3.0, High resistance
(7) Mibellon, PermaNet 3.0, moderate resistance, *An funestus*
(8) Mibellon, Olyset Plus, Moderate resistance, *An funestus*
(9) Ifakara, Veeralin, Unclassified, *An funestus*
(10) Ifakara, Veeralin, Susceptible, *An arabiensis*
(11) Akron, Permanet 3.0, Moderate resistance
(12) Malanville, Olyset Plus, High resistance
(13) Vallee du Kou 5 ,Olyset Plus, High resistance
(14) Tengrela, Permanet 3.0, High resistance
(15) Tengrela, Olyset Plus, High resistance
(16) Vallee du Kou 5, Permanet 3.0, High resistance
(17) Zeneti, Permanet 3.0, Susceptible

Analysis 2.4. Comparison 2: Commercial pyrethroid-PBO nets versus commercial LLINs: hut trials, Outcome 4: Mosquito mortality (high resistance) hut/night (adjusted ICC 0.1)

Study or Subgroup	PBO-LLINs Events	PBO-LLINs Total	LLINs Events	LLINs Total	Weight	Risk Ratio M-H, Random, 95% CI
2.4.1 Unwashed						
Bayili 2017 (1)	130	302	83	553	7.3%	2.87 [2.26 , 3.64]
Bayili 2017 (2)	92	425	72	482	6.9%	1.45 [1.10 , 1.92]
Corbel 2010 (3)	181	232	73	165	7.7%	1.76 [1.47 , 2.12]
Koudou 2011 (4)	117	214	78	224	7.4%	1.57 [1.26 , 1.95]
Pennetier 2013 (5)	53	66	39	95	7.0%	1.96 [1.49 , 2.56]
Toé 2018 (6)	62	221	57	293	6.5%	1.44 [1.05 , 1.97]
Toé 2018 (7)	116	249	52	269	6.9%	2.41 [1.83 , 3.18]
Toé 2018 (8)	125	199	116	325	7.8%	1.76 [1.47 , 2.11]
Toé 2018 (9)	146	272	97	310	7.6%	1.72 [1.41 , 2.09]
Subtotal (95% CI)		**2180**		**2716**	**65.0%**	**1.84 [1.60 , 2.11]**
Total events:	1022		667			
Heterogeneity: Tau² = 0.03; Chi² = 25.39, df = 8 (P = 0.001); I² = 68%						
Test for overall effect: Z = 8.65 (P < 0.00001)						
2.4.2 Washed						
Bayili 2017 (2)	83	474	86	515	6.9%	1.05 [0.80 , 1.38]
Bayili 2017 (1)	65	420	92	550	6.7%	0.93 [0.69 , 1.24]
Corbel 2010 (3)	119	241	79	263	7.4%	1.64 [1.31 , 2.06]
Koudou 2011 (3)	72	224	77	201	7.1%	0.84 [0.65 , 1.09]
Pennetier 2013 (3)	64	96	43	117	6.9%	1.81 [1.38 , 2.39]
Subtotal (95% CI)		**1455**		**1646**	**35.0%**	**1.20 [0.88 , 1.63]**
Total events:	403		377			
Heterogeneity: Tau² = 0.11; Chi² = 27.69, df = 4 (P < 0.0001); I² = 86%						
Test for overall effect: Z = 1.14 (P = 0.25)						
Total (95% CI)		**3635**		**4362**	**100.0%**	**1.58 [1.34 , 1.86]**
Total events:	1425		1044			
Heterogeneity: Tau² = 0.08; Chi² = 84.86, df = 13 (P < 0.00001); I² = 85%						
Test for overall effect: Z = 5.46 (P < 0.00001)						
Test for subgroup differences: Chi² = 6.11, df = 1 (P = 0.01), I² = 83.6%						

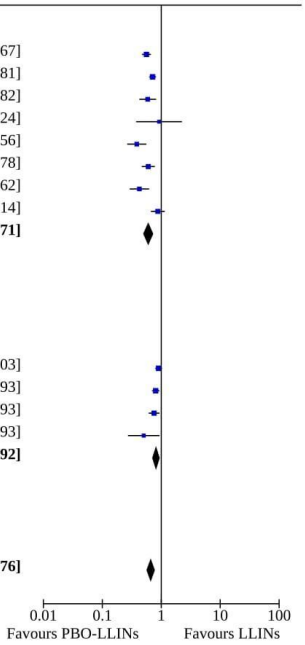


Footnotes

(1) Valle du Kou, DawaPlus 4.0, High resistance
(2) Valle du Kou, DawaPlus 3.0, High resistance
(3) Vallée du Kou, Permanet 3.0, High resistance
(4) Yaokoffikro, Permanet 3.0, High resistance
(5) Malanaville, Olyset Plus, High resistance
(6) Tengrela, Olyset Plus, High resistance
(7) Tengrela, Permanet 3.0, High resistance
(8) Vallee du Kou 5, Permanet 3.0, High resistance
(9) Vallee du Kou 5, Olyset Plus, High resistance

Analysis 2.5. Comparison 2: Commercial pyrethroid-PBO nets versus commercial LLINs: hut trials, Outcome 5: Mosquito blood-feeding success (high resistance) hut/night (adjusted ICC 0.1)

Study or Subgroup	PBO-LLINs Events	PBO-LLINs Total	LLINs Events	LLINs Total	Weight	Risk Ratio M-H, Random, 95% CI
2.5.1 Unwashed						
Bayili 2017 (1)	102	302	333	553	10.8%	0.56 [0.47 , 0.67]
Bayili 2017 (2)	182	425	290	482	11.5%	0.71 [0.62 , 0.81]
Corbel 2010 (3)	48	232	58	165	7.6%	0.59 [0.42 , 0.82]
Pennetier 2013 (4)	7	66	11	95	2.1%	0.92 [0.37 , 2.24]
Toé 2018 (5)	28	199	119	325	6.8%	0.38 [0.26 , 0.56]
Toé 2018 (6)	63	249	113	269	9.0%	0.60 [0.47 , 0.78]
Toé 2018 (7)	31	272	83	310	6.7%	0.43 [0.29 , 0.62]
Toé 2018 (8)	62	221	94	293	8.8%	0.87 [0.67 , 1.14]
Subtotal (95% CI)		**1966**		**2492**	**63.3%**	**0.60 [0.50 , 0.71]**
Total events:	523		1101			
Heterogeneity: Tau² = 0.04; Chi² = 23.11, df = 7 (P = 0.002); I² = 70%						
Test for overall effect: Z = 5.76 (P < 0.00001)						
2.5.2 Washed						
Bayili 2017 (2)	215	474	259	515	11.5%	0.90 [0.79 , 1.03]
Bayili 2017 (1)	170	420	277	550	11.3%	0.80 [0.70 , 0.93]
Corbel 2010 (3)	88	241	127	263	10.0%	0.76 [0.61 , 0.93]
Pennetier 2013 (4)	12	96	29	117	3.7%	0.50 [0.27 , 0.93]
Subtotal (95% CI)		**1231**		**1445**	**36.7%**	**0.81 [0.72 , 0.92]**
Total events:	485		692			
Heterogeneity: Tau² = 0.01; Chi² = 5.07, df = 3 (P = 0.17); I² = 41%						
Test for overall effect: Z = 3.24 (P = 0.001)						
Total (95% CI)		**3197**		**3937**	**100.0%**	**0.66 [0.57 , 0.76]**
Total events:	1008		1793			
Heterogeneity: Tau² = 0.04; Chi² = 48.33, df = 11 (P < 0.00001); I² = 77%						
Test for overall effect: Z = 5.72 (P < 0.00001)						
Test for subgroup differences: Chi² = 7.70, df = 1 (P = 0.006), I² = 87.0%						

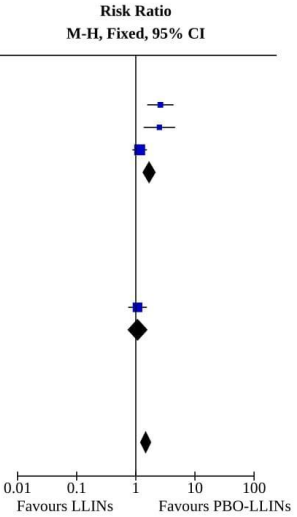


Footnotes

(1) Vallee du Kou, DawaPlus 4.0, High resistance
(2) Vallee du Kou, DawaPlus 3.0, High resistance
(3) Vallée du Kou, Permanet 3.0, High resistance
(4) Malanville, Olyset Plus, High resistance
(5) Vallee du Kou 5, Permanet 3.0, High resistance
(6) Tengrela, Permanet 3.0, High resistance
(7) Vallee du Kou 5, Olyset Plus, High resistance
(8) Tengrela, Olyset Plus, High resistance

Analysis 2.6. Comparison 2: Commercial pyrethroid-PBO nets versus commercial LLINs: hut trials, Outcome 6: Mosquito mortality (moderate resistance) hut/night (adjusted ICC 0.1)

Study or Subgroup	PBO-LLINs Events	PBO-LLINs Total	LLINs Events	LLINs Total	Weight	Risk Ratio M-H, Fixed, 95% CI
2.6.1 Unwashed						
Menze 2020 (1)	32	106	19	164	13.0%	2.61 [1.56 , 4.35]
Menze 2020 (2)	34	140	12	124	11.1%	2.51 [1.36 , 4.63]
N'Guessan 2010 (3)	59	115	45	102	41.5%	1.16 [0.88 , 1.54]
Subtotal (95% CI)		**361**		**390**	**65.6%**	**1.68 [1.33 , 2.11]**
Total events:	125		76			
Heterogeneity: Chi² = 10.98, df = 2 (P = 0.004); I² = 82%						
Test for overall effect: Z = 4.35 (P < 0.0001)						
2.6.2 Washed						
N'Guessan 2010 (3)	40	130	42	146	34.4%	1.07 [0.74 , 1.54]
Subtotal (95% CI)		**130**		**146**	**34.4%**	**1.07 [0.74 , 1.54]**
Total events:	40		42			
Heterogeneity: Not applicable						
Test for overall effect: Z = 0.36 (P = 0.72)						
Total (95% CI)		**491**		**536**	**100.0%**	**1.47 [1.21 , 1.78]**
Total events:	165		118			
Heterogeneity: Chi² = 13.31, df = 3 (P = 0.004); I² = 77%						
Test for overall effect: Z = 3.86 (P = 0.0001)						
Test for subgroup differences: Chi² = 4.17, df = 1 (P = 0.04), I² = 76.0%						

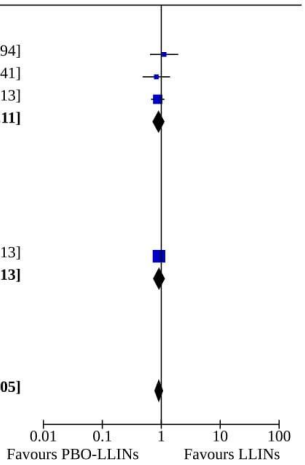


Footnotes

(1) Mibellon, PermaNet 3.0, moderate resistance, *An funestus*
(2) Mibellon, Olyset Plus, Moderate resistance, *An funestus*
(3) Akron, Permanet 3.0, Moderate resistance

Analysis 2.7. Comparison 2: Commercial pyrethroid-PBO nets versus commercial LLINs: hut trials, Outcome 7: Mosquito blood-feeding success (moderate resistance) hut/night (adjusted ICC 0.1)

Study or Subgroup	PBO-LLINs Events	PBO-LLINs Total	LLINs Events	LLINs Total	Weight	Risk Ratio M-H, Random, 95% CI
2.7.1 Unwashed						
Menze 2020 (1)	24	140	19	124	7.4%	1.12 [0.64 , 1.94]
Menze 2020 (2)	17	106	32	165	7.8%	0.83 [0.48 , 1.41]
N'Guessan 2010 (3)	55	115	56	102	33.3%	0.87 [0.67 , 1.13]
Subtotal (95% CI)		**361**		**391**	**48.5%**	**0.90 [0.72 , 1.11]**
Total events:	96		107			
Heterogeneity: Tau² = 0.00; Chi² = 0.77, df = 2 (P = 0.68); I² = 0%						
Test for overall effect: Z = 0.99 (P = 0.32)						
2.7.2 Washed						
N'Guessan 2010 (3)	71	133	87	149	51.5%	0.91 [0.74 , 1.13]
Subtotal (95% CI)		**133**		**149**	**51.5%**	**0.91 [0.74 , 1.13]**
Total events:	71		87			
Heterogeneity: Not applicable						
Test for overall effect: Z = 0.84 (P = 0.40)						
Total (95% CI)		**494**		**540**	**100.0%**	**0.91 [0.78 , 1.05]**
Total events:	167		194			
Heterogeneity: Tau² = 0.00; Chi² = 0.77, df = 3 (P = 0.86); I² = 0%						
Test for overall effect: Z = 1.29 (P = 0.20)						
Test for subgroup differences: Chi² = 0.01, df = 1 (P = 0.90), I² = 0%						

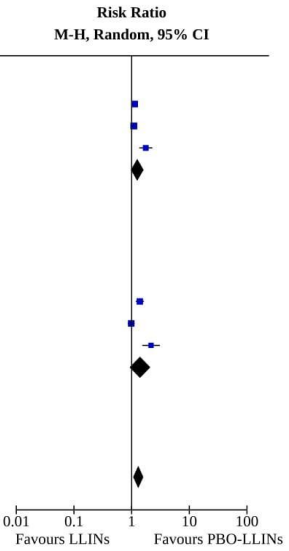


Footnotes

(1) Mibellon, Olyset Plus, Moderate resistance, *An funestus*
(2) Mibellon, PermaNet 3.0, moderate resistance, *An funestus*
(3) Akron, Permanet 3.0, Moderate resistance

Analysis 2.8. Comparison 2: Commercial pyrethroid-PBO nets versus commercial LLINs: hut trials, Outcome 8: Mosquito mortality (low resistance) hut/night (adjusted ICC 0.1)

Study or Subgroup	PBO-LLINs Events	PBO-LLINs Total	LLINs Events	LLINs Total	Weight	Risk Ratio M-H, Random, 95% CI
2.8.1 Unwashed						
Corbel 2010 (1)	110	117	70	84	18.9%	1.13 [1.01 , 1.25]
Corbel 2010 (2)	170	176	177	200	19.8%	1.09 [1.03 , 1.16]
Oumbouke 2019	79	156	62	215	14.2%	1.76 [1.35 , 2.28]
Subtotal (95% CI)		**449**		**499**	**53.0%**	**1.25 [0.99 , 1.57]**
Total events:	359		309			
Heterogeneity: Tau² = 0.03; Chi² = 23.55, df = 2 (P < 0.00001); I² = 92%						
Test for overall effect: Z = 1.91 (P = 0.06)						
2.8.2 Washed						
Corbel 2010 (1)	82	105	112	199	17.5%	1.39 [1.18 , 1.63]
Corbel 2010 (2)	124	178	117	165	18.1%	0.98 [0.86 , 1.13]
Oumbouke 2019	60	158	38	217	11.4%	2.17 [1.53 , 3.08]
Subtotal (95% CI)		**441**		**581**	**47.0%**	**1.39 [0.95 , 2.04]**
Total events:	266		267			
Heterogeneity: Tau² = 0.10; Chi² = 23.97, df = 2 (P < 0.00001); I² = 92%						
Test for overall effect: Z = 1.70 (P = 0.09)						
Total (95% CI)		**890**		**1080**	**100.0%**	**1.30 [1.09 , 1.56]**
Total events:	625		576			
Heterogeneity: Tau² = 0.04; Chi² = 53.37, df = 5 (P < 0.00001); I² = 91%						
Test for overall effect: Z = 2.86 (P = 0.004)						
Test for subgroup differences: Chi² = 0.23, df = 1 (P = 0.63), I² = 0%						



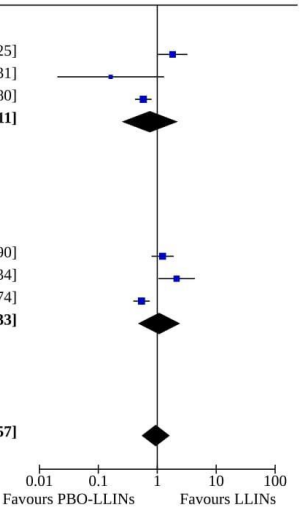
Footnotes

(1) Pitoa, Permanet 3.0, Low resistance

(2) Malanville, Permanet 3.0, Low resistance

Analysis 2.9. Comparison 2: Commercial pyrethroid-PBO nets versus commercial LLINs: hut trials, Outcome 9: Mosquito blood-feeding success (low resistance) hut/night (adjusted ICC 0.1)

Study or Subgroup	PBO-LLINs Events	PBO-LLINs Total	LLINs Events	LLINs Total	Weight	Risk Ratio M-H, Random, 95% CI
2.9.1 Unwashed						
Corbel 2010 (1)	33	117	13	84	17.7%	1.82 [1.02 , 3.25]
Corbel 2010 (2)	1	176	7	200	4.8%	0.16 [0.02 , 1.31]
Oumbouke 2019	37	156	88	215	20.9%	0.58 [0.42 , 0.80]
Subtotal (95% CI)		**449**		**499**	**43.5%**	**0.75 [0.27 , 2.11]**
Total events:	71		108			
Heterogeneity: Tau² = 0.61; Chi² = 13.59, df = 2 (P = 0.001); I² = 85%						
Test for overall effect: Z = 0.55 (P = 0.58)						
2.9.2 Washed						
Corbel 2010 (1)	26	105	40	199	19.6%	1.23 [0.80 , 1.90]
Corbel 2010 (2)	23	178	10	165	15.9%	2.13 [1.05 , 4.34]
Oumbouke 2019	37	158	94	217	21.0%	0.54 [0.39 , 0.74]
Subtotal (95% CI)		**441**		**581**	**56.5%**	**1.07 [0.49 , 2.33]**
Total events:	86		144			
Heterogeneity: Tau² = 0.40; Chi² = 16.87, df = 2 (P = 0.0002); I² = 88%						
Test for overall effect: Z = 0.18 (P = 0.86)						
Total (95% CI)		**890**		**1080**	**100.0%**	**0.94 [0.56 , 1.57]**
Total events:	157		252			
Heterogeneity: Tau² = 0.30; Chi² = 30.72, df = 5 (P < 0.0001); I² = 84%						
Test for overall effect: Z = 0.24 (P = 0.81)						
Test for subgroup differences: Chi² = 0.30, df = 1 (P = 0.59), I² = 0%						



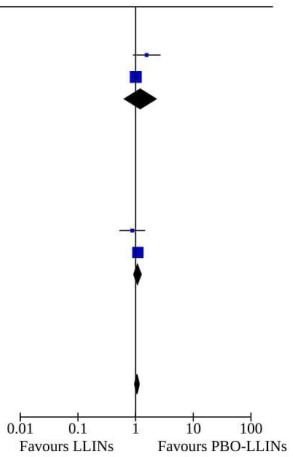
Footnotes

(1) Pitoa, Permanet 3.0, Low resistance

(2) Malanville, Permanet 3.0, Low resistance

Analysis 2.10. Comparison 2: Commercial pyrethroid-PBO nets versus commercial LLINs: hut trials, Outcome 10: Mosquito mortality (susceptible) hut/night (adjusted ICC 0.1)

Study or Subgroup	PBO-LLINs Events	PBO-LLINs Total	LLINs Events	LLINs Total	Weight	Risk Ratio M-H, Random, 95% CI
2.10.1 Unwashed						
Moore 2016 (1)	23	161	22	239	2.7%	1.55 [0.90 , 2.69]
Tungu 2010 (2)	223	233	300	315	48.8%	1.00 [0.97 , 1.04]
Subtotal (95% CI)		**394**		**554**	**51.5%**	**1.20 [0.64 , 2.26]**
Total events:	246		322			
Heterogeneity: Tau² = 0.18; Chi² = 5.47, df = 1 (P = 0.02); I² = 82%						
Test for overall effect: Z = 0.57 (P = 0.57)						
2.10.2 Washed						
Moore 2016 (3)	23	186	28	198	3.0%	0.87 [0.52 , 1.46]
Tungu 2010 (2)	271	285	260	299	45.5%	1.09 [1.04 , 1.15]
Subtotal (95% CI)		**471**		**497**	**48.5%**	**1.07 [0.92 , 1.25]**
Total events:	294		288			
Heterogeneity: Tau² = 0.01; Chi² = 1.16, df = 1 (P = 0.28); I² = 14%						
Test for overall effect: Z = 0.93 (P = 0.35)						
Total (95% CI)		**865**		**1051**	**100.0%**	**1.05 [0.96 , 1.15]**
Total events:	540		610			
Heterogeneity: Tau² = 0.00; Chi² = 11.29, df = 3 (P = 0.01); I² = 73%						
Test for overall effect: Z = 1.08 (P = 0.28)						
Test for subgroup differences: Chi² = 0.11, df = 1 (P = 0.74), I² = 0%						



Footnotes

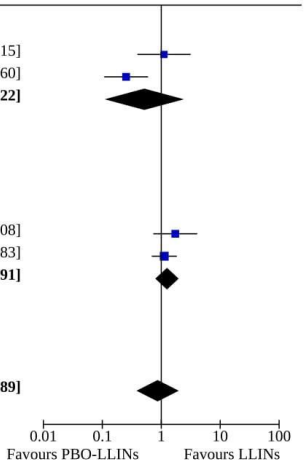
(1) Ifakara, Veeralin, Susceptible, *An arabiensis.* The population was resistant to deltamethrin and permethrin.

(2) Zeneti, Permanet 3.0, Susceptible

(3) Ifakara, Veeralin, Susceptible, *An. arabiensis.* The population was resistant to deltamethrin and permethrin.

Analysis 2.11. Comparison 2: Commercial pyrethroid-PBO nets versus commercial LLINs: hut trials, Outcome 11: Mosquito blood-feeding success (susceptible) hut/night (adjusted ICC 0.1)

Study or Subgroup	PBO-LLINs Events	PBO-LLINs Total	LLINs Events	LLINs Total	Weight	Risk Ratio M-H, Random, 95% CI
2.11.1 Unwashed						
Moore 2016 (1)	6	161	8	239	21.3%	1.11 [0.39 , 3.15]
Tungu 2010 (2)	6	233	32	315	24.3%	0.25 [0.11 , 0.60]
Subtotal (95% CI)		**394**		**554**	**45.6%**	**0.52 [0.12 , 2.22]**
Total events:	12		40			
Heterogeneity: Tau² = 0.88; Chi² = 4.73, df = 1 (P = 0.03); I² = 79%						
Test for overall effect: Z = 0.89 (P = 0.37)						
2.11.2 Washed						
Moore 2016 (3)	13	186	8	198	24.2%	1.73 [0.73 , 4.08]
Tungu 2010 (2)	30	285	28	299	30.2%	1.12 [0.69 , 1.83]
Subtotal (95% CI)		**471**		**497**	**54.4%**	**1.25 [0.82 , 1.91]**
Total events:	43		36			
Heterogeneity: Tau² = 0.00; Chi² = 0.73, df = 1 (P = 0.39); I² = 0%						
Test for overall effect: Z = 1.03 (P = 0.30)						
Total (95% CI)		**865**		**1051**	**100.0%**	**0.87 [0.40 , 1.89]**
Total events:	55		76			
Heterogeneity: Tau² = 0.46; Chi² = 11.86, df = 3 (P = 0.008); I² = 75%						
Test for overall effect: Z = 0.36 (P = 0.72)						
Test for subgroup differences: Chi² = 1.30, df = 1 (P = 0.25), I² = 23.0%						

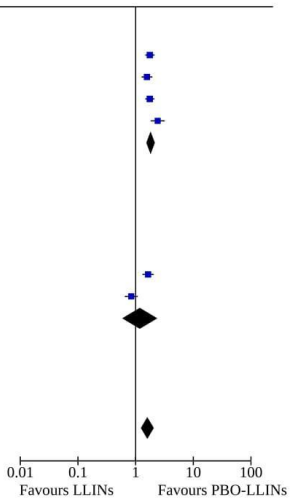


Footnotes

(1) Ifakara, Veeralin, Susceptible, *An arabiensis*. The population was resistant to deltamethrin and permethrin.
(2) Zeneti, Permanet 3.0, Susceptible
(3) Ifakara, Veeralin, Susceptible, *An. arabiensis*. The population was resistant to deltamethrin and permethrin.

Analysis 2.12. Comparison 2: Commercial pyrethroid-PBO nets versus commercial LLINs: hut trials, Outcome 12: Mosquito mortality (high resistance/Permanet) hut/night (adjusted ICC 0.1)

Study or Subgroup	PBO-LLINs Events	PBO-LLINs Total	LLINs Events	LLINs Total	Weight	Risk Ratio M-H, Random, 95% CI
2.12.1 Not Washed						
Corbel 2010 (1)	181	232	73	165	17.6%	1.76 [1.47 , 2.12]
Koudou 2011 (2)	117	214	78	224	16.9%	1.57 [1.26 , 1.95]
Toé 2018 (3)	125	199	116	325	17.6%	1.76 [1.47 , 2.11]
Toé 2018 (4)	116	249	52	269	15.4%	2.41 [1.83 , 3.18]
Subtotal (95% CI)		**894**		**983**	**67.4%**	**1.81 [1.56 , 2.10]**
Total events:	539		319			
Heterogeneity: Tau² = 0.01; Chi² = 5.95, df = 3 (P = 0.11); I² = 50%						
Test for overall effect: Z = 7.88 (P < 0.00001)						
2.12.2 Washed						
Corbel 2010 (1)	119	241	79	263	16.7%	1.64 [1.31 , 2.06]
Koudou 2011 (1)	72	224	77	201	15.9%	0.84 [0.65 , 1.09]
Subtotal (95% CI)		**465**		**464**	**32.6%**	**1.18 [0.61 , 2.28]**
Total events:	191		156			
Heterogeneity: Tau² = 0.21; Chi² = 14.81, df = 1 (P = 0.0001); I² = 93%						
Test for overall effect: Z = 0.49 (P = 0.63)						
Total (95% CI)		**1359**		**1447**	**100.0%**	**1.59 [1.26 , 2.01]**
Total events:	730		475			
Heterogeneity: Tau² = 0.07; Chi² = 34.38, df = 5 (P < 0.00001); I² = 85%						
Test for overall effect: Z = 3.90 (P < 0.0001)						
Test for subgroup differences: Chi² = 1.57, df = 1 (P = 0.21), I² = 36.2%						

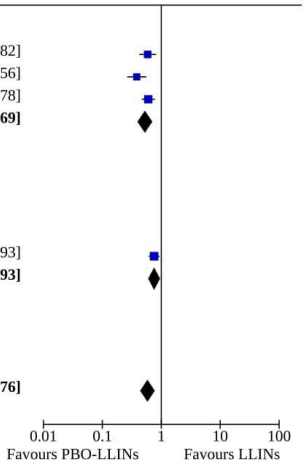


Footnotes

(1) Vallée du Kou, Permanet 3.0, High resistance
(2) Yaokoffikro, Permanet 3.0, High resistance
(3) Vallee du Kou 5, Permanet 3.0, High resistance
(4) Tengrela, Permanet 3.0, High resistance

Analysis 2.13. Comparison 2: Commercial pyrethroid-PBO nets versus commercial LLINs: hut trials, Outcome 13: Mosquito blood-feeding success (high resistance/Permanet) hut/night (adjusted ICC 0.1)

Study or Subgroup	PBO-LLINs Events	PBO-LLINs Total	LLINs Events	LLINs Total	Weight	Risk Ratio M-H, Random, 95% CI
2.13.1 Unwashed						
Corbel 2010 (1)	48	232	58	165	23.1%	0.59 [0.42 , 0.82]
Toé 2018 (2)	28	199	119	325	20.8%	0.38 [0.26 , 0.56]
Toé 2018 (3)	63	249	113	269	26.8%	0.60 [0.47 , 0.78]
Subtotal (95% CI)		**680**		**759**	**70.7%**	**0.53 [0.40 , 0.69]**
Total events:	139		290			
Heterogeneity: Tau² = 0.03; Chi² = 4.29, df = 2 (P = 0.12); I² = 53%						
Test for overall effect: Z = 4.72 (P < 0.00001)						
2.13.2 Washed						
Corbel 2010 (1)	88	241	127	263	29.3%	0.76 [0.61 , 0.93]
Subtotal (95% CI)		**241**		**263**	**29.3%**	**0.76 [0.61 , 0.93]**
Total events:	88		127			
Heterogeneity: Not applicable						
Test for overall effect: Z = 2.63 (P = 0.009)						
Total (95% CI)		**921**		**1022**	**100.0%**	**0.58 [0.45 , 0.76]**
Total events:	227		417			
Heterogeneity: Tau² = 0.05; Chi² = 10.34, df = 3 (P = 0.02); I² = 71%						
Test for overall effect: Z = 4.04 (P < 0.0001)						
Test for subgroup differences: Chi² = 4.40, df = 1 (P = 0.04), I² = 77.2%						

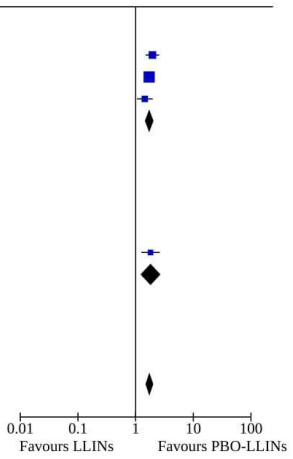


Footnotes

(1) Vallée du Kou, Permanet 3.0, High resistance
(2) Vallee du Kou 5, Permanet 3.0, High resistance
(3) Tengrela, Permanet 3.0, High resistance

Analysis 2.14. Comparison 2: Commercial pyrethroid-PBO nets versus commercial LLINs: hut trials, Outcome 14: Mosquito mortality (high resistance/Olyset) hut/night (adjusted ICC 0.1)

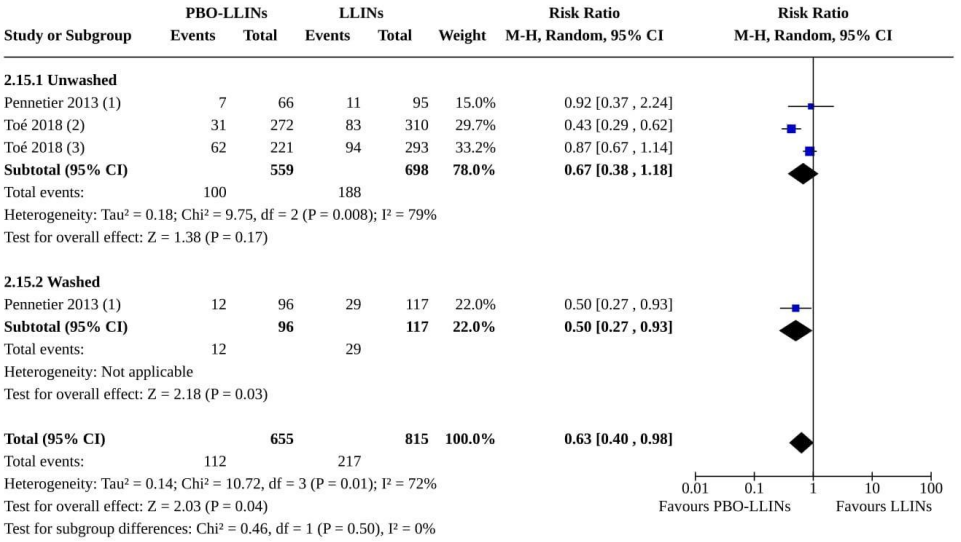
Study or Subgroup	PBO-LLINs Events	PBO-LLINs Total	LLINs Events	LLINs Total	Weight	Risk Ratio M-H, Random, 95% CI
2.14.1 Unwashed						
Pennetier 2013 (1)	53	66	39	95	24.4%	1.96 [1.49 , 2.56]
Toé 2018 (2)	146	272	97	310	44.8%	1.72 [1.41 , 2.09]
Toé 2018 (3)	62	221	57	293	17.8%	1.44 [1.05 , 1.97]
Subtotal (95% CI)		**559**		**698**	**87.0%**	**1.72 [1.48 , 1.99]**
Total events:	261		193			
Heterogeneity: Tau² = 0.00; Chi² = 2.17, df = 2 (P = 0.34); I² = 8%						
Test for overall effect: Z = 7.08 (P < 0.00001)						
2.14.2 Washed						
Pennetier 2013	64	96	21	57	13.0%	1.81 [1.25 , 2.61]
Subtotal (95% CI)		**96**		**57**	**13.0%**	**1.81 [1.25 , 2.61]**
Total events:	64		21			
Heterogeneity: Not applicable						
Test for overall effect: Z = 3.16 (P = 0.002)						
Total (95% CI)		**655**		**755**	**100.0%**	**1.73 [1.51 , 1.97]**
Total events:	325		214			
Heterogeneity: Tau² = 0.00; Chi² = 2.22, df = 3 (P = 0.53); I² = 0%						
Test for overall effect: Z = 8.08 (P < 0.00001)						
Test for subgroup differences: Chi² = 0.07, df = 1 (P = 0.79), I² = 0%						



Footnotes

(1) Malanaville, Olyset Plus, High resistance
(2) Vallee du Kou 5, Olyset Plus, High resistance
(3) Tengrela, Olyset Plus, High resistance

Analysis 2.15. Comparison 2: Commercial pyrethroid-PBO nets versus commercial LLINs: hut trials, Outcome 15: Mosquito blood-feeding success (high resistance/Olyset) hut/night (adjusted ICC 0.1)



Footnotes

(1) Malanville, Olyset Plus, High resistance
(2) Vallee du Kou 5, Olyset Plus, High resistance
(3) Tengrela, Olyset Plus, High resistance

ADDITIONAL TABLES

Table 1. World Health Organization Pesticide Evaluation Scheme (WHOPES) classification

WHOPES Phase	Definition
WHOPES Phase I. Laboratory bioassays	Cone bioassays: these studies are conducted in the laboratory setting and use standard WHO protocols (WHO 2013, Section 2.2.1), when mosquitoes are exposed to a suitable LLIN (treated intervention or untreated control) for three minutes using a standard plastic WHO cone. Following net exposure, mosquitoes are transferred to a holding container and are maintained on a sugar solution diet while entomological outcomes (mosquitoes knocked down 1 hour post exposure, and mosquito mortality 24 hours post exposure) are measured. Tunnel tests: these studies are conducted in the laboratory setting and use standard WHO protocols (WHO 2013, Section 2.2.2). Mosquitoes are released into a glass tunnel covered at each end with untreated netting. The intervention or control LLIN net sample is placed one-third down the length of the tunnel, and the net contains 9 holes that enable mosquitoes to pass through. A suitable bait is immobilized in the shorter section of the tunnel, where it is available for mosquito biting. Mosquitoes are released into the opposite end of the tunnel and must make contact with the net and locate holes before they are able to feed on the bait. After 12 to 15 hours, mosquitoes are removed from both sections of the tunnel, and entomological outcomes (the number of mosquitoes in each section, mortality, and blood-feeding inhibition at the end of the assay and 24 hours post exposure) are recorded.

Table 1. World Health Organization Pesticide Evaluation Scheme (WHOPES) classification *(Continued)*

	Wire-ball bioassays: these studies are conducted in the laboratory setting, where mosquitoes are introduced into a wire-ball frame that has been covered with the intervention or control LLIN. Mosquitoes are exposed for 3 minutes, after which they are transferred to a holding container, and entomological outcomes (mosquitoes knocked down 1 hour post exposure, and mosquito mortality 24 hours post exposure) are measured.
WHOPES Phase II. Experimental hut trials	WHOPES Phase II experimental hut trials are field trials conducted in Africa where wild mosquito populations or local colonized populations are evaluated. Volunteers or livestock sleep in experimental huts under a purposefully holed LLIN, with 1 person or animal per hut. Huts are designed to resemble local housing based on a West or East African design (WHO 2013; Section 3.3.1-2). However these trials have identical design features, such as eave gaps or entry slits to allow mosquitoes to enter, and exit traps to capture exiting mosquitoes. LLINs and volunteers are randomly allocated to huts and are rotated in a Latin square to avoid bias, with huts cleaned between rotations to avoid contamination. Several nets, including an untreated control net, can be tested at the same time. Dead and live mosquitoes are collected each morning from inside the net, inside the hut, and inside the exit traps. They are then scored as blood-fed or non-blood-fed, and as alive or dead, and live mosquitoes are maintained for a further 24 hours to assess delayed mosquito mortality.
WHOPES Phase III. Village trials	WHOPES Phase III village trials are conducted in Africa where wild mosquito populations are evaluated. Villages chosen to be included in the study are similar in terms of size, housing structure, location, and data available on insecticide resistance status of local malaria vectors. Households are assigned as conventional LLINs or PBO-LLINs. Randomization can be done at the household or village level. Adult mosquitoes are collected from study houses, and mosquito density is measured. An indication of malaria transmission is measured at the study sites by recording infections in mosquitoes, parasite prevalence, or malaria incidence.

LLIN: long-lasting insecticidal nets; PBO: piperonyl butoxide; WHOPES: World Health Organization Pesticide Evaluation Scheme.

Table 2. World Health Organization (WHO)-recommended long-lasting insecticidal nets (LLINs)

Product name	Product type	Status of WHO recommendation
DawaPlus 2.0	Deltamethrin coated on polyester	Interim
DawaPlus 3.0	Combination of deltamethrin coated onto polyester (side panels) and deltamethrin and PBO incorporated into polyester (roof)	Interim
DawaPlus 4.0	Deltamethrin and PBO incorporated into polyester	Interim
Duranet	Alpha-cypermethrin incorporated into polyethylene	Full
Interceptor	Alpha-cypermethrin coated on polyester	Full
Interceptor G2	Alpha-cypermethrin and chlorfenapyr incorporated into polyester	Interim
LifeNet	Deltamethrin incorporated into polypropylene	Interim
MAGNet	Alpha-cypermethrin incorporated into polyethylene	Full
MiraNet	Alpha-cypermethrin incorporated into polyethylene	Interim
Olyset Net	Permethrin incorporated into polyethylene	Full
Olyset Plus	Permethrin (20 g/kg) and PBO (10 g/kg) incorporated into polyethylene	Interim
Panda Net 2.0	Deltamethrin incorporated into polyethylene	Interim

Table 2. World Health Organization (WHO)-recommended long-lasting insecticidal nets (LLINs) *(Continued)*

PermaNet 2.0	Deltamethrin coated on polyester	Full
PermaNet 3.0	Combination of deltamethrin coated on polyester with strengthened border (side panels) and deltamethrin and PBO incorporated into polyethylene (roof)	Interim
Royal Sentry	Alpha-cypermethrin incorporated into polyethylene	Full
SafeNet	Alpha-cypermethrin coated on polyester	Full
Veeralin	Alpha-cypermethrin and PBO incorporated into polyethylene	Interim
Yahe	Deltamethrin coated on polyester	Interim
Yorkool	Deltamethrin coated on polyester	Full

LLIN: long-lasting insecticidal net; PBO: piperonyl butoxide; WHO: World Health Organization.

Table 3. World Health Organization (WHO)-recommended insecticide products for treatment of mosquito nets for malaria vector control

Insecticide	Formulation	Dosage[a]
Alpha-cypermethrin	SC 10%	20 to 40
Cyfluthrin	EW 5%	50
Deltamethrin	SC 1% WT 25% WT 25% + binder[b]	15 to 25
Etofenprox	EW 10%	200
Lambda-cyhalothrin	CS 2.5%	10 to 15
Permethrin	EC 10%	200 to 500

EC: emulsifiable concentrate; EW: emulsion, oil in water; CS: capsule suspension; SC: suspension concentrate; WT: water dispersible tablet.
[a]Active ingredient/netting (mg/m^2).
[b]K-O TAB 1-2-3.

Table 4. Definition of resistance level

Outcome	Confirmed resistance	Suspected resistance	Susceptible	Unclassified
WHO mosquito mortality[a]	< 90%	90% to 97%	98% to 100%	Unknown
CDC knock-down[b]	< 90%	80% to 97%	98% to 100%	Unknown

CDC: Centers for Disease Control and Prevention; WHO: World Health Organization.
[a]Definition of resistance level based on mosquito mortality (%) after exposure to insecticide in a WHO diagnostic dose assay.
[b]Definition of resistance level based on mosquito mortality (%) after exposure to insecticide in a CDC bottle bioassay using the methods, diagnostic doses, and diagnostic times recommended by each test respectively.

Table 5. Stratification of resistance level

Outcome	Low	Moderate	High	Unclassified
Mosquito mortality[a]	61% to 90%	31% to 60%	< 30%	Unknown

[a]24-hour post-exposure mortality (%).

Table 6. Study inclusion screening form

Criteria	Assessment			Comments
	Yes	No	Unclear	
Mosquito population				
Did the study test *Anopheles gambiae* complex or *Anopheles funestus* group mosquitoes?	↓	—	↓	State mosquito species
Were a minimum of 50 mosquitoes tested per study arm?	↓	—	↓	
Intervention				
Did the study include a long-lasting insecticidal net (LLIN) or insecticide-treated net (ITN)?	↓	—	↓	State net LLIN or ITN
Was the intervention net either of the following? 1. A piperonyl butoxide (PBO) LLIN that received a minimum of interim World Health Organization (WHO) approval.	↓	—	↓	State net type
Was the control net either of the following? 1. A pyrethroid LLIN of the same fabric impregnated with the same insecticide and dose as the intervention net. 2. A pyrethroid LLIN impregnated with the same insecticide at any dose.	↓	—	↓	State which objective study meets
Study design				
Was the study one of the following? 1. Experimental hut study 2. Village trial	↓	—	↓	State study type
For experimental hut study and village trial. Was the study conducted in Africa?	↓	—	↓	State country
Outcome				
Did the study include at least 1 of the following outcome measures? 1. Mortality	↓	—	↓	

Table 6. Study inclusion screening form *(Continued)*

2. Blood feeding
3. Sporozoite rate
4. Not passed through the net
5. Deterrence
6. Exophily
7. Mosquito density
8. Parity rate

Decision				
Is the study eligible for inclusion?	—	—	↓	State reason(s) for exclusion
	Discuss with authors			

ITN: insecticide-treated net; LLIN: long-lasting insecticidal net; PBO: piperonyl butoxide; WHO: World Health Organization.

Table 7. Experimental hut trials: deterrence data

Study ID	Locality	Net type	Net washed	Total number in ITN hut	Total number in UTN hut	Deterrence (%) reported	Deterrence (%) calculated
Bayili 2017	Vallée du Kou	DawaPlus 2.0	No	1548	1848	16.23	16.23
Bayili 2017	Vallée du Kou	DawaPlus 2.0	Yes	2155	1848	0	-16.61
Bayili 2017	Vallée du Kou	DawaPlus 3.0	No	1365	1848	26.13	26.14
Bayili 2017	Vallée du Kou	DawaPlus 3.0	Yes	1981	1848	0	-7.20
Bayili 2017	Vallée du Kou	DawaPlus 4.0	No	846	1848	54.22	54.22
Bayili 2017	Vallée du Kou	DawaPlus 4.0	Yes	1646	1848	10.93	10.93
Corbel 2010	Malanville	Permanet 2.0	Yes	195	285	31.58	31.58
Corbel 2010	Malanville	Permanet 3.0	Yes	210	285	26.32	26.32
Corbel 2010	Malanville	Permanet 2.0	No	243	285	14.74	14.74
Corbel 2010	Malanville	Permanet 3.0	No	214	285	24.91	24.91
Corbel 2010	Pitoa	Permanet 2.0	Yes	310	401	22.69	22.69
Corbel 2010	Pitoa	Permanet 3.0	Yes	163	401	59.35	59.35
Corbel 2010	Pitoa	Permanet 2.0	No	105	401	73.82	73.82
Corbel 2010	Pitoa	Permanet 3.0	No	146	401	63.59	63.59
Corbel 2010	Vallée du Kou	Permanet 2.0	Yes	788	908	13.22	13.22
Corbel 2010	Vallée du Kou	Permanet 3.0	Yes	724	908	20.26	20.26
Corbel 2010	Vallée du Kou	Permanet 2.0	No	329	908	63.77	63.77
Corbel 2010	Vallée du Kou	Permanet 3.0	No	463	908	49.01	49.01
Koudou 2011	Yaokoffikro	Permanet 3.0	No	303	796	62.1	61.93

Table 7. Experimental hut trials: deterrence data *(Continued)*

Koudou 2011	Yaokoffikro	Permanet 2.0	No	317	796	60.4	60.18
Koudou 2011	Yaokoffikro	Permanet 3.0	Yes	313	796	60.1	60.68
Koudou 2011	Yaokoffikro	Permanet 2.0	Yes	281	796	64.4	64.70
Menze 2020	Mibellon	PermaNet 2.0	No	237	390	39.2	39.2
Menze 2020	Mibellon	PermaNet 3.0	No	153	390	60.8	60.8
Menze 2020	Mibellon	Olyset Net	No	176	390	54.9	54.9
Menze 2020	Mibellon	Olyset Plus	No	199	390	49	49
Moore 2016	Ifakara	Veeralin LN	No	722	810	11	10.86
Moore 2016	Ifakara	Veeralin LN	Yes	727	810	10	10.25
Moore 2016	Ifakara	MAGNet LN	No	1070	810	0	-32.10
Moore 2016	Ifakara	MAGNet LN	Yes	773	810	5	4.57
Moore 2016	Ifakara	Veeralin LN	No	89	170	48	47.65
Moore 2016	Ifakara	Veeralin LN	Yes	85	170	50	50.00
Moore 2016	Ifakara	MAGNet LN	No	114	170	33	32.94
Moore 2016	Ifakara	MAGNet LN	Yes	103	170	39	39.41
N'Guessan 2010	Akron	Permanet 3.0	No	128	185	31	30.81
N'Guessan 2010	Akron	Permanet 3.0	Yes	155	185	NR	16.22
N'Guessan 2010	Akron	Permanet 2.0	No	114	185	38	38.38
N'Guessan 2010	Akron	Permanet 2.0	Yes	174	185	NR	5.95
Pennetier 2013	Malanville	Olyset Plus	No	67	69	NR	2.90
Pennetier 2013	Malanville	Olyset Plus	Yes	101	69	NR	-46.38

Table 7. Experimental hut trials: deterrence data *(Continued)*

Pennetier 2013	Malanville	Olyset Net	No	96	69	NR	-39.13
Pennetier 2013	Malanville	Olyset Net	Yes	124	69	NR	-79.71
Toé 2018	Tengrela	Olyset Net	No	923	480	-92.29	-92.29
Toé 2018	Tengrela	Olyset Plus	No	695	480	-44.79	-44.79
Toé 2018	Tengrela	Permanet 2.0	No	858	480	-78.75	-78.75
Toé 2018	Tengrela	Permanet 3.0	No	794	480	-65.42	-65.42
Toé 2018	VK5	Olyset Net	No	1458	1095	-33.15	-33.15
Toé 2018	VK5	Olyset Plus	No	1278	1095	-16.71	-16.71
Toé 2018	VK5	Permanet 2.0	No	1075	1095	1.83	1.83
Toé 2018	VK5	Permanet 3.0	No	657	1095	40	40.00
Tungu 2010	Zeneti	PermaNet 3.0	No	425	723	41	41.22
Tungu 2010	Zeneti	PermaNet 2.0	No	574	723	21	20.61
Tungu 2010	Zeneti	PermaNet 3.0	Yes	558	723	23	22.82
Tungu 2010	Zeneti	PermaNet 2.0	Yes	586	723	19	18.95

ITN: insecticide-treated net; LLIN: long-lasting insecticidal net; NR: not reported; PBO: piperonyl butoxide; UTN: untreated net; WHO: World Health Organization.

Table 8. Village trials: mosquito density data

Study ID	Net type	Species	Density measurement	Collection method	Baseline density	Post-intervention density	Reduction (%)
Awolola 2014	Untreated	*An gambiae* s.l.	Mean number caught per house	WT, IRC	16.2	17.1	-5.56
Awolola 2014	PermaNet 2.0	*An gambiae* s.l.	Mean number caught per house	WT, IRC	21.3	7.2	66.20
Awolola 2014	PermaNet 3.0	*An gambiae* s.l.	Mean number caught per house	WT, IRC	20.1	1.4	93.03

Table 8. Village trials: mosquito density data *(Continued)*

Cisse 2017	PermaNet 2.0	*An gambiae* s.l.	Resting density per room per day	IRC	-	1.92	-
Cisse 2017	PermaNet 3.0	*An gambiae* s.l.	Resting density per room per day	IRC	-	3.05	-
Cisse 2017	Olyset	*An gambiae* s.l.	Resting density per room per day	IRC	-	3.21	-
Cisse 2017	Olyset Plus	*An gambiae* s.l.	Resting density per room per day	IRC	-	3.7	-
Mzilahowa 2014	Olyset	*An gambiae*	Mean number caught per catch	PSC	-	0.10	-
Mzilahowa 2014	Olset Plus	*An gambiae*	Mean number caught per catch	PSC	-	0.12	-
Mzilahowa 2014	PermaNet 2.0	*An gambiae*	Mean number caught per catch	PSC	-	0.13	-
Mzilahowa 2014	PermaNet 3.0	*An gambiae*	Mean number caught per catch	PSC	-	0.09	-
Mzilahowa 2014	Olyset	*An funestus*	Mean number caught per catch	PSC	-	0.08	-
Mzilahowa 2014	Olyset Plus	*An funestus*	Mean number caught per catch	PSC	-	0.16	-
Mzilahowa 2014	PermaNet 2.0	*An funestus*	Mean number caught per catch	PSC	-	0.27	-
Mzilahowa 2014	PermaNet 3.0	*An funestus*	Mean number caught per catch	PSC	-	0.13	-
Mzilahowa 2014	Olyset	*An gambiae*	Mean number caught per catch	LT	-	1.23	-
Mzilahowa 2014	Olset Plus	*An gambiae*	Mean number caught per catch	LT	-	0.27	-
Mzilahowa 2014	PermaNet 2.0	*An gambiae*	Mean number caught per catch	LT	-	0.96	-
Mzilahowa 2014	PermaNet 3.0	*An gambiae*	Mean number caught per catch	LT	-	1.44	-
Mzilahowa 2014	Olyset	*An funestus*	Mean number caught per catch	LT	-	2.02	-
Mzilahowa 2014	Olset Plus	*An funestus*	Mean number caught per catch	LT	-	2.1	-
Mzilahowa 2014	PermaNet 2.0	*An funestus*	Mean number caught per catch	LT	-	5.76	-
Mzilahowa 2014	PermaNet 3.0	*An funestus*	Mean number caught per catch	LT	-	3.76	-
Protopopoff 2018	Olyset (2015)	*Anopheles* species	Mean number caught per house per night	LT	-	2.61	-

Table 8. Village trials: mosquito density data *(Continued)*

Protopopoff 2018	Olyset Plus (2015)	*Anopheles* species	Mean number caught per house per night	LT	-	1.85	-
Protopopoff 2018	Olyset (2016)	*Anopheles* species	Mean number caught per house per night	LT	-	3.60	-
Protopopoff 2018	Olyset Plus (2016)	*Anopheles* species	Mean number caught per house per night	LT	-	2.68	-
Staedke 2020	Permanet 2.0 (6 months)	*An gambiae* s.l.	Mean density per house	IRC	0.3	0.67	
Staedke 2020	Permanet 3.0 (6 months)	*An gambiae* s.l.	Mean density per house	IRC	0.8	0.17	78.75
Staedke 2020	Olyset (6 months)	*An gambiae* s.l.	Mean density per house	IRC	0.3	0.81	
Staedke 2020	Olyset Plus (6 months)	*An gambiae* s.l.	Mean density per house	IRC	0.1	0.16	
Staedke 2020	Permanet 2.0 (12 months)	*An gambiae* s.l.	Mean density per house	IRC	0.3	1.35	
Staedke 2020	Permanet 3.0 (12 months)	*An gambiae* s.l.	Mean density per house	IRC	0.8	0.52	35
Staedke 2020	Olyset (12 months)	*An gambiae* s.l.	Mean density per house	IRC	0.3	1.1	
Staedke 2020	Olyset Plus (12 months)	*An gambiae* s.l.	Mean density per house	IRC	0.1	0.23	
Staedke 2020	Permanet 2.0 (18 months)	*An gambiae* s.l.	Mean density per house	IRC	0.3	1.65	
Staedke 2020	Permanet 3.0 (18 months)	*An gambiae* s.l.	Mean density per house	IRC	0.8	1.57	
Staedke 2020	Olyset (18 months)	*An gambiae* s.l.	Mean density per house	IRC	0.3	0.66	
Staedke 2020	Olyset Plus (18 months)	*An gambiae* s.l.	Mean density per house	IRC	0.1	0.19	

Table 8. Village trials: mosquito density data *(Continued)*

Stiles-Ocran 2013	No intervention	*An gambiae* s.s.	Mean number caught per village	IRC	230	79	65.65
Stiles-Ocran 2013	Permanet 2.0	*An gambiae* s.s.	Mean number caught per village	IRC	39	36	7.69
Stiles-Ocran 2013	Permanet 2.0	*An gambiae* s.s.	Mean number caught per village	IRC	82	45	45.12
Stiles-Ocran 2013	Permanet 3.0	*An gambiae* s.s.	Mean number caught per village	IRC	77	12	84.42
Stiles-Ocran 2013	Permanet 3.0	*An gambiae* s.s.	Mean number caught per village	IRC	178	15	91.57
Stiles-Ocran 2013	No intervention	*An gambiae* s.s.	Mean number caught per person per night per village	Indoor & outdoor HLC	415	72	82.65
Stiles-Ocran 2013	Permanet 2.0	*An gambiae* s.s.	Mean number caught per person per night per village	Indoor & outdoor HLC	33	31	6.06
Stiles-Ocran 2013	Permanet 2.0	*An gambiae* s.s.	Mean number caught per person per night per village	Indoor & outdoor HLC	79	64	18.99
Stiles-Ocran 2013	Permanet 3.0	*An gambiae* s.s.	Mean number caught per person per night per village	Indoor & outdoor HLC	98	19	80.61
Stiles-Ocran 2013	Permanet 3.0	*An gambiae* s.s.	Mean number caught per person per night per village	Indoor & outdoor HLC	156	36	76.92

An funestus: *Anopheles funestus*; *An gambiae*: *Anopheles gambiae*; HLC: human landing catch; IRC: indoor resting catch; LT: light trap; PSC: pyrethrum spray catch; WT: window trap.

APPENDICES

Appendix 1. Detailed search strategies

The Cochrane Library

Description:

#1 piperonyl butoxide

#2 MeSH descriptor: [Piperonyl Butoxide] explode all trees

#3 #1 or #2

#4 Net* or bednet* or hammock* or curtain* or ITN* or LLIN* or "Insecticide-Treated Bednet*" or "Insecticide-Treated net*"

#5 Olyset* or PermaNet* or Veeralin

#6 DawaPlus* or Tsara* or Duranet*

#7 MeSH descriptor: [Insecticide-Treated Bednets] explode all trees

#8 #4 or #5 or #6 or #7

#9 #3 and #8

MEDLINE (PubMed)

	Query
#1	Search "Piperonyl Butoxide"[Mesh]
#2	Search piperonyl butoxide or PBO Field: Title/Abstract
#3	Search ("Piperonyl Butoxide"[MESH]) OR #2
#4	Search Net* OR bednet* OR curtain* OR ITN* OR LLIN* or "Insecticide-Treated Bednet*" or "Insecticide-Treated net*" Field: Title/Abstract
#5	Search "Olyset* or Permanet* or Veeralin Field: Title/Abstract
#6	Search DawaPlus* or Tsara* or Duranet* Field: Title/Abstract
#7	Search "Insecticide-Treated Bednets" [MESH]
#8	Search (((#4) OR #) OR #6) OR #7
#9	Search (#8) AND (#3)

Embase (OVID)

1 piperonyl butoxide/

2 piperonyl butoxide.tw.

3 1 or 2

4 PBO.tw.

5 3 or 4

6 (Net* or bednet* or hammock* or curtain* or ITN* or LLIN* or "Insecticide-Treated Bednet*" or "Insecticide-Treated net*").mp.

7 (Olyset* or Permanet* or Veeralin).mp.

8 (DawaPlus* or Tsara* or Duranet*).mp.

9 insecticide treated net/

10 6 or 7 or 98 or 9

11 5 and 10

Web of Science™ Core Collection

Set	
# 5	#3 AND #4 Indexes=SCI-EXPANDED, SSCI, CPCI-S, CPCI-SSH Timespan=All years
# 4	#1 OR #2 Indexes=SCI-EXPANDED, SSCI, CPCI-S, CPCI-SSH Timespan=All years
# 3	**TOPIC:** (Net* OR bednet* OR ITN* OR LLIN* or "Insecticide-Treated Bednet*" or "Insecticide-Treated net*") *OR* **TOPIC:** (Olyset* or PermaNet* or Veeralin) *OR* **TOPIC:** (DawaPlus*or Tsara* or Duranet*) Indexes=SCI-EXPANDED, SSCI, CPCI-S, CPCI-SSH Timespan=All years
# 2	**TOPIC:** (PBO) *NOT* **TOPIC:** (placebo) Indexes=SCI-EXPANDED, SSCI, CPCI-S, CPCI-SSH Timespan=All years
# 1	**TOPIC:** ("Piperonyl Butoxide") Indexes=SCI-EXPANDED, SSCI, CPCI-S, CPCI-SSH Timespan=All years

CABI: CAB Abstracts®

Set	
# 3	#2 AND #1 Indexes=CAB Abstracts Timespan=All years
# 2	**TOPIC:** (Net* OR bednet* OR hammock* OR curtain* OR ITN* OR LLIN* or "Insecticide-Treated Bednet*" or "Insecticide-Treated net*") *OR* **TOPIC:** (Olyset* or PermaNet* or Veeralin) Indexes=CAB Abstracts Timespan=All years
# 1	**TOPIC:** (PBO or "Piperonyl Butoxide") Indexes=CAB Abstracts Timespan=All years

ClinicalTrials.gov and WHO ICTRP

piperonyl butoxide and malaria

Appendix 2. Study characteristics extraction form

Table 2.1 Trial characteristics of the included experimental hut trials

Trial ID	Trial name	Trial location	Mosquito species (strain/origin)	Resistance level	Resistance status	Trial start/end date	Intervention	Net washed	Net holed	Measured outcome			
										M	BF	D	E

BF; blood feeding; D: deterrence; E: exophily; M: mortality.

Table 2.2 Trial characteristics of the included village trials

Trial ID	Trial name	Trial location	Mosquito species (strain/origin)	Resistance level	Resistance status	Trial start/end date	Intervention	Net washed	Net holed	Measured outcome						
										M	BF	SR	MD	PR	PP	CMC

BF: blood feeding; CMC: clinical malaria confirmation; M: mortality; MD: mosquito density; PP: parasite presence; PR: parity rate; SR: sporozoite rate.

Appendix 3. Data extraction form

Table 3.1 Data extracted from experimental hut trials

Trial ID	Trial name	Net type	Net washed	Net holed	Mosquito species	Resistance level	Resistance status	Total mosquitoes	Dead	Mosquito mortality (%)	BF	BF (%)	BFI	Number of mosquitoes deterred	Deterrence (%)	Exit trap	Exophily (%)	Total number of people (N)

BF: blood feeding; BFI: blood feeding inhibition; N: number of people.

Table 3.2 Data extracted from village trials

Trial ID	Trial name	Net type	Net washed	Net holed	Mosquito species	Resistance level	Resistance status	Total mosquitoes	Dead	Mosquito mortality (%)	BF	BF (%)	BFI	Sporozoite (%)	Mosquito density (%)	Parity (%)	Total number of people (N)	PP (%)	CMC (%)

BF: blood feeding; CMC: clinical malaria confirmation; N: number of people; PP: parasite prevalence.

Appendix 4. 'Risk of bias' assessment form

Table 4.2 'Risk of bias' assessment for experimental hut trials

Trial ID	Trial-name	Comparability of mosquitoes in LLIN and LLIN + PBO groups	Collectors blinded	Sleepers blinded	Sleeper bias	Treatment allocation	Treatment rotation	Standardized hut design	Hut cleaning between treatments	Incomplete outcome data	Raw data reported for both treatment groups	Authors' conflicting interest

LLIN: long-lasting insecticidal nets; PBO: piperonyl butoxide.

Table 4.3 'Risk of bias' assessment for village trials

Trial ID	Trial name	Comparability of mosquitoes in LLIN and LLIN + PBO households	Collectors blinded	Household blinded	Allocation of treatments	Incomplete outcome data	Raw data reported for both groups	Authors' conflicting interest

LLIN: long-lasting insecticidal nets; PBO: piperonyl butoxide.

Appendix 5. 'Risk of bias' assessment: experimental hut trials

'Risk of bias' component	Low	Unclear	High
Mosquito group comparability	Huts accessible to the same mosquito population	No or unclear information reported	Huts not accessible to the same mosquito population
Collectors blinded	Outcomes assessed blinded	No or unclear information reported If outcomes assessed were not blinded, but this is unlikely to influence the results, we will judge this to be low risk	Outcomes assessed not blinded, and this is likely to influence the results If outcomes assessed were not blinded, but this is unlikely to influence the results, we will judge this to be low risk
Sleepers blinded	Outcomes assessed blinded	No or unclear information reported If outcomes assessed were not blinded, but this is unlikely to influence the results, we will judge this to be low risk	Outcomes assessed not blinded, and this is likely to influence the results If outcomes assessed were not blinded, but this is unlikely to influence the results, we will judge this to be low risk
Sleeper bias	Sleepers were rotated between huts according to a Latin square design	No or unclear information reported	Sleepers not rotated between huts
Treatment allocation	Treatments randomized Treatments not randomized; however equal attractiveness demonstrated	No or unclear information reported	Treatments not randomized, and equal attractiveness not demonstrated
Treatment rotation	Treatments rotated through huts according to a Latin square design	No or unclear information reported	Treatments not rotated
Standardized hut design	Huts of West or East African design	No or unclear information reported	Huts of non-standardized design
Cleaning	Huts cleaned between treatments	No or unclear information reported	Huts not cleaned between treatments
Incomplete outcome data addressed	No or low missing data; reason for missing data is unlikely to be related to the true outcome	No or unclear information reported	High missing data; reason for missing data is likely to be related to the true outcome
Raw data reported	Raw data reported	No or unclear information reported	Raw data not reported
Conflicting interests	No conflict of interest stated	No or unclear information reported	Conflict of interest stated

Appendix 6. 'Risk of bias' assessment: village trials

'Risk of bias' component	Low	Unclear	High
Recruitment bias	No participants recruited after clusters randomized	No or unclear information reported Recruitment bias not applicable to trial design, as it is related to human participants	Paricipants recruited to trial after clusters randomized
Mosquito group comparability	Mosquito populations comparable	No or unclear information reported	Mosquito populations comparable
Collectors blinded	Outcomes assessed blinded	No or unclear information reported Outcomes assessed not blinded, but this is unlikely to influence the results	Outcomes assessed not blinded, and this is likely to influence the results
Household blinded	Outcomes assessed blinded	No or unclear information reported If outcomes assessed were not blinded, but this is unlikely to influence the results, we will judge this to be low risk	Outcomes assessed not blinded, and this is likely to influence the results If outcomes assessed were not blinded, but this is unlikely to influence the results, we will judge this to be low risk
Treatment allocation	Treatments randomized	No or unclear information reported	Treatments not randomized
Allocation concealment	Allocation concealment procedures were adhered to	No or unclear information reported Allocation concealment procedures were not adhered to; however this is unlikely to affect the results	Allocation procedures were not adhered to, and this is likely to have affected the results
Incomplete outcome data addressed	No or low missing data; reason for missing data is unlikely to be related to the true outcome	No or unclear information reported	High missing data; reason for missing data is likely to be related to the true outcome
Raw data reported	Raw data reported	No or unclear information reported	Raw data not reported
Clusters lost to follow-up	No complete clusters lost from trial	No or unclear information as to whether clusters were lost from trial	At least 1 cluster lost from trial
Selective reporting	No selective reporting; all measured outcomes reported in results	No or unclear information on whether all measured outcomes were reported in results	Selective reporting; not all measured outcomes were reported in results
Correct statistical methods; adjusted for clustering	Clustering was taken into account and statistical methods adjusted for clustering	No or unclear information as to whether clustering was taken into account for statistical methods	Trial did not take clustering into account for statistical methods
Conflicting interests	No conflict of interest stated	No or unclear information reported	Conflict of interest stated

Appendix 7. Prespecified changes for review update 2021

Protocol section	Protocol changes
Background and research question	We will update any references and background information
Inclusion criteria	We propose to remove objective 1 (evaluate whether adding PBO to pyrethroid LLINs increases the epidemiological and entomological effectiveness of the nets' and focus instead on comparing pyrethroid-PBO nets with their non-PBO equivalent (objective 2). As a result, laboratory studies will be excluded. We make this decision as we only identified two studies meeting the inclusion criteria for objective 1 in Gleave 2018, both of which were laboratory assays; results from these cannot readily be translated into public health outcomes.
Methods	We will subgroup our analysis on epidemiological data by follow-up time. We will update the search strategy terms as one brand of bednet has changed name, and we will perform a new search to identify all possible trials.

This table was approved by the CIDG editorial team on 26 Oct 2020.

WHAT'S NEW

Date	Event	Description
30 June 2021	Amended	The author team corrected minor spelling problems in the abstract and summary of findings tables. They also corrected raw participant numbers in the summary of findings tables for moderate- and low-resistance settings. These edits do not alter the review findings or outcomes.

HISTORY

Protocol first published: Issue 8, 2017
Review first published: Issue 11, 2018

Date	Event	Description
21 June 2021	Amended	The author team made minor edits to Summary of findings 1. Under 'Patient or population' they added "adults and children living in malaria-endemic areas". The corrections to parasite prevalence numbers reported do not impact the odds ratios reported, or review findings or interpretation.
24 May 2021	New citation required and conclusions have changed	This is an update of the first Cochrane Review of pyrethroid-PBO nets (Gleave 2018). The date of search is 25 September 2020.
24 May 2021	New search has been performed	The prespecified changes to the protocol (before the review update commenced) are given in Appendix 7. We excluded studies using only laboratory assays from this review update due to the challenges in extrapolating public health value from laboratory

Date	Event	Description
		bioassays alone. We amended the search strategy including different search terms due to a bed net brand name change. A new search was undertaken to capture all relevant trials for this update.
6 June 2019	Amended	Abstract amended. Authors' conclusions section: changed from "reduce mosquito mortality and blood feeding rates" to "increase mosquito mortality and reduce blood feeding rates"

CONTRIBUTIONS OF AUTHORS

KG, NL, and HR conceived and designed the protocol.

KG, NL, and LC conducted trial screening, data extraction, and analysis.

MC and LC provided statistical support.

KG, NL, LC, and HR wrote the final manuscripts, and all review authors approved the final manuscript.

HR is the guarantor of the review.

DECLARATIONS OF INTEREST

KG has no known conflicts of interest.
NL has acted as rapporteur since 2015 for the Innovative Vector Control Consortium (IVCC) at its External Scientific Advisory Committee (ESAC) meetings.
MC has no known conflicts of interest.
LC has no known conflicts of interest.
HR has served on a WHO committee to consider the evidence for PBO nets in malaria control. Preparation of the background work presented at this WHO meeting was funded by the Global Fund for AIDS, TB, and Malaria. Although HR interacts regularly with bed net manufacturers through her own research and her previous role on IVCC's advisory panels, neither HR nor her research group have received direct funding from these companies.

SOURCES OF SUPPORT

Internal sources

- Liverpool School of Tropical Medicine, UK

External sources

- Foreign, Commonwealth and Development Office (FCDO), UK

  Project number 300342-104
- World Health Organization (WHO), Switzerland

  WHO Global Malaria Programme Agreement for Performance of Work (APW) Grant 2017 (number 709319)

DIFFERENCES BETWEEN PROTOCOL AND REVIEW

Previously, PBO-nets were classified as PBO-LLINs; however as the durability of PBO on nets has not been classified as long-lasting, these were subsequently referred to as pyrethroid-PBO nets. As a result of this, our review title changed from 'Piperonyl butoxide (PBO) combined with pyrethroids in long-lasting insecticidal nets (LLINs) to prevent malaria in Africa' to 'Piperonyl butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa'.

We added Leslie Choi as a review author.

Additional criteria for assessing the risk of bias of village trials were added. These are in line with the Cochrane 'Risk of bias' tool (Higgins 2017), as well as the five additional criteria listed in Section 16.3.2 of the *Cochrane Handbook for Systematic Reviews of Interventions* that relate specifically to cluster-randomized trials (Higgins 2011).

The published protocol stated all stratified analysis factors under subgroup analysis (Gleave 2017). We have corrected this to state that subgroup analysis was performed only on whether nets were unwashed or washed.

Differences between review (2018) and review update (2021)

The prespecified changes to the protocol (before the review update commenced) are given in Appendix 7. In brief, the published review included laboratory bioassay studies (n = 2) (Gleave 2018). We excluded studies using only laboratory assays from this review update due to the challenges in extrapolating public health value from laboratory bioassays alone. We amended the search strategy including different search terms due to a bed net brand name change. A new search was undertaken to capture all relevant trials for this update.

INDEX TERMS

Medical Subject Headings (MeSH)

Africa [epidemiology]; Culicidae; Drug Combinations; Feeding Behavior; Insecticide Resistance [*drug effects]; *Insecticide-Treated Bednets; Malaria [epidemiology] [*prevention & control]; Mortality; Mosquito Control [*methods]; *Pesticide Synergists; *Piperonyl Butoxide; *Pyrethrins; Randomized Controlled Trials as Topic

MeSH check words

Animals; Humans

Behaviour of pyrethroid resistant *Anopheles gambiae* at the interface of two dual active-ingredient bed nets, assessed by room-scale infrared video tracking

K. Gleave, A. Guy, F. Mechan, A. Matope, M. Emery, A, Murphy, V. Voloshin, C. E. Towers, D. Towers, H. Ranson, G. M. Foster, P. J. McCall *

*corresponding author

Background

Resistance to insecticides has emerged in mosquitoes across the globe and threatens the future use of insecticides to control many vector-borne diseases. The most effective malaria control method in Africa, where the vast majority of malaria cases occur, is the widespread use of insecticide-treated nets (ITNs) (Pryce *et al*., 2018). The first generation of ITNs use fast-acting pyrethroids, and pyrethroid resistance has spread at an alarming rate through *Anopheles* populations in Africa (Hancock *et al*., 2020; Hemingway, 2017; Ranson & Lissenden, 2016) reducing ITN efficacy (Churcher *et al*., 2016). Several types of 'next-generation ITNs' are now available and used in many malaria-endemic countries; these all contain pyrethroids plus an additional active ingredient (AI) with a different mode of action (MoA). Currently, the most widely used next-generation nets are pyrethroid-piperonyl butoxide nets (pyrethroid-PBO nets); PBO increases the potency of pyrethroids by blocking enzymes that break down insecticides. In 2021, pyrethroid-PBO

nets constituted 42.8% of the nets distributed in Sub-Saharan Africa with public funds (The Alliance for Malaria Prevention, 2022). Recent clinical trials of ITNs with two insecticides (Interceptor G2®, BASF, containing a pyrethroid plus the pyrrole insecticide chlorfenapyr) (Mosha *et al*., 2022) or containing pyrethroid plus pyriproxyfen (a chemical that sterilises female adults) (Tiono *et al*., 2018) have shown improved clinical outcomes over standard ITNs. However, improved epidemiological outcomes have only been demonstrated in a single setting with pyriproxyfen nets showing no improved public health value over standard ITNs in the Tanzanian trial (Mosha *et al*, 2022). Further evidence of their efficacy in different ecological and epidemiological environments is needed prior to national or global policy changes.

The success of ITNs relies predominantly on the daily behaviour of the major malaria vectors in Africa, where *Anopheles* species are largely anthropophagic, endophagic, endophilic and feed during the night when people are more likely to be underneath their bed nets (Pates & Curtis, 2005; Killeen *et al*., 2006). Multiple types of mosquito behavioural alterations in response to widespread ITN use at the population level could decrease their efficacy (Gatton *et al*., 2013; Killeen, 2014), and several examples of this behavioural resistance have been described after multiple years of net use. For example following a mass ITN distribution programme in Benin, *An. funestus* have shown a shift in biting time from a peak late at night to early morning when people emerge from their protective ITNs (Moiroux *et al*., 2012). Monitoring these population changes induced by widespread deployment of ITNs, or any other vector control tool, is essential to explain and predict their epidemiological impact. Indeed, modelling studies have indicated that behavioural resistance and physiological resistance (caused, for example, by target site modifications or enhanced detoxification) could be equally detrimental to the efficacy of ITNs (Gatton *et al*.,

2013). Therefore, surveillance of vector behaviour is an essential component of resistance management programmes.

In addition to population surveillance, critical insights into the behaviour of mosquitoes in response to ITNs can be gained by laboratory and semi-field studies that quantify important traits This includes net contact time and blood-feeding volumes and relates these to key endpoints such as longevity and reproductive outputs. Performing these tests on mosquito populations with different levels, and mechanisms of pyrethroid resistance, may inform predictions on the efficacy of standard and next-generation ITNs in different environments. Standard WHO assays, designed to measure the performance of a single, fast-acting insecticide in ITNs (*i.e* pyrethroids) are not suitable for measuring the impact of combining AIs with differing MoAs and endpoints. We have therefore been developing and evaluating a series of benchtop and room-scale assays to record mosquito responses to a more diverse range of ITNs.

The 'baited box' assay allows for close-range observation of mosquitoes attempting to take a blood meal through an ITN, with results from Hughes *et al*., reporting that the accumulated duration of net contact by *Anopheles gambiae* was 50% lower on ITNs compared to untreated nets, with no difference in contact duration between susceptible and resistant mosquitoes (Hughes *et al*., (2020). Benchtop tests are undoubtedly informative, but the impacts of ITNs extend beyond the close range captured in these assays. Parker *et al*., (2015, 2017) used an infrared tracking system to characterise mosquito behaviour at mid-range*, i.e.* host-seeking events around an entire human-baited PermaNet® 2.0 bed net (Vestergaard Sarl), from room entry to arrival at the ITN. The initial behaviour of insecticide-susceptible *An. gambiae* and wild *An. arabiensis* did not differ between an untreated or pyrethroid ITN; mosquitoes continued to respond to the host without any

evidence of repellency until they contacted the insecticide on the net surface. After this time, activity decayed rapidly, reaching zero after around 30 minutes, demonstrating the highly efficient rapid action of pyrethroid-treated ITNs. Here we apply this method to studying the behaviour of insecticide-resistant mosquitoes to next-generation bed nets to gain initial insights into the utility of this method in comparing responses between mosquito populations and net types.

This study investigated the mosquito response to two next-generation nets, PermaNet® 3.0 (Vestergaard Sarl) and Interceptor® G2 (BASF AGRO B.V Arnhem [NL] Freienbach Branch) performed in comparison with a standard pyrethroid only ITN (Olyset™ Net, Sumitomo Chemical Co., Ltd) and an untreated net, as measured by impacts on both pyrethroid susceptible and resistant mosquitoes. This study also sought evidence for any altered behaviours during host-seeking at the net which may be attributed to the new nets.

Materials and Methods

Mosquitoes from two insecticide-susceptible (Kisumu and N'gousso) and two insecticide-resistant (VK7 and Banfora) *An. gambiae s.l* strains were maintained under standard insectary-controlled conditions (27°C ± 2°C, and 80% relative humidity (RH)) at the Liverpool School of Tropical Medicine (LSTM). Susceptible *An. gambiae s.s* Kisumu colony originates from Kenya (Shute, 1956) and has been maintained in colony since 1975. *An. coluzzii* N'gousso was colonised from Cameroon in 2006 (Harris *et al*., 2010). *An. coluzzii* VK7 and Banfora strains originated from Burkina Faso, have been reared at LSTM since 2014 and 2015, respectively, and are highly resistant to pyrethroids with susceptibility only partially restored by PBO pre-exposure (Williams *et al*., 2019, 2022). The VK7 strain is fixed

for the knockdown resistant (Kdr) 995F allele in the voltage-gated sodium channel (*Vgsc*), whereas the Banfora strain has a more complex set of *Vgsc* mutants (Ingham *et al*., 2021). Both strains have elevated cytochrome P450 expression, but additional resistance mechanisms are present in the Banfora strain including an increased respiratory rate (Ingham *et al*., 2021). All mosquitoes were reared under an altered 12:12 light/dark cycle to allow for testing to be conducted during the 'night' phase of the circadian rhythm.

The ITNs used are shown in Table 1. Nets were obtained directly from the manufacturer, aired at room temperature for four weeks prior to testing and then adjusted in size to fit the custom-made bed net frame, ensuring maximum visualisation of mosquito activity. A single net was used for each treatment, each stored at 4°C between testing replicates and acclimatised at 27±2°C and 70±10% humidity for at least one hour prior to testing.

Table 1. Insecticide treated nets used in room scale tracking assays.

Net type	***Specification***	***Manufacturer***
Polyester control	Untreated	Bayer AG, Leverkusen, Germany
Olyset Net (OL)	150 denier polyethylene net incorporated with permethrin at 800 mg/m^2	Sumitomo Chemical Company, Tokyo, Japan
PermaNet 3.0 (P3)	Roof: 100 denier polyethylene net incorporated with deltamethrin at 120mg/m^2 and PBO at 750mg/m^2, Sides: 75 denier polyethylene net with deltamethrin at 84mg/m^2	Vestergaard Sarl, South Africa
Interceptor G2 (IG2)	75 denier polyester net coated with alphacypermethrin at 100mg/m^2 and chlorfenapyr at 200 mg/m^2	BASF AGRO B.V Arnhem (NL), Germany

All experiments required a human volunteer to act as bait under the net. Volunteers were asked to wear light clothing, not to wear any strong scented products and not to bathe for at least four hours prior to testing. During the experiment, volunteers were asked to lie as motionless as possible, while still being comfortable. To control for any effect of body positioning, volunteer orientation was randomly assigned either with head or feet nearest to the mosquito release point.

A total of 25, three-to-five-day old un-fed female mosquitoes were used per test replicate, as per Parker 2015 (Parker *et al* 2015). Mosquito access to 10% sugar solution was removed by 16:00 the day prior to testing and replaced with distilled water; this was removed three hours prior to testing.

Experimental set-up

All experiments were performed in the LSTM Accelerator building, using a custom built free-flight testing room (7m x 4.8m in area, 2.5m high) which is climate controlled (27±2°C and 70% ±10% RH), while recording is operated from an adjacent room. Assays were performed during the afternoon to coincide with the 'night' phase of the mosquito's circadian rhythm when they would be host-seeking in the wild. Frames made of carbon rods with roofs tilted towards the recording equipment were constructed for each bed net type to allow accurate observations of mosquito activity (dimensions: front height 45cm, rear height 75cm, roof width 90cm, roof length 180cm).

Mosquitoes were placed into a holding cup one hour prior to testing to acclimatise within the testing room. The cup was attached to a long cord allowing mosquitoes to be released remotely by the operator outside the tracking room. Fifteen minutes before the test began the volunteer entered the ITN; to start the test, the release cord was pulled. After two-hour recording, free flying and knocked down mosquitoes were collected using a HEPA filter

mouth aspirator (John. W. Hock, USA) to avoid any insect damage and placed into a fresh collection cup. Mortality was recorded at 24 hours, with all mosquitoes individually monitored for sub-lethal insecticide effects (see below).

ITN treatments were changed approximately every three weeks and the testing room decontaminated between each ITN type, using 5% Decon90 solution (Decon Laboratories Conway Street, UK), followed by two water washes and a final wash with 70% ethanol. World Health Organisation (WHO) cone tests (World Health Organization, 2006) using susceptible *An. gambiae* were performed on the walls 24hours after decontamination for quality control (QC). During testing, no WHO cone assays resulted in >20% mortality, therefore all cleaning procedures were considered to pass the QC process.

Mosquito Tracking

Mosquitoes were tracked using paired identical recording systems, positioned 1050 mm apart and consisting of the following: each recording system used one camera (12 MPixel Ximea CB120RG-CM with a 14mm focal length lens), aligned with a single Fresnel lens (1400 x 1050mm and 3mm thick, 1.2m focal length; NTKJ Co., Ltd, Japan) placed approximately 12100 mm away. Cameras recorded with an exposure time of 5ms and -3.5 dB gain with a lens aperture of F#8.0 (Voloshin *et al*., 2020). As experiments were carried out in the dark, infrared light was provided using custom ring light sources constructed by colleagues at Warwick university (12 OSRAM™ SFH 4235 infrared LEDs with a peak wavelength of 850nm) which illuminated the total recording volume of 2 x 2 x 1.4m. To reflect light back towards the cameras a custom designed Retroreflective screen (2.4 x 2.1 m, material: 3M™ Scotchlite™ High Gain Reflective Sheeting 7610) was placed 2m from the Fresnel lenses, with the bed and ITN placed in between both. The reflected light is focused by the Fresnel lens and forms a telecentric lens pair with an imaging optic mounted on the camera which

allows illumination and imaging to occur from one side of the experimental set up. More information on signal processing can be found in *Voloshin et al*., (2020). Recordings were captured for both cameras over the two-hour assay using StreamPix recording software (StreamPix V7, Norpix, Montreal, Canada) at 50 frames per second (fps) onto a Windows PC (Intel® Xeon® Silver 4114 CPU 2.20 GHz, 24 Gigabytes RAM, Windows 10 Pro; 12 configured into 2 RAID arrays of 24 Terabytes each, at 1 array per camera.

Video analysis

All video analysis was carried out using bespoke software written in Matlab (Mathworks) developed by collaborators at Warwick University (Angarita-Jaimes *et al*., 2016). Video segmentation, then compression to .mp4 files was performed before all videos were manually reviewed and cleaned to remove false tracks and human movement using 'Seq File Processing' software. Data extracted includes trajectory duration, distance travelled, the number, duration and location of contacts with the bed net, time to first contact and track velocity, all of which have been previously described by Parker *et al.*, (2015). Additional track joining and the deletion of false tracks created by volunteer and camera noise was performed in 'Post Processing' along with categorising activity into behavioural modes using existing quantification algorithms (Table 2) and dividing the field of view into 10 distinct regions to quantify net contact location and duration at 10 different regions of the bed net. Since multiple mosquitoes were released into the room in all tests, tracking individual mosquitoes was not possible, hence analysis was performed on flight tracks with each track from entry into and exit out of the field of view analysed separately. One flight track could consist of three different behavioural modes (visiting, bouncing and resting as they all involve net contact), upon which the time spent in each mode were recorded separately.

Table 2. Definition of mosquito behavioural modes (adapted from (Parker *et al*., 2017).

Behavioural mode	***Definition***
Swooping	Flight tracks without net contact.
Visiting	Tracks where extended periods of flight were interspersed with infrequent contacts with the bed net. Contacts were characterized as sharp 80° turns or more in the trajectory, and when multiple contacts occurred with the net, the minimum interval between each contact was 0.4 seconds (*i.e.,* an interval of at least 20- frames, at 50 frames per second).
Bouncing	Tracks where the mosquito made multiple contacts at intervals of less than 0.4 seconds with the bed net surface; including tracks with short flights between the contacts, or tracks maintaining contact with the bed net surface without being static. This includes 'walking' or 'probing' the net with gaps in movement lasting less than 0.75 seconds
Resting	Tracks where the mosquitoes were static for at least 0.75 seconds on the net surface, or where the velocity of mosquito movement was less than 1.33 mm/s. Dead mosquitoes were excluded by limiting resting periods to a maximum of 300 seconds, however, no dead mosquitoes were found on nets at the end of each test

Sub-lethal pipeline

The methods for sub-lethal pipeline monitoring have been previously described in Hughes *et al*., (in press). After each tracking assay, the following were measured for each individual mosquito: 24hour mortality, willingness to feed at 60 minutes, or 24hours (by exposure to the arm of a human volunteer), longevity and wing length (Figure 1).

Data analysis

A sample size for comparing net contact times at three different ITNs was calculated using the mean difference in net contact time for a single strain between untreated and treated nets generated in an earlier study in the statistical program R (R Development Team, 2017), and using the phia (Rosario-Martinez, 2015) and pwr (Champely, 2017) packages. With a significance level of 0.05 that gives at least a power of 90%, a minimum sample size was determined inflating the sample size with 30% to adjust for any potential confounding factors. A common standard deviation was assumed for all groups used was 562.14 (obtained from the previous study based on the ANOVA or t-test (Parker *et al*., 2017)). A total of 6 replicates per strain and treatment was the minimum requirement determined to compare net contact times at different ITNs. This sample size does not account for the correlation of the measurements from the same volunteer, although this correlation may still exist.

ITN bioefficacy and mosquito longevity

Bioefficacy of nets was assessed through measuring mosquito mortality post-exposure. Mosquitoes were transferred to individual falcon tubes, provided with a source of 10% sugar water and mortality measured daily until all mosquitoes had died.

Quantifying mosquito activity and behaviour

Total activity per strain (seconds of movement), per net treatment was calculated as the sum of all mosquito activity, regardless of behavioural mode and binned into 5-minute intervals for analysis. Further analyses were performed using the total activity stratified into the four described behavioural modes (swooping, visiting, resting and bouncing).

Defining and quantifying mosquito contact with the bed net interface

Total contact number and total contact duration with a net was calculated from the sum of all contacts obtained from visits, bounces or resting tracks. Total duration of contact in the first 10 minutes of the assay was calculated as a percentage from overall contact duration along with an average of mosquito duration. As it was not possible to determine individual mosquito contact, we calculated the possible minimum and maximum values of net contact as in Parker *et al*., (2015): for the maximum value, total contact duration was divided by the maximum number of mosquitoes seen simultaneously contacting the net in any one frame of the recording; the minimum value assumed that all 25 mosquitoes released into the assay responded at the same time.

Determination of contact location

The recording field of view was divided into 16 regions using previously described software (Angarita-Jaimes *et al*., 2016). Ten of these regions were on the net surface; six on top of the bed net, two on the front of the net and one at either side.

Speed around the bed nets

Flight speed was analysed using whole swooping tracks around the bed nets to investigate any changes in mosquito flight.

Mosquito activity decay over the 2hour assay

Exponential decay modelling was considered for analysis of activity over time, as reported previously by Parker *et al*., (2015) but many of the test replicates violated the equation constraints, so an alternative method was used whereby total activity in the first 5 minutes of recording was subtracted from total activity in the final 5 minutes of recording. A negative value indicated that activity decayed over time and a positive value represented an increase in activity between the two timepoints.

Determining willingness to refeed and mosquito size

Wing length was used as an estimate for mosquito body size and to control for potential size differences between cohorts. The right wing was removed, and an image taken using GXCAM ECLIPSE Wi-Fi camera attached to a GX Stereo microscope (GT Vision Ltd). The length of the wing was measured from the axial vein to the distal end of the R1 vein using GXCAM software (GXCAM Ver.6.7).

To assess any effects of sub-lethal insecticide exposure, mosquitoes were offered a blood meal at 1-hour post-exposure and longevity measured. Blood feeding inhibition was calculated by considering all mosquitoes in each replicate and assessing whether they were able to take a blood meal or not.

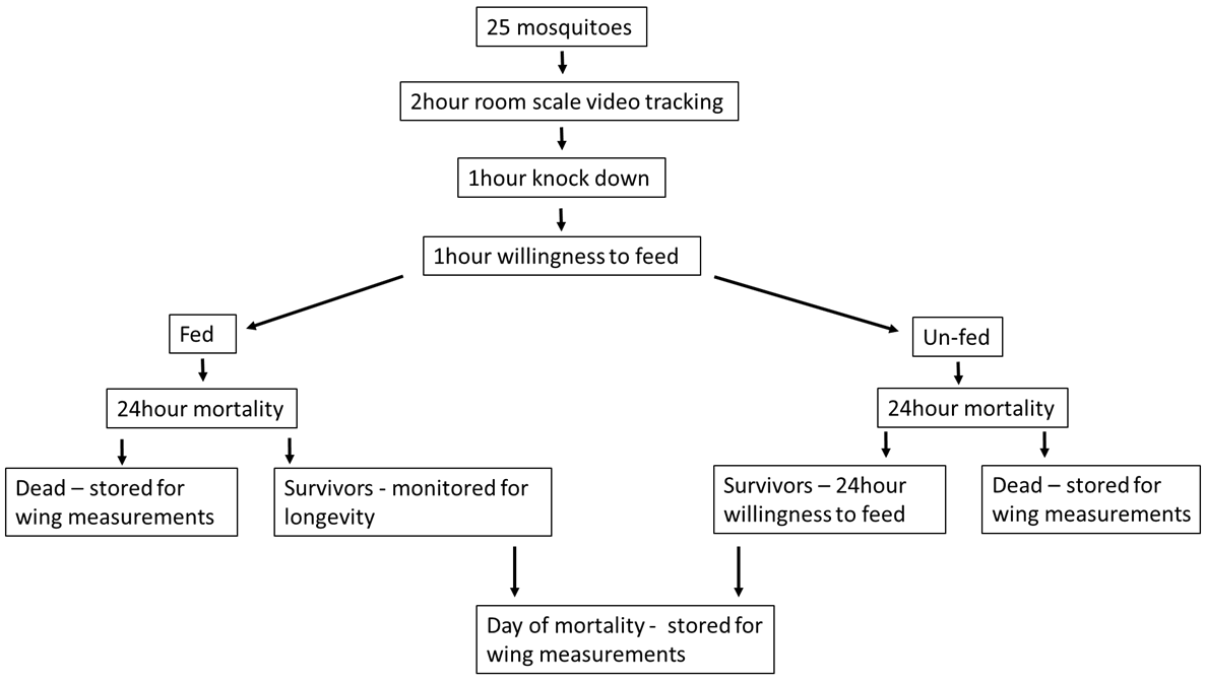


Figure 1. Measured sub-lethal pipeline outcomes per room scale video tracking assay.

Statistical analysis

Statistical analysis was performed used Prism 6 (GraphPad) and R (R Core Team 2019). 24hour mortality was assessed using t-tests for the comparison of observed means, and mosquito longevity was analysed using Kaplan Meir Long-rank (Mantel-Cox) tests. Shapiro-Wilk tests were carried out on all activity data to check for normality. Total activity was analysed used Welch's ANOVA as we did not assume that all groups sampled were from populations with equal variance. Generalised linear models (GLMs) with normal probability distribution were used to analyse pairwise comparisons of mosquito strain and net type for: behavioural mode, contact number, contact duration, duration of contact in first 10 minutes, average contact duration, swooping speed, activity decay, willingness to refeed and wing length. Post-hoc analysis used the Tukey method of adjustment for comparing a family of four estimates. We used a binomial GLM to look for any interactions that might explain a relationship between net contact duration and mortality, however the model showed that there was no interaction between net type and contact duration or strain and contact duration. We used a GLM to investigate the relationship between mosquito wing size and blood feeding success, considering interactions with mosquito strain and net type. For all statistical comparisons, the α threshold used was 0.05. Unless stated otherwise, 95% confidence intervals are reported.

Ethical permission

With no infection risk and no exposure to untested chemicals, the procedures involved in generating these data results did not require clearance by LSTM Research Ethics Committee. We obtained written consent from all volunteers.

Results

A total of 1690 mosquitoes were tested across 73 assays, with 18 different volunteers being used as a human 'bait'. The total number of replicates performed for each strain and

treatment are shown in Table 3. It was not possible to reach the target replicate number of six for all strain and net treatment combinations because several video files were corrupted during a computer failure resulting in missing videos, time constraints due to national COVID-19 restrictions and the LSTM Banfora colony which lost its high level of resistance before PermaNet 3.0 and Interceptor G2 replicates could be completed. All room scale recordings were completed between June 2019 and February 2020.

Table 3. Total number of replicates performed per ITN, per mosquito strain. (UT = untreated net, OL = Olyset Net, P3 = PermaNet 3.0, IG2 = Interceptor G2)

Strain	***Recording dates***	***Long-Lasting Insecticidal Nets***			
		UT	***OL***	***P3***	***IG2***
Kisumu	**Jun 2019 –Jan 2020**	5	6	6	6
N'gousso	**Jun 2019 – Nov 2019**	4	6	2	6
VK7	**Jun 2019 – Feb 2020**	4	5	5	5
Banfora	**Jul 2019 – Dec 2020**	4	6	3	0

Mosquito survival

Bioefficacy

Mortality at 24h after the two-hour room scale tracking assay on untreated net (UT) was below 20% for all strains (Figure 2). OL, P3 and IG2 all killed more than 90% of susceptible strains within 24hours. Mortality rates at 24hours were significantly lower for resistant VK7 and Banfora strains with OL, P3 and IG2 nets (Figure 2) (Additional Table 1) compared to susceptible strains Kisumu and N'gousso (OL: VK7 v Kisumu $p<0.0001$, VK7 v N'gousso $p<0.0001$, Banfora v Kisumu $p=0.0013$, Banfora v N'gousso $p=0.0014$; P3: VK7 v Kisumu $p=0.0042$, N'gousso v VK7 $p=0.0903$, N'gousso v Banfora $p=0.0602$ Banfora v Kisumu

p=0.0007; IG2: VK7 v Kisumu p<0.0001, VK7 v N'gousso p<0.0001) (Additional Table 2). Note that the N'gousso results derive from only 2 test repeats, which may account for the non-significant *P*-values, despite the differences in mean mortalities. The highest 24hour mortality observed for VK7 strain was following P3 tests, which was significantly higher than that of OL (p=0.0009) and IG2 (p<0.0001). There was no significant difference in mortality rates between OL and IG2. Twenty-four-hour mortalities of the Banfora strain ranged between 45.34% and 72.38% and were not significantly different between ITNs.

Cumulative mortality rates 72hr after exposure to IG2 (containing the slower acting pyrrole insecticide chlorfenapyr) were lower in VK7 than in both susceptible strains (VK7 25.25%, 95% CI 10.29, 40.21]; Kisumu 95.91%, 95% CI [86.91, 100]; N'gousso 98.86%, 95% CI [95.25, 100]; VK7 v Kisumu t(8)= 9.28, p<0.0001; VK7 v N'gousso t(8)= 10.04, p<0.0001). Cumulative 72hr mortality for VK7 and Banfora after exposure to OL increased to 35.04% and 61.42% respectively, and after P3 exposure to 79.29% and 73.53% respectively. The increase in mortality between 24 and 72 hours seen after all ITN exposure was not significantly different to the increase seen in this time frame after exposure to UT nets for either resistant strains.

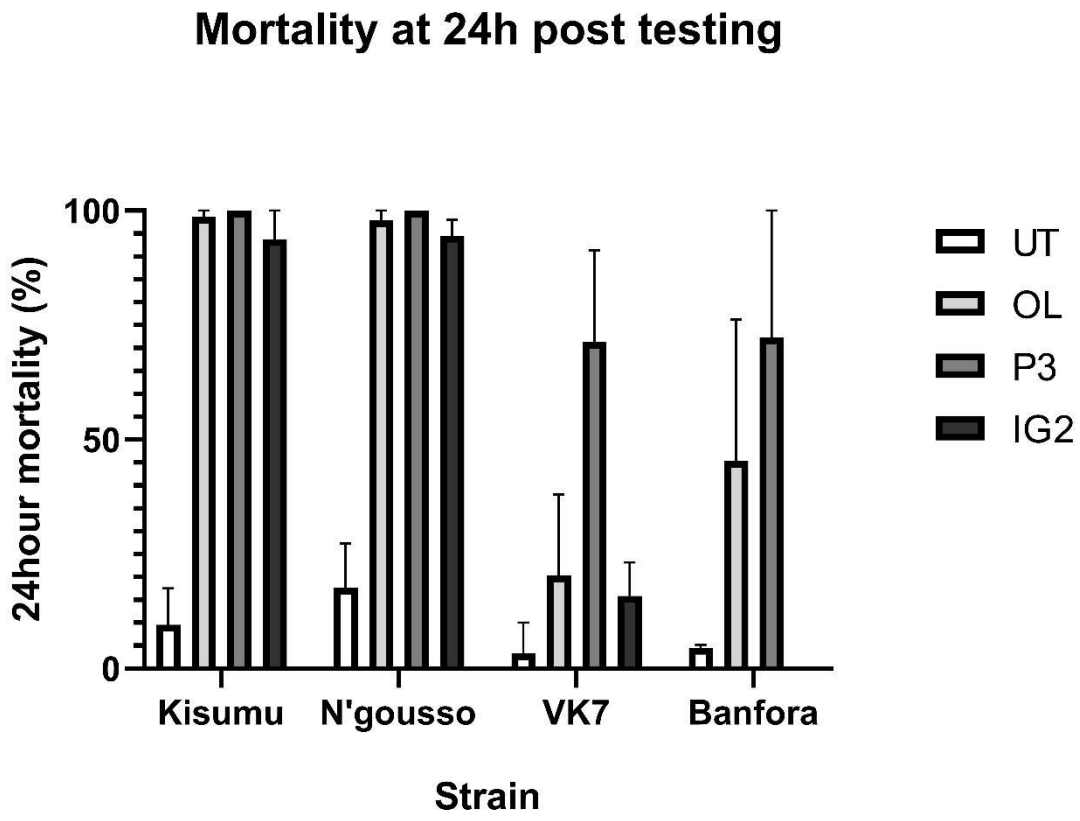


Figure 2. Mean mortality of two susceptible (Kisumu, and N'gousso) and two resistant (VK7 and Banfora) *Anopheles gambiae* strains at 24h after a two-hour exposure during room scale tracking to untreated net (UT), Olyset Net (OL), PermaNet 3.0 (P3) and Interceptor G2 (IG2) with 95% Confidence Intervals.

Longevity

For VK7, median survival time after IG2 exposure was identical to that recorded after UT exposure IG2 10days [95% CI 7.53, 12.48]; UT 10days [95% CI 8.23, 11.77]] with no significant difference in overall longevity [VK7 UT v IG2 p=0.2150]. For the same strain, median survival times following OL exposure was five days [95% CI 3.20, 6.80] and following P3 was one day [95% CI 0, 1]. In both resistant strains, P3 exposure had the largest impact in reducing longevity (VK7: UT v OL p=0.0198, UT v P3 p<0.0001; Banfora: UT v OL p=0.0026, UT v P3 p=0.0099) (Figure 3). Both resistant strains survived significantly longer after

exposure to all three ITNs compared to the susceptible strains (Additional Table 3). The median survival time after exposure to UT nets varied between strains (Kisumu 7 days [95% CI 5.58, 8.33]; N'gousso 12 days [95% CI 10.25, 13.76]; VK7 10 days [95% CI 8.23, 11.77]; Banfora 8 days [95% CI 6.49, 9,51]).

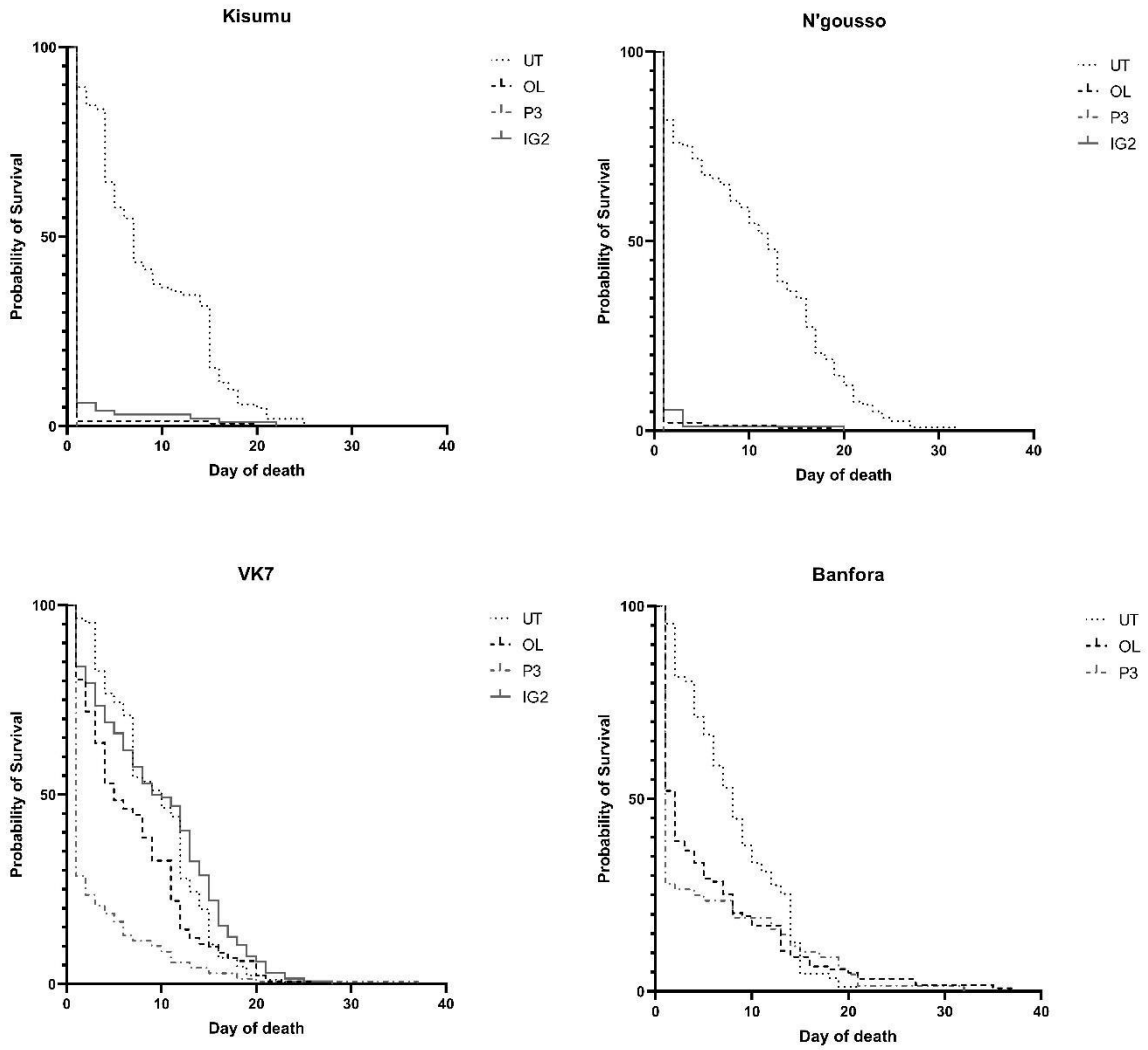


Figure 3. Survival curves for susceptible (Kisumu and N'gousso) and resistant (VK7 and Banfora) *Anopheles gambia*e after exposure in the room scale tracking room to either untreated net (UT), Olyset Net (OL), PermaNet 3.0 (P3) or Interceptor G2 (IG2). Day 0 is day of exposure.

Mosquito activity and behaviour

Total activity and behavioural mode

Figure 4 shows mean total mosquito activity for each strain and net combination, across a two-hour recording, with activity separated into the four distinct behavioural modes: swooping, visiting, bouncing or resting defined by Parker *et al* (2015). Across all treatments, flight track length ranged from 2.5mm to 20,249mm and track duration ranged from 0.08 seconds to 1,010 seconds. For all four strains, total activity was significantly longer at an UT net than at any of the three ITNs (Kisumu Welch's $F(3.0, 8.71)=44.44$, $p<0.0001$; N'gousso Welch's $F(3.0, 3.59)=24.15$, $p=0.0074$; VK7 Welch's $F(3.0, 7.27)=20.82$, $p=0.0006$; Banfora Welch's $F(2.0, 5.29)=32.17$, $p=0.0011$). Comparing net types showed no significant differences in total activity between any of the strains (UT Welch's $F(3.0, 6.90)=3.94$, $p=0.0626$; OL Welch's $F(3.0, 9.38)=2.21$, $p=0.1543$; P3 Welch's $F(3.0, 4.11)=2.23$, $p=0.2240$; IG2 Welch's $F(2.0, 9.30)=0.60$, $p=0.5709$).

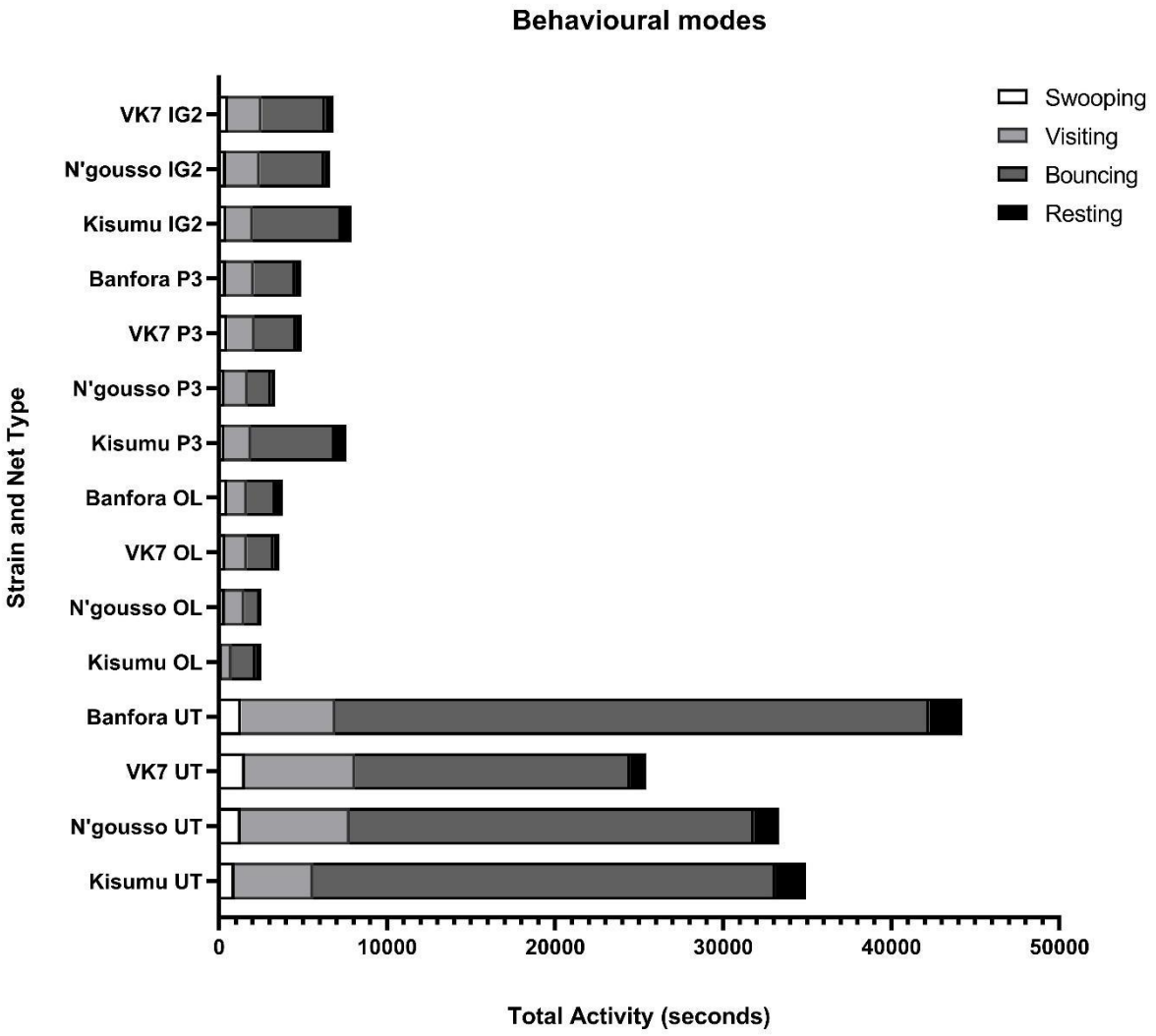


Figure 4. Behaviour of *Anopheles gambiae* at human baited bed nets. Mean total activity time of *Anopheles gambiae* recorded for each behavioural mode over two-hour recording period. As multiple mosquitoes were active simultaneously in the field of view, the total activity time could exceed the total recording time of 2 hours (7,200 seconds).

Breaking down total mosquito activity to look at time spent in each of the four distinct behavioural modes, revealed that both susceptible and resistant mosquitoes always spent more time swooping, visiting, bouncing and resting at an UT net than at any of the three ITNs (Additional Table 4;he one exception to this was comparing VK7 on UT and IG2, where there was no difference in total time spent resting (VK7 UT v IG2 p=0.1591)). However,

there were no significant differences in the proportionate amounts of time spent swooping, visiting, bouncing, or resting between different ITNs (Additional Table 5).

Results comparing total activity changes on each net between strains for the four behavioural modes, showed that there was no difference in swooping activity between any strains on any nets, bar VK7 showing more activity than Kisumu around an UT net (UT Kisumu v VK7 $p=0.0010$). Analysis of total visiting time showed that N'gousso and VK7 spent more time in this behavioural mode than Kisumu when an UT net was present (UT Kisumu v N'gousso $p=0.0352$, Kisumu v VK7 $p=0.0248$), but there were no differences when comparing between any other nets. Banfora spent significantly more time bouncing on UT net than all other strains (UT Kisumu v Banfora $p=0.0014$, N'gousso v Banfora $p<0.0001$, VK7 v Banfora $p<0.0001$), and both susceptible strains spent more time bouncing than resistant VK7 (Kisumu v VK7 $p<0.0001$ N'gousso v VK7 $p=0.0032$). There was no difference in time spent bouncing between any strains on any of the ITNs. Kisumu and Banfora spent more time resting on an UT net than VK7 (UT Kisumu v VK7 $p=0.0004$, VK7 v Banfora $p=0.0001$), but there were no other significant differences in total time spent resting with an UT net or any of the ITNs (Additional Table 6).

Quantifying number and duration of net contact

Contact number

All strains showed significantly greater mean total number of contacts with the UT net than with any of the ITNs (Additional Table 7). There were significant differences in the mean number of contacts with an UT net between some strains: Banfora had significantly more contact with the UT net than N'gousso and VK7, while Kisumu and N'gousso had more contact than VK7. Within strain comparisons showed there was no significant difference in

the number of contacts made with any of the ITNs (Additional Table 8). There was also no difference in the number of contacts made between any of the strains on any of the ITNs (Additional Table 9) (Figure 5, panel A).

Contact duration

Both susceptible and resistant mosquitoes spent significantly more time in contact with the UT net than any of the ITNs. Kisumu spent significantly more time in contact with IG2 than OL, but there were no other differences between nets (Additional Table 10). Between strain comparisons showed that Banfora spent significantly more time on UT net than all other strains, and both susceptible strains had longer contact duration than VK7. There was no significant difference in net contact duration for any strain combinations on treated nets (Additional Table 11) (Figure 5, panel B).

We calculated that during the 120-minute recording period each mosquito had between 285.62 seconds and 1041.79 seconds of contact with the UT net. There were no significant differences in the minimum and maximum time that susceptible and resistant mosquitoes spent on any of the three ITNs (OL: susceptible strains between 7.58 seconds and 101.39 seconds, resistant strains between 3.39 seconds and 255.53 seconds; P3: susceptible strains between 40.30 seconds to 241.77 seconds, resistant strains 33.35 seconds to 273.47 seconds; IG2: susceptible strains between 40.45 seconds and 403.39 seconds, resistant strain between 34.44 seconds and 378.73 seconds). The only notable differences we observed were that the minimum time that one Kisumu mosquito could have spent on OL was significantly lower than IG2 ($p=0.0344$), and the maximum time that N'gousso spent on IG2 was longer than on OL ($p=0.0243$) (Table 4).

Table 4. Minimum and maximum individual mosquito net contact duration (seconds) for entire 120minute recording.

Treatment	*Strain*	*Minimum contact duration (s)*	*Maximum contact duration (s)*
Untreated	Kisumu	301.45	952.28
	N'gousso	398.72	962.83
	VK7	285.62	714.06
	Banfora	542.17	1041.79
Olyset	Kisumu	7.58	101.39
	N'gousso	9.96	64.28
	VK7	18.7	77.93
	Banfora	3.39	255.53
P3	Kisumu	40.3	241.77
	VK7	33.35	273.47
	Banfora	46.65	323.24
IG2	Kisumu	52.44	403.39
	N'gousso	40.45	341.07
	VK7	34.44	378.73

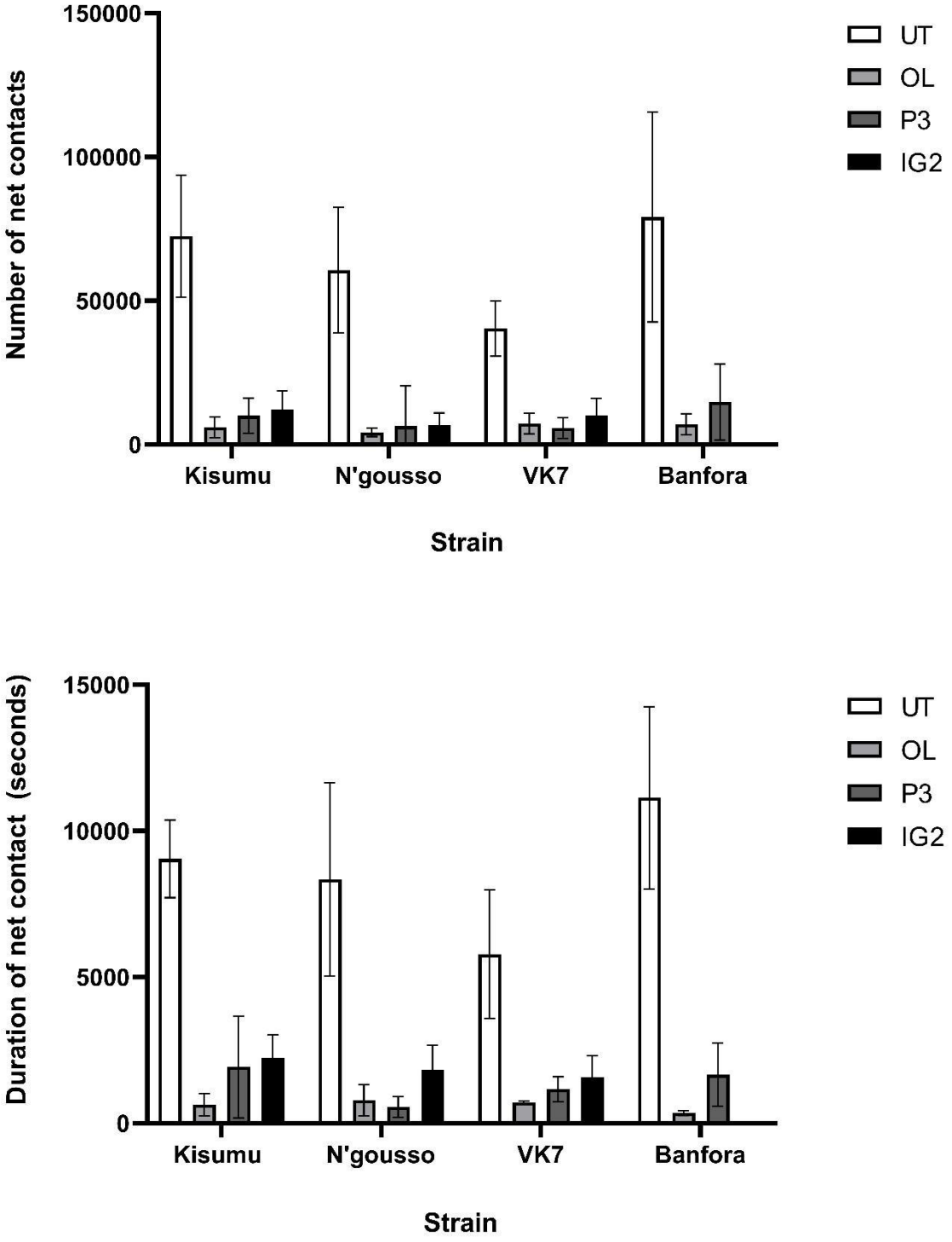


Figure 5. Mean total number of net contacts (A) and mean total duration net contact (B) with 95% Confidence Intervals for susceptible (Kisumu and N'gousso) and resistant (VK7 and Banfora) *Anopheles gambiae* strains on untreated net (UT), Olyset Net (OL), PermaNet 3.0 (P3) and Interceptor G2 (IG2).

Net interactions in first 10minutes of assay

We investigated net contact in the first 10 minutes of the video tracking to examine if there was any suggestion of immediate repellent effects of the ITNs. While contact number and contact duration was lower at ITNs than UT nets, a higher percentage of overall contact duration occurred in the first 10 minutes of the assay on ITNs for the susceptible strains (Table 5). In the first 10 minutes, Kisumu spent significantly more time in contact with the ITNs than UT, and more time in contact with IG2 than OL or P3. Similarly, N'gousso had a higher percentage of contact time occurring in the first part of the assays when OL and IG2 were present, compared to the UT net. Again, N'gousso also had a longer contact duration on IG2 than OL. For resistant VK7, the highest initial 10-minute contact duration was observed on P3, whereas Banfora showed similar time spent across all three treatments. Despite differences within strains on different nets, there were no differences observed between susceptible and resistant strains for 10-minute contact duration when an UT net or P3 was present. There was, however, a difference with OL, as both susceptible strains had a higher percentage of their overall contact duration occurring in this first period than both resistant strains. Susceptible strains also spent considerably more time contacting IG2 than VK7 (Additional Table 12, 13, 14, 15).

Table 5. Percentage of overall contact duration occurring in the first 10minutes of the 2hour assay [95% Confidence Intervals].

Net	***Strain***	***% 10mins [95% CI]***
UT	Kisumu	5.49 [3.43, 7.55]
	N'gousso	8.58 [-0.26, 17.42]
	VK7	1.81 [0.16, 3.46]
	Banfora	4.65 [1.91, 7.40]

OL	Kisumu	48.13 [21.76, 74.50]
	N'gousso	55.86 [38.31, 73.41]
	VK7	1.27 [-1.93, 4.47]
	Banfora	6.39 [-1.09, 13.87]
P3	Kisumu	29.68 [10.70, 48.65]
	N'gousso	31.73 [-59.07, 122.53]
	VK7	23.73 [5.20, 42.26]
	Banfora	11.75 [3.64, 19.85]
IG2	Kisumu	38.57 [33.29. 43.85]
	N'gousso	34.67 [17.65, 51.68]
	VK7	6.00 [1.01, 10.98]

Location of activity at the bed net interface

The distribution of total activity was heavily focused on the roof of the bed net for all strains and all net treatments (>90% on UT, >85% OL, >72% P3 and >87% IG2) as described in previous studies on standard ITNS (Lynd & Mccall, 2013; Parker *et al*., 2017) (Table 6). There was no significant difference in the percentage of contact occurring on the roof of the net for any strain or net combinations.

Table 6. Percentage of overall contact across different regions of the bed net (%).

Treatment	Strain	Roof	Front	Sides
Untreated	Kisumu	93.91	5.81	0.28
	N'gousso	96.49	2.83	0.69
	VK7	91.64	7.51	0.86
	Banfora	95.73	3.47	0.80
Olyset Net	Kisumu	92.58	7.09	0.33
	N'gousso	86.39	10.27	3.34

	VK7	86.59	11.99	1.42
	Banfora	85.22	13.15	1.63
PermaNet 3.0	Kisumu	72.19	25.66	2.15
	VK7	78.67	16.22	5.11
	Banfora	91.61	5.84	2.55
Interceptor G2	Kisumu	92.33	6.64	1.03
	N'gousso	92.23	6.53	1.24
	VK7	87.87	9.77	2.36

Mosquito velocity during interaction with host within bed nets

Average speed of whole swooping tracks was analysed to assess changes in speed between strains around different bed nets. Only susceptible Kisumu showed any difference in flight speed around different net treatments, flying significantly faster around OL and IG2 than UT nets. Resistant strains did now show any difference in flight speed between different net types. Between strains, both resistant strains flew faster around an UT net than Kisumu and Banfora was significantly faster than Kisumu around P3. There was so difference in overall swooping speed between strains when OL or IG2 were present (Additional Table 16, 17).

Mosquito interaction with the bed nets over time

We observed a steep decay in activity over the duration of the assay for susceptible strains with P3 and OL compared to UT net (Kisumu: UT v OL $p=0.0023$, UT v P3 $p=0.0020$). Kisumu also showed a dramatic decrease in activity in the presence of IG2 (UT v IG2 $p<0.0001$), which was not replicated in N'gousso activity decay around the same net. Resistant strains showed a less dramatic decay in activity when P3 and OL present, however decay was still more pronounced than with UT (VK7 UT v OL $p=0.0128$, UT v P3 $p=0.0010$), and there was no significant activity decay when VK7 was exposed to IG2. All strains exhibited no activity decay in the presence of an UT net (Figure 6) (Additional Table 18, 19).

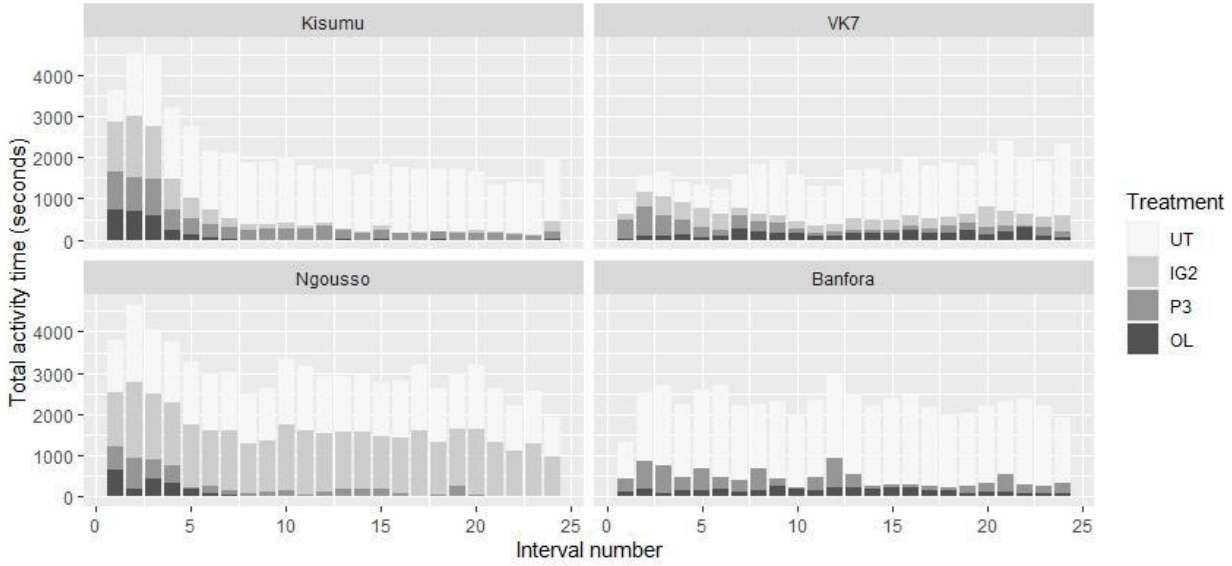


Figure 6. Rates of *Anopheles gambiae* activity across all four behavioural modes combined, throughout 120minute recording test period. Total activity is shown for untreated net (UT), Olyset Net (OL), PermaNet 3.0 (P3) and Interceptor G2 (IG2) for Kisumu, N'gousso, VK7 and Banfora.

Sub-lethal pipeline – wing size and willingness to feed

Wing size was measured as a proxy for mosquito body size. There was a negative correlation between wing size and blood-feeding inhibition, with smaller mosquitoes less likely to survive and accept a bloodmeal. However, there was no significant interaction between wing size and strain (p=0.9447), indicating that the relationship between wing size and blood feeding success was the same for all strains.

The majority of susceptible mosquitoes exposed to the three ITNs were either knocked-down or dead and hence unable to blood feed. OL reduced resistant strain feeding by up to 83% (VK7 71% [95% CI 62, 80], Banfora 83% [95% CI, 76, 91]), P3 reduced VK7 feeding by 97% [95% CI 94, 99], whereas IG2 had a smaller effect, reducing VK7 blood feeding success by 41% [95% CI 31, 51] (Figure 7). Between 14% and 70% of mosquitoes were unable to blood feed after exposure to UT net.

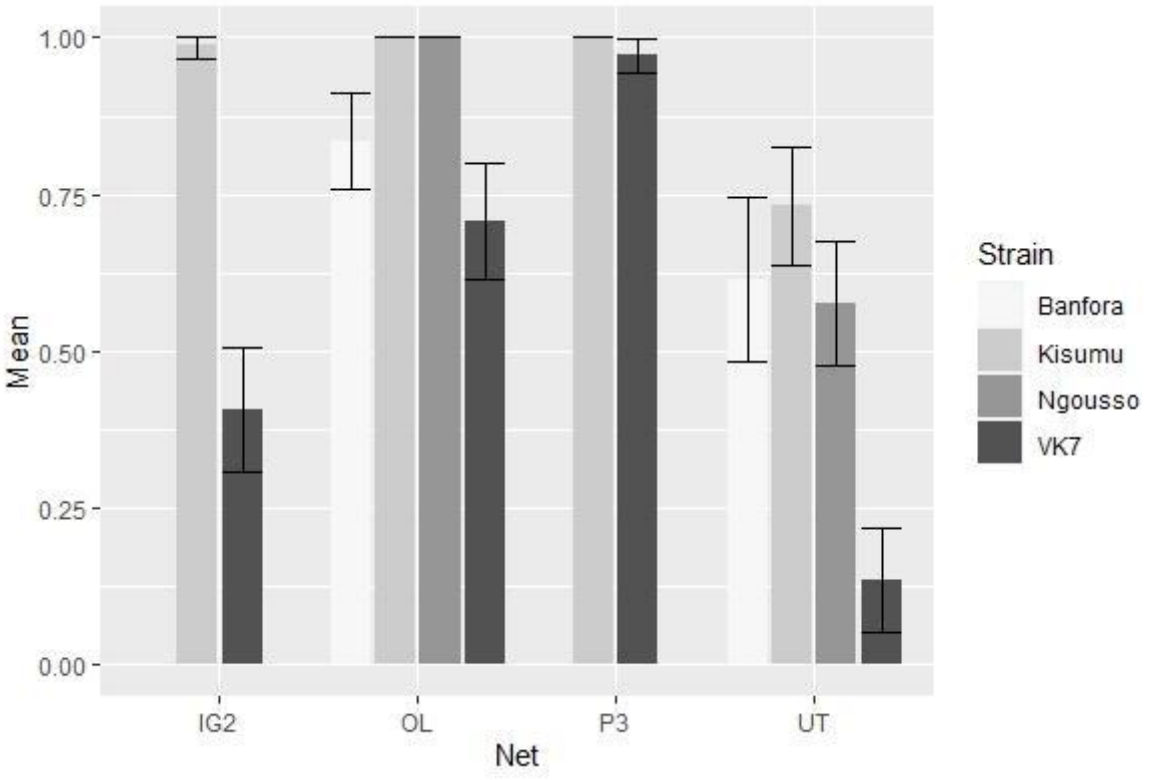


Figure 7. Predicted mean reduction in blood-feeding success of four strains after exposure to untreated (UT), Olyset Net (OL), PermaNet 3.0 (P3) and Interceptor G2 (IG2) (95% CI).

Discussion

These results provide a first in-depth description of the behaviour of susceptible and resistant *Anopheles gambiae* strains around next-generation bed nets and the impact of these new nets on them. As insecticide resistance continues to be a growing threat to the success of African vector control programmes, there is an urgent need for safe novel treatments suitable for use on ITNs. The first of the next-generation nets using these treatments are now being evaluated in field trials (Mosha *et al*., 2022; Tungu *et al.*, 2021) and deployed at scale in pilot studies in several countries (IVCC, 2020). Determining how mosquitoes interact with the nets, and the consequences of net contact for mosquitoes, will aid in interpretation of the results of clinical trials, and extrapolation to alternative settings with different mosquito populations.

OL, P3 and IG2 all killed more than 90% of susceptible mosquitoes 24 hours after a 2-hour exposure, but this effect was not seen with resistant mosquitoes where only 20.4% of VK7 and 45.4% of Banfora on OL, 71.4% of VK7 and 72.4% of Banfora on P3, and 15.9% of VK7 on IG2 (the Banfora strain was not tested on this net) were dead at 24hours. Total mosquito activity was higher around an UT net than all ITNs, which is comparable with results obtained in previous studies (Parker *et al*., 2015). Interestingly, there was no difference in total activity observed between susceptible and resistant strains around any of the ITNs tested, the number and duration times of net contact was also similar for all strains. Net contact was focussed predominantly on the roof for all types of bed net and did not change throughout the assay (Parker *et al*., 2015; James F. Sutcliffe & Yin, 2014). Through comparing the difference in the first and last 10 minutes of recording activity, we observed a steep decay in activity for both susceptible strains when P3 and OL were present, but only a decrease in activity around IG2 for susceptible Kisumu. Resistant strains showed a less

dramatic decay in activity when P3 and OL present, however decay was still more pronounced than with UT. The activity decay in susceptible strains most likely reflects that mosquitoes are being knocked down and killed by the active-ingredients, however, the lack of decay observed with resistant strains is surprising, particularly for dual-treated nets.

The behaviour of the strains as measured by tracking was remarkably similar for all the strains tested with no significant differences observed in the number of contacts, or the duration of time spent contacting ITNs between susceptible and resistant mosquito strains. We did not observe evidence of a repellent effect on susceptible mosquitoes for any ITN as a higher percentage of overall contact duration occurred during the first 10 minutes of the assay on all ITNs compared to untreated net.

The low mortality results in resistant strains from our study do not match those from recent experimental hut studies reporting promising results with the Interceptor G2 net (Bayili *et al*., 2017; Camara *et al*., 2018; N'Guessan *et al*., 2016; Tungu *et al*., 2021) where mortality in huts with IG2 was significantly higher than for standard pyrethroid only ITNs in all settings. A recent clinical trial by Mosha *et al.,* (2022) reported after two years IG2 provided significantly better protection from malaria than an alpha-cypermethrin only ITN in areas where mosquito populations are resistant to pyrethroids (Mosha *et al*., 2022). Nevertheless, when tested in a laboratory under standard conditions, the results from ours and other studies are not dissimilar, with low mortalities of insecticide resistant mosquitoes at both 24hours and 72hours post IG2 exposure. We recorded 25.6% mortality at 72hours in the resistant VK7 strains and others have reported ~5-26% mortality using a 3minute WHO cone assay and a wider range of between ~18% - 100% after a 30minute exposure in a WHO tube assay(Camara *et al*., 2018; N'Guessan *et al.*, 2016). The reasons for differences in performance of IG2 under laboratory and field settings are unclear but differences in the

mosquito population assessed may be important. Unpublished data from multiple experimental hut studies in southwest Burkina Faso (the region of origin of the VK7 and Banfora strains used in the current study) show relatively poor performance of IG2 nets compared to data from other settings (Sanou, A, Sagnon N, Guelbeogo M).

Moreover, the complete entomological mode of action of chlorfenapyr has not yet been determined and reproducing in the level of mortality seen in hut trials in laboratory assays has proven challenging. This severely limits our ability to apply lab tests, including video tracking, in the evaluation of products containing this insecticide.

Mosquitoes were given the opportunity to blood feed one-hour post-assay and we observed a reduction in blood feeding success with resistant strains after exposure to all ITNs. Despite lower mortality with the pyrethroid only Olyset Net and next-generation Interceptor G2, blood feeding success in resistant strains was reduced by up to 83% and 41% respectively. A reduction in blood-feeding after insecticide exposure was also found by Barreaux *et al.*, (2022) who reported that that after forced exposure to ITNs the blood feeding success of highly insecticide resistant *An. gambiae* strains was reduced. The authors suggest that this was not a result of mosquitoes avoiding the net or being repelled by it, but instead because contact with insecticides reduced feeding capacity.

As previously observed (Parker *et al*., 2017), both susceptible and resistant strains showed a much higher level of overall activity when an UT net was present, with activity levels reducing dramatically in the presence of all tested ITNs. This reduction in activity was observed for all strains with no significant differences in total activity level between any of the strain and ITN comparisons. This suggests that even if this measurable reduction in activity is attributable to the pyrethroid component on the net the additional AI.s do not

alter it. Moreover, the novel chemistries do not affect mosquitoes of differing resistance status differently. One result to note, is that despite the low mortality rate of VK7 when exposed to IG2, the time spent resting on this ITN was similar to that of when an UT net was present. One explanation for these results could be that there is a currently unknown interaction occurring between the two insecticides, which is reducing the efficacy of chlorfenapyr. We believe that this could be due to the pyrethroid suppressing chlorfenapyr activation by preferentially binding cytochrome p450s and hence delaying activation to the lethal metabolite tralopyril.

There are a few limitations to this study which are important to consider. (Voloshin *et al*., 2020). While the environment in which the tracking assay data are collected reproduces as much as possible the conditions in the interior of a hut, there are important omissions and differences. Firstly, the (apparent) repellent properties of some nets that reduce initial eave entry cannot be measured here nor can the proportion of mosquitoes that leave the room after contacting the net. Hence all 25 mosquitoes must enter and remain in the room potentially delivering an overestimate of the lethality of the net being tested. Environmental conditions also remained static throughout the test whilst in reality air disturbances, and changes in temperature during the night may affect net contact.

It was not possible to determine individual mosquito contact, and total net contact was calculated based on the maximum number of mosquitoes seen simultaneously contacting the net in any one frame of the recording. Although this method provides a more realistic estimate of mosquito/ITN contact times than other standard bioassays, the measurement does not account for mosquitoes that make zero contact or that return to make multiple contacts with the net. This is especially important for the interpretation of sublethal results with contact duration varying between the individuals exposed. There is therefore a strong argument for collecting data to determine LD50 equivalents for duration of net contact, determined for each ITN. The video recordings in this study were limited to 2hours as the

data files produced are extremely large (2-3Tb per camera, per recording), but recording mosquito behaviour for longer periods to assess any delayed effects on mosquito behaviour could prove important when evaluating impacts of nets with poorly understood AIs. Future studies would benefit from more replicates with multiple different resistant mosquito strains, to investigate the potential effect of different resistance mechanisms, an aspect of evaluating ITNs already supported by many (Lees *et al*., 2022).

Overall, these findings expand our knowledge of how mosquitoes interact with ITNs, particularly with regards to behaviour around new chemistries. These results indicate that the effects of a range of ITNs on mosquito behaviour is remarkably consistent with no major alterations in mosquito responses, particularly ITN contact resulting from exposure to the nets by strains of differing pyrethroid susceptibilities. It also appears that lower ITN contact is not the reason for observed lower mortality in resistant strains. Ongoing work in multiple field sites will continue to explore the effects of new ITNs on the behaviour of wild mosquito populations and will undoubtedly contribute to the body of work gathering as a foundation for understanding behavioural mechanisms of resistance.

Author contributions

Conceptualisation: PJM, HR, GMF. Funding acquisition: PJM, HR, GMF. Hardware and software: DT, CET, VV. Data collection: KG, AG, ME, AnM. Data analysis: KG, AG, FM, AM. Writing, original draft: KG. Writing, review and editing: all authors.

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References

Angarita-Jaimes, N. C., Parker, J. E. A., Abe, M., Mashauri, F., Martine, J., Towers, C. E., … Towers, D. P. (2016). A novel video-tracking system to quantify the behaviour of nocturnal mosquitoes attacking human hosts in the field. *Journal of the Royal Society Interface*, *13*(117). https://doi.org/10.1098/rsif.2015.0974

Balabanidou, V., Kampouraki, A., MacLean, M., Blomquist, G. J., Tittiger, C., Juárez, M. P., … Vontas, J. (2016). Cytochrome P450 associated with insecticide resistance catalyzes cuticular hydrocarbon production in *Anopheles gambiae*. *Proceedings of the National Academy of Sciences*, *113*(33), 9268–9273. https://doi.org/10.1073/pnas.1608295113

Bayili, K., Severin, N., Namountougou, M., Sanou, R., Ouattara, A., Dabiré, R. K., … Diabaté, A. (2017). Evaluation of efficacy of IG2 , a long - lasting insecticide net coated with a mixture of chlorfenapyr and alpha - cypermethrin , against pyrethroid resistant Anopheles gambiae s . l . in Burkina Faso. *Malaria Journal*, 1–9. https://doi.org/10.1186/s12936-017-1846-4

Churcher, T. S., Lissenden, N., Griffin, J. T., Worrall, E., & Ranson, H. (2016). The impact of pyrethroid resistance on the efficacy and effectiveness of bednets for malaria control in Africa. *Elife*, *5*. https://doi.org/10.7554/eLife.16090

Edi, C. V., Djogbénou, L., Jenkins, A. M., Regna, K., Muskavitch, M. A. T., Poupardin, R., … Weetman, D. (2014). CYP6 P450 Enzymes and ACE-1 Duplication Produce Extreme and Multiple Insecticide Resistance in the Malaria Mosquito Anopheles gambiae. *PLoS Genetics*, *10*(3). https://doi.org/10.1371/journal.pgen.1004236

Gatton, M. L., Chitnis, N., Churcher, T., Donnelly, M. J., Ghani, A. C., Godfray, H. C. J., … Lindsay, S. W. (2013). The importance of mosquito behavioural adaptations to malaria control in Africa. *Evolution*, *67*(4), 1218–1230. https://doi.org/10.1111/evo.12063

Hancock, P. A., Hendriks, C. J. M., Tangena, J. A., Gibson, H., Hemingway, J., Coleman, M., … Moyes, C. L. (2020). Mapping trends in insecticide resistance phenotypes in African malaria vectors. *PLoS Biology*, *18*(6), 1–23. https://doi.org/10.1371/journal.pbio.3000633

Harris, C., Lambrechts, L., Rousset, F., Abate, L., Nsango, S. E., Fontenille, D., … Cohuet, A. (2010). Polymorphisms in Anopheles gambiae immune genes associated with natural resistance to plasmodium falciparum. *PLoS Pathogens*, *6*(9). https://doi.org/10.1371/journal.ppat.1001112

Hauser, G., Thiévent, K., & Koella, J. C. (2019). *the ability of Anopheles gambiae mosquitoes to bite through a permethrin-treated net and the consequences for their fitness*. https://doi.org/10.1038/s41598-019-44679-1

Hemingway, J. (2017). The way forward for vector control. *Science*, *358*(6366), 998–999. https://doi.org/10.1126/science.aaj1644

Hughes, A., Foster, G. M., Guy, A., Matope, A., Abe, M., Towers, D., & McCall, P. J. (2020). Quantifying late-stage host-seeking behaviour of Anopheles gambiae at the insecticidal net interface using a baited-box bioassay. *Malaria Journal*, *19*(1), 1–11. https://doi.org/10.1186/s12936-020-03213-9

Hughes, A. J., Matope, A., Emery, M., Steen, K., Murray, G. P. D., Ranson, H., … Foster, G. M. (n.d.). *A closer look at the WHO cone bioassay: video analysis of the hidden effects of a human host on mosquito behaviour and insecticide contact*.

Killeen, G. F. (2014). Characterizing, controlling and eliminating residual malaria transmission. *Malaria Journal*, *13*(1), 1–22. https://doi.org/10.1186/1475-2875-13-330

Killeen, G. F., Kihonda, J., Lyimo, E., Oketch, F. R., Kotas, M. E., Mathenge, E., … Drakeley, C. J. (2006). Quantifying behavioural interactions between humans and mosquitoes: Evaluating the protective efficacy of insecticidal nets against malaria transmission in

rural Tanzania. *BMC Infectious Diseases*, *6*, 1–10. https://doi.org/10.1186/1471-2334-6-161

Liu, N. (2015). Insecticide resistance in mosquitoes: Impact, mechanisms, and research directions. *Annual Review of Entomology*, *60*, 537–559. https://doi.org/10.1146/annurev-ento-010814-020828

Martinez-Torres, D., Chandre, F., Williamson, M. S., Darriet, F., Bergeâ, J. B., Devonshire, A. L., … Pauron, D. (1998). Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector Anopheles gambiae s.s. *Insect Molecular Biology*, *7*(2), 179–184.

Moiroux, N., Gomez, M. B., Pennetier, C., Elanga, E., Djènontin, A., Chandre, F., … Corbel, V. (2012). *Changes in Anopheles funestus Biting Behavior Following Universal Coverage of Long-Lasting Insecticidal Nets in Benin*. https://doi.org/10.1093/infdis/jis565

Murray, G. P. D., Lissenden, N., Jones, J., Voloshin, V., Toé, K. H., Sherrard-Smith, E., … McCall, P. J. (2020). Barrier bednets target malaria vectors and expand the range of usable insecticides. *Nature Microbiology*, *5*(1), 40–47. https://doi.org/10.1038/s41564-019-0607-2

Ngufor, C., N'Guessan, R., Fagbohoun, J., Odjo, A., Malone, D., Akogbeto, M., & Rowland, M. (2014). Olyset Duo® (a pyriproxyfen and permethrin mixture net): An experimental hut trial against pyrethroid resistant Anopheles gambiae and Culex quinquefasciatus in southern Benin. *PLoS ONE*, *9*(4). https://doi.org/10.1371/journal.pone.0093603

Parker, Angarita-Jaimes, N., Abe, M., Towers, C. E., Towers, D., & McCall, P. J. (2015). Infrared video tracking of Anopheles gambiae at insecticide-treated bed nets reveals rapid decisive impact after brief localised net contact. *Scientific Reports*, *5*(July), 1–14. https://doi.org/10.1038/srep13392

Parker, J. E. A., Angarita Jaimes, N. C., Gleave, K., Mashauri, F., Abe, M., Martine, J., … McCall, P. J. (2017). Host-seeking activity of a Tanzanian population of Anopheles

arabiensis at an insecticide treated bed net. *Malaria Journal*, *16*(1). https://doi.org/10.1186/s12936-017-1909-6

Pates, H., & Curtis, C. (2005). Mosquito behaviour and vector control. *Annual Review of Entomology*, *50*, 53–70.

Pryce, J., Richardson, M., & Lengeler, C. (2018). Insecticide-treated nets for preventing malaria. *Cochrane Database of Systematic Reviews*, *2018*(11). https://doi.org/10.1002/14651858.CD000363.pub3

Sanou, A., Nelli, L., Moussa Guelbéogo, W., Cissé, F., Tapsoba, M., Ouédraogo, P., … Ferguson, H. M. (2020). Insecticide resistance and behavioural adaptation as a response to long-lasting insecticidal net deployment in malaria vectors in the Cascades region of Burkina Faso. *Scientific Reports |*, *11*, 17569. https://doi.org/10.1038/s41598-021-96759-w

Shute, G. T. (1956). A method of maintaining colonies of east african strains of anopheles gambiae. In *Annals of Tropical Medicine and Parasitology* (Vol. 50). https://doi.org/10.1080/00034983.1956.11685743

Staedke, S. G., Gonahasa, S., Dorsey, G., Kamya, M. R., Maiteki-Sebuguzi, C., Lynd, A., … Donnelly, M. J. (2020). Effect of long-lasting insecticidal nets with and without piperonyl butoxide on malaria indicators in Uganda (LLINEUP): a pragmatic, cluster-randomised trial embedded in a national LLIN distribution campaign. *Lancet*, *395*(10232), 1292–1303. https://doi.org/10.1016/s0140-6736(20)30214-2

Toé, K. H., Jones, C. M., N'fale, S., Ismai, H. M., Dabiré, R. K., & Ranson, H. (2014). Increased pyrethroid resistance in malaria vectors and decreased bed net effectiveness Burkina Faso. *Emerging Infectious Diseases*, *20*(10), 1691–1696. https://doi.org/10.3201/eid2010.140619

Toé, K. H., N'Falé, S., Dabiré, R. K., Ranson, H., & Jones, C. M. (2015). The recent escalation in strength of pyrethroid resistance in Anopheles coluzzi in West Africa is linked to

increased expression of multiple gene families. *BMC Genomics*, *16*(1), 1–11. https://doi.org/10.1186/s12864-015-1342-6

Voloshin, V., Kröner, C., Seniya, C., Murray, G. P. D., Guy, A., Towers, C. E., … Towers, D. P. (2020). *Diffuse retro-reflective imaging for improved video tracking of mosquitoes at human baited bednets*. https://doi.org/10.1098/rsos.191951

Williams, J., Flood, L., Praulins, G., Ingham, V. A., Morgan, J., Lees, R. S., & Ranson, H. (2019). Characterisation of Anopheles strains used for laboratory screening of new vector control products. *Parasites and Vectors*, *12*(1), 1–14. https://doi.org/10.1186/s13071-019-3774-3

Williams, J., Ingham, V. A., Morris, M., Toe, H. K., Hien, A. S., Morgan, J. C., … Ranson, H. (2022). Sympatric populations of the Anopheles gambiae complex in southwest Burkina Faso evolve multiple diverse resistance mechanisms in response to intense selection pressure with pyrethroids. *Insects*.

Additional material

Additional Table 1. Mean 24hour mortality [95% CI]

Treatment	Strain	24hour mortality (%) [95% CI]
Untreated	Kisumu	9.5 [1.47, 17,54]
	N'gousso	17.64 [7.87, 27.40]
	VK7	3.36 [0, 10.05]
	Banfora	4.52 [3.85, 5.20]
Olyset Net	Kisumu	98.67 [95.24, 100]
	N'gousso	97.97 [94.44, 100]
	VK7	20.35 [2.09, 38.01]
	Banfora	45.34 [14.52, 76.17]
PermaNet 3.0	Kisumu	100 [100, 100]
	N'gousso	100 [100, 100]
	VK7	71.37 [51.39,91.36]
	Banfora	72.38 [41.13, 100]
Interceptor G2	Kisumu	93.88 [81.53, 100]
	N'gousso	94.56 [91.10, 98.02]
	VK7	15.90 [8.62, 23.21]

Additional Table 2. Mean 24hour mortality comparisons between three insecticide treated nets and four mosquito strains, two susceptible (Kisumu and N'gousso) and two resistant (VK7 and Banfora).

Strain comparison	ITN		
	Olyset Net	PermaNet 3.0	Interceptor G2
Kisumu v VK7	t(9)= 12.80, p<0.0001	t(10)= 3.68, p=0.0042	t(8)= 16.64, p<0.0001

Kisumu v Banfora	t(10)= 4.42, p0.0013	t(7)= 5.81, p=0.0007	N/A
N'gousso v VK7	t(9)= 12.67, p<0.0001	t(6)= 2.07, p=0.0903	t(8)= 21.54, p<0.0001
N'gousso v Banfora	t(10)= 4.36, p=0.0014	t(3)= 2.95, p=0.0602	N/A

Additional Table 3. Comparison of median survival times of susceptible (Kisumu and N'gousso) and resistant (VK7 and Banfora) strains on four different net treatments.

Strain comparison	ITN			
	Untreated net	Olyset Net	PermaNet 3.0	Interceptor G2
Kisumu v N'gousso	χ^2 (1, N=221) = 6.68, p=0.0098	χ^2 (1, N=285) = 0.12, p=0.7241	χ^2 (1, N=188) = 0.00, p>0.9999	χ^2 (1, N=190) = 0.42, p=0.5191
Kisumu v VK7	χ^2 (1, N=192) = 0.01, p=0.9733	χ^2 (1, N=267) = 134.40, p<0.0001	χ^2 (1, N=284) = 47.72, p<0.0001	χ^2 (1, N=234) = 102.80, p<0.0001
Kisumu v Banfora	χ^2 (1, N=191) = 2.55, p=0.1102	χ^2 (1, N=268) = 67.23, p<0.0001	χ^2 (1, N=212) = 43.99, p<0.0001	N/A
N'gousso v VK7	χ^2 (1, N=205) = 7.11, p=0.0077	χ^2 (1, N=272) = 133.50, p<0.0001	χ^2 (1, N=184) = 15.98, p<0.0001	χ^2 (1, N=228) = 124.10, p<0.0001
N'gousso v Banfora	χ^2 (1, N=204) = 15.67, p<0.0001	χ^2 (1, N=263) = 65.16, p<0.0001	χ^2 (1, N=112) = 14.67, p=0.0001	N/A
VK7 v Banfora	χ^2 (1, N=175) = 3.12, p=0.0773	χ^2 (1, N=255) = 3.70, p=0.0545	χ^2 (1, N=208) = 3.63, p=0.0568	N/A

Additional Table 4. Statistically significant differences (p values) in total activity time split into four different behavioural modes (swooping, visiting, bouncing and resting), comparing untreated (UT) net to either Olyset Net (OL), PermaNet 3.0 (P3) or Interceptor G2 (IG2), for susceptible (Kisumu and N'gousso) and resistant (VK7 and Banfora) mosquitoes.

Strain	Behaviour	Insecticide treated net		
		Olyset Net	PermaNet 3.0	Interceptor G2
Kisumu	swooping	<0.0001	0.0006	0.0067
	visiting	<0.0001	<0.0001	<0.0001
	bouncing	<0.0001	<0.0001	<0.0001
	resting	<0.0001	<0.0001	<0.0001
N'gousso	swooping	<0.0001	N/A	<0.0001
	visiting	<0.0001	N/A	<0.0001
	bouncing	<0.0001	N/A	<0.0001
	resting	<0.0001	N/A	<0.0001
VK7	swooping	<0.0001	<0.0001	<0.0001
	visiting	<0.0001	<0.0001	<0.0001
	bouncing	<0.0001	<0.0001	<0.0001
	resting	0.0300	0.0264	0.1591
Banfora	swooping	<0.0001	<0.0001	N/A
	visiting	<0.0001	<0.0001	N/A
	bouncing	<0.0001	<0.0001	N/A
	resting	<0.0001	<0.0001	N/A

Additional Table 5. Within strain comparisons (p-value) of total activity time split into four different behavioural modes (swooping, visiting, bouncing and resting) between three ITNs (Olyset Net = OL, PermaNet 3.0 = P3, Interceptor G2 = IG2).

Behaviour	ITN comparison	Strain			
		Kisumu	N'gousso	VK7	Banfora
Swooping	OL v P3	0.5855	N/A	0.8362	0.9811
	OL v IG2	0.1778	0.9800	0.5898	N/A
	P3 v IG2	0.8577	N/A	0.9740	N/A

Visiting	OL v P3	0.3119	N/A	0.9528	0.9015
	OL v IG2	0.2388	0.3841	0.6678	N/A
	P3 v IG2	0.9985	N/A	0.9275	N/A
Bouncing	OL v P3	0.1818	N/A	0.9674	0.8342
	OL v IG2	0.0961	0.3402	0.6559	N/A
	P3 v IG2	0.9897	N/A	0.8978	N/A
Resting	OL v P3	0.2797	N/A	0.9999	0.2268
	OL v IG2	0.3265	0.5880	0.8416	N/A
	P3 v IG2	0.9997	N/A	0.8657	N/A

Additional Table 6. Within treatment comparisons (p-value) of total activity split into for behavioural modes (swooping, visiting, bouncing and resting) on four ITNs (Untreated net = UT, Olyset Net = OL, PermaNet 3.0 = P3, Interceptor G2 = IG2) between four mosquito strains

Behaviour	Strain comparison	ITN			
		UT	OL	P3	IG2
Swooping	Kisumu v N'gousso	0.0950	0.4483	N/A	0.9978
	Kisumu v VK7	0.0010	0.4572	0.6651	0.8395
	Kisumu v Banfora	0.0640	0.0879	0.9477	N/A
	N'gousso v VK7	0.4166	0.9999	N/A	0.7475
	N'gousso v Banfora	0.9984	0.8000	N/A	N/A
	VK7 v Banfora	0.5157	0.8484	0.9748	N/A
Visiting	Kisumu v N'gousso	0.0352	0.7484	N/A	0.9844
	Kisumu v VK7	0.0248	0.6266	0.9997	0.9377
	Kisumu v Banfora	0.5026	0.7043	0.9989	N/A
	N'gousso v VK7	0.9994	0.9946	N/A	0.9997

Bouncing	N'gousso v Banfora	0.5523	0.9998	N/A	N/A
	VK7 v Banfora	0.4796	0.9980	0.9999	N/A
	Kisumu v N'gousso	0.3291	0.9915	N/A	0.7701
	Kisumu v VK7	<0.0001	0.9997	0.5171	0.7801
	Kisumu v Banfora	0.0014	0.9985	0.8952	N/A
	N'gousso v VK7	0.0032	0.9822	N/A	1.000
	N'gousso v Banfora	<0.0001	0.9697	N/A	N/A
	VK7 v Banfora	<0.0001	0.9999	0.9669	N/A
Resting	Kisumu v N'gousso	0.1368	0.5673	N/A	0.3099
	Kisumu v VK7	0.0004	1.0000	0.3601	0.8771
	Kisumu v Banfora	0.8988	0.9949	0.8801	N/A
	N'gousso v VK7	0.0668	0.5917	N/A	0.7962
	N'gousso v Ban	0.1368	0.3588	N/A	N/A
	VK7 v Banfora	0.0001	0.9891	0.1704	N/A

Additional Table 7. Mean total number of bed net contacts [95% CI], mean total contact duration [95% CI] and maximum number of mosquitoes seen in one frame of video recording.

ITN	Strain	Replicates	Mean total number of contacts [95% CI]	Mean total contact duration [95% CI]	Maximum number of mosquitoes
UT	Kisumu	5	74885 [53016.58, 96753.42]]	9044.20 [7723.32, 10202.13]	29
	N'gousso	4	62162.25 [39731.46, 84593.04]	8254.66 [5049.89, 11459.43]	17
	VK7	4	41811.25 [31737, 51885.5]	5783.19 [3589.78, 7976.59]	14

	Banfora	4	80824 [43804.85, 117843.20]	11005.31 [7866.99, 14143.62]	14
OL	Kisumu	6	6169.17 [2521.05, 9817.29]	622.03 [238.49, 1005.57]	16
	N'gousso	5	4531.17 [3175.86, 5886.47]	342.37 [256.65, 428.09]	10
	VK7	6	7393.2 [3465.90, 11320.50]	682.53 [532.04, 833.02]	9
	Banfora	6	7413.5 [3695.42, 11167.58]	787.19 [268.22, 1306.16]	14
P3	Kisumu	6	10909.33 [3149.47, 18669.20]	1929.23 [187.19, 3671.27]	27
	VK7	5	6219 [2576.16, 9861.84]	1164.04 [[736.89, 1591.20]	9
	Banfora	3	14772 [1453.14, 28090.86]	1668.92 586.24, 2751.60]	8
IG2	Kisumu	6	12759.5 [6312.24, 19206.76]	2236.16 [1434.93, 3037.40]	14
	N'gousso	6	6686.5 [2384.28, 10988.75]	1822.44 [971.85, 2673.03]	16
	VK7	5	10488 [4366.25, 16609.75]	1587.40 [853.88, 2320.90]	11

Additional Table 8. Within strain statistical comparisons (p value) of total number of net contacts for susceptible (Kisumu and N'gousso) and resistant (VK7 and Banfora) mosquitoes between ~~three ITNs~~four nets (UT = untreated, OL = Olyset Net, P3 = PermaNet 3.0, IG2 = Interceptor G2).

Net comparison	Strain			
	Kisumu	N'gousso	VK7	Banfora
UT v OL	<0.0001	<0.0001	<0.0001	<0.0001
UT v P3	<0.0001	N/A	<0.0001	<0.0001
UT v IG2	<0.0001	<0.0001	<0.0001	N/A
OL v P3	0.7873	N/A	0.9966	0.6402
OL v IG2	0.5684	0.9741	0.9445	N/A
P3 v IG2	0.9833	N/A	0.8689	N/A

Additional Table 9. Within treatment statistical comparisons (p value) of total number of net contacts for four nets between four mosquito strains.

Strain comparison	ITN			
	Untreated	Olyset Net	PermaNet 3.0	Interceptor G2
Kisumu v N'gousso		0.9883	N/A	0.6322
Kisumu v VK7	<0.0001	0.9957	0.8151	0.9738
Kisumu v Banfora		0.9948	0.9250	N/A
N'gousso v VK7	0.0095	0.9496	N/A	0.8914
N'gousso v Banfora	0.0202	0.9414	N/A	N/A
VK7 v Banfora	<0.0001	1.0000	0.5473	N/A

Additional Table 10. Within strain comparisons (p-value) of total duration of net contact for susceptible (Kisumu and N'gousso) and resistant (VK7 and Banfora) mosquitoes between three ITNs (OL = Olyset Net, P3 = PermaNet 3.0, IG2 = Interceptor G2).

Net comparison	Strain			
	Kisumu	N'gousso	VK7	Banfora
UT v OL	<0.0001	<0.0001	<0.0001	<0.0001
UT v P3	<0.0001	N/A	<0.0001	<0.0001
UT v IG2	<0.0001	<0.0001	<0.0001	<0.0001
OL v P3	0.1265	N/A	0.8889	0.6018
OL v IG2	0.0373	0.0617	0.5123	N/A
P3 v IG2	0.9514	N/A	0.9088	N/A

Additional Table 11. Within treatment comparison (p-value) of total net contact duration for three ITNs between four mosquito strains.

Strain comparison	ITN			
	Untreated	Olyset Net	PermaNet 3.0	Interceptor G2
Kisumu v N'gousso		0.9567	N/A	0.8908
Kisumu v VK7	0.0001	0.9994	0.5914	0.7097
Kisumu v Banfora	0.0252	0.9938	0.9829	N/A
N'gousso v VK7	0.0051	0.9310	N/A	0.9801
N'gousso v Banfora	0.0015	0.8683	N/A	N/A
VK7 v Banfora	<0.0001	0.9992	0.9006	N/A

Additional Table 12. Percentage of contact duration in first the 10minutes of room scale tracking assay – within strain, between net differences.

Net comparison	Strain			
	Kisumu	N'gousso	VK7	Banfora
UT v OL	<0.0001	<0.0001	0.9999	0.9965
UT v P3	0.0121	N/A	0.0547	0.8800
UT v IG2	0.0003	0.0108	0.9592	N/A
OL v P3	0.0626	N/A	0.0312	0.9302
OL v IG2	0.5533	0.0243	0.9327	N/A
P3 v IG2	0.6108	N/A	0.1253	N/A

Additional Table 13. Percentage of contact duration in first 10mins of assay – within net, between strain differences

Strain comparison	ITN			
	Untreated	Olyset Net	PermaNet 3.0	Interceptor G2
Kisumu v N'gousso	0.9829	0.7099	N/A	0.9489
Kisumu v VK7	0.9717	<0.0001	0.8614	0.0004
Kisumu v Banfora	0.9996	<0.0001	0.1913	N/A
N'gousso v VK7	0.8703	<0.0001	N/A	0.0021
N'gousso v Banfora	0.9707	<0.0001	N/A	N/A
VK7 v Banfora	0.9884	0.9062	0.5609	N/A

Additional Table 14. Average contact duration in first 10minutes – within strain, between net comparisons.

Net comparison	Strain			
	Kisumu	N'gousso	VK7	Banfora
UT v OL	0.8368	0.0083	0.9488	0.0607

UT v P3	0.9476	N/A	0.7547	0.3962
UT v IG2	0.1146	0.9217	1.0000	N/A
OL v P3	0.9899	N/A	0.3730	0.9217
OL v IG2	0.0092	0.0199	0.9347	N/A
P3 v IG2	0.0223	N/A	0.7299	N/A

Additional Table 15. Average contact duration in first 10minutess – within net, between strain comparisons.

Strain comparison	**ITN**			
	Untreated	**Olyset Net**	**PermaNet 3.0**	**Interceptor G2**
Kisumu v N'gousso	0.3666	0.7884	N/A	0.6326
Kisumu v VK7	0.1950	0.2028	0.9356	0.0002
Kisumu v Banfora	0.9943	0.6882	0.7480	N/A
N'gousso v VK7	0.0054	0.6882	N/A	0.0075
N'gousso v Banfora	0.5587	0.8701	N/A	N/A
VK7 v Banfora	0.1498	0.9818	0.9622	N/A

Additional Table 16. Comparison (p-value) of average swooping speeds across 2hour assay within four different strains, between four different net treatments.

Net comparison	**Strain**			
	Kisumu	**N'gousso**	**VK7**	**Banfora**
UT v OL	0.0226	0.4931	0.9972	0.2854
UT v P3	0.0937	N/A	0.9910	0.2929
UT v IG2	0.0092	0.8099	0.9995	N/A

OL v P3	0.9276	N/A	0.9996	0.9920
OL v IG2	0.9861	0.9345	0.9879	N/A
P3 v IG2	0.7756	N/A	0.9735	N/A

Additional Table 17. Comparison of average swooping speeds across 2hour assay within four net treatments, between four strains.

Strain comparison	**ITN**			
	Untreated	**Olyset Net**	**PermaNet 3.0**	**Interceptor G2**
Kisumu v N'gousso	0.0013	0.0173	N/A	0.1576
Kisumu v VK7	0.0240	0.9555	0.6271	0.9987
Kisumu v Banfora	0.0164	0.0736	0.0332	N/A
N'gousso v VK7	0.7782	0.0882	N/A	0.1414
N'gousso v Banfora	0.8472	0.9390	N/A	N/A
VK7 v Banfora	0.9991	0.2601	0.3216	N/A

Additional Table 18. Comparison of activity decay over time (p-value), within strain, between net treatment.

Net comparison	**Strain**			
	Kisumu	**N'gousso**	**VK7**	**Banfora**
UT v OL	0.0023	0.8774	0.0128	0.1454
UT v P3	0.0020	N/A	0.0010	0.2103
UT v IG2	<0.0001	0.1902	0.0387	N/A
OL v P3	1.000	N/A	0.8049	0.9987

OL v IG2	0.3361	0.4861	0.9708	N/A
P3 v IG2	0.3894	N/A	0.5401	N/A

Additional Table 19. Comparison of activity decay over time (p-value), within net treatment, between strains.

Strain comparison	**ITN**			
	Untreated	**Olyset Net**	**PermaNet 3.0**	**Interceptor G2**
Kisumu v N'gousso	0.0734	0.9965	N/A	0.9745
Kisumu v VK7	0.4510	0.2427	0.7543	0.0013
Kisumu v Banfora	0.9962	0.2987	0.5513	N/A
N'gousso v VK7	0.0021	0.3397	N/A	0.0047
N'gousso v Banfora	0.0609	0.4128	N/A	N/A
VK7 v Banfora	0.6268	0.9969	0.9675	N/A

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The effects of temephos, permethrin and malathion selection on the fitness and fecundity of *Aedes aegypti*

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Abstract. The recent scale-up of insecticide use has led to the rapid spread of insecticide resistance (IR) in mosquito populations across the world. Previous work has suggested that IR mechanisms could influence mosquito life-history traits, leading to alterations in fitness and key physiological functions. This study investigates to what extent mosquito fitness may be affected in a colony of *Aedes aegypti* after selection with temephos, permethrin or malathion insecticides. We measured immature development, sex ratio, adult longevity, energetic reserves under different rearing conditions and time points, ingested bloodmeal volume, mosquito size, male and female reproductive fitness and flight capability in the unexposed offspring of the three selected strains and unselected strain. We found that insecticide selection does have an impact on mosquito fitness traits in both male and female mosquitoes, with our temephos-exposed strain showing the highest immature development rates, improved adult survival, larger females under crowded rearing and increased sperm number in males. In contrast, this strain showed the poorest reproductive success, demonstrating that insecticide selection leads to trade-offs in life-history traits, which have the potential to either enhance or limit disease transmission potential.

Key words. Energetic resources, flight, insecticide resistance, larvicide, life-history parameters, mosquito.

Introduction

Insecticide resistance (IR) in disease vectors is at a crucial tipping point. The recent scale-up of insecticide-based vector control has protected hundreds of millions of people from disease exposure (Bhatt *et al.*, 2016), but has also resulted in the emergence and rapid spread of IR mechanisms across the world (Vontas *et al.*, 2012; Ranson & Lissenden 2016; WHO 2018). Within the major arbovirus vector *Aedes aegypti*, resistance has evolved to the four insecticide classes most commonly used for public health (Ranson *et al.*, 2010; Moyes *et al.*, 2017), with resistance to both larval and adult insecticides well documented in field populations (Montella *et al.*, 2007). This has led to a reduction in the efficacy of current insecticide-based control strategies (Moyes *et al.*, 2017). However, IR is energetically costly and can reduce mosquito fitness in the absence of insecticides, with effects ranging from minimal to highly damaging (Martins *et al.*, 2012; Brito *et al.*, 2013; Belinato & Martins 2016). Resistance mechanisms cause significant changes to key physiological functions in the vector, such as depleting energy resources (Diniz *et al.*, 2015), affecting development time (Martins *et al.*, 2012; Rahim *et al.*, 2017; Ramos *et al.*, 2018) or altering immune functions (Vontas *et al.* 2005), which can lead to changes in disease transmission. Metabolic resistance, caused by elevated enzyme activity, can be energetically costly with resources diverted for sequestration, metabolism and detoxification of insecticides (Saingamsook *et al.*, 2019). Previous studies have shown that metabolic resistance to temephos is associated with a reduction in egg batch size (Martins *et al.*, 2012; Diniz *et al.*, 2015; Viana-Medeiros *et al.*, 2017). Removing insecticide pressures from an environment results in lower frequencies of resistant alleles in mosquito populations, suggesting there is a fitness cost to maintaining these alleles in the absence of insecticide (Coustau *et al.*, 2000; David *et al.*, 2018).

Lipids and glycogen are important energy resources used for processes such as flight, vitellogenesis and immune responses

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(Steele, 1981). Glycogen stores are released from within cells and provide a source of energy for immediate flight, whereas ingested carbohydrates are converted to lipids that are directly involved in oogenesis, moulting and sustained flight (Beenakkers *et al.*, 1981). Resource-based trade-offs have been previously observed in insecticide-resistant mosquito populations, with the over-production of detoxifying enzymes requiring an extensive investment of resources. This can lead to depleted lipid stores, likely because lipids play a vital role in amino acid synthesis, thus leading to a knock-on negative impact on life-history traits, which rely on stored energy reserves (Rivero *et al.*, 2010). If the availability of these resources is altered at either the larval or adult stage then development, reproduction and movement will be affected.

Research into mosquito behaviour, fitness and fecundity tends to focus on measurements of females and their offspring. However, the physiological and behavioural traits observed in females post-mating (egg development, oviposition rates and host-seeking behaviours) are partially attributed to the receipt of male seminal fluid proteins and sperm (Hiss & Fuchs, 1972; Downe, 1975; Adlakha & Pillai, 1976; Klowden, 1993; Villarreal *et al.*, 2018). Both positive and negative associations between resistance and male reproductive success have been demonstrated, with Arnaud *et al.* (2005) reporting that insecticide-resistant beetles have improved reproductive success and are superior sperm competitors, whereas, in resistant mosquitoes, Belinato *et al.* (2012) saw a reduced frequency of female insemination.

While many studies have reported negative effects of IR on fitness and fecundity, a few studies have documented positive effects. Chan & Zairi (2013) demonstrated that permethrin-resistant *Aedes albopictus* survived longer when starved and produced larger females under crowded rearing densities than their susceptible counterparts. If resistant female mosquitoes show increased longevity, they are more likely to survive through a pathogen's extrinsic incubation period, increasing transmission potential (Kramer & Ebel, 2003).

Numerous limitations from previous studies likely contribute to poor concordance in study outcomes. Often only one or two fitness-related phenotypes were measured, despite the interdependency between longevity, male and female fecundity and energy resources. Furthermore, there are very few comparable pairs of resistant and susceptible strains, which only differ in resistance phenotype.

Our study aimed to investigate the fitness costs associated with IR by measuring energetic reserves, development, longevity, reproduction and flight in four strains of *A. aegypti* with different histories of insecticide exposure.

Materials and Methods

Establishment and maintenance of four A. aegypti *strains*

An *A. aegypti* colony from Recife, Brazil, was used to create four strains via exposure over 10 generations to either the larval organophosphate temephos (REC-R), adult pyrethroid permethrin (REC-P), adult organophosphate malathion (REC-M), or no insecticide exposure (REC-U) (Thornton *et al.*, 2020). All four strains were established and maintained under standard controlled conditions (27 °C ± 2 °C, and 80% relative humidity, 12:12 light/dark cycle) in an insectary at the Liverpool School of Tropical Medicine. Eggs were obtained by feeding mated adult females on human blood using a Hemotek feeder (Hemotek Ltd, Blackburn, U.K.). To standardize rearing conditions, 200 first instar larvae were counted and placed in plastic larval rearing trays (23.5 × 34.5 × 7.5 cm) containing 1 L of deionized (DI) water and one Brewer's yeast tablet (500 mg). To mimic high larval density rearing, 500 first instar larvae were counted and placed in rearing trays with 1 L of DI water and 1 yeast tablet. For each strain, four larval trays at each density were reared to use for testing and larvae were fed with one yeast tablet every other day. Adults were maintained on 10% sugar solution.

Resistance profiles. Resistance ratios after 1 year of selection, using lethal concentration (LC) 50 and LC95, were previously examined and compared to a fully susceptible New Orleans colony (Thornton *et al.*, 2020). For permethrin, REC-P was five times more resistant than REC-U, REC-M and REC-R. For malathion, REC-R and REC-M were slightly more resistant (∼2×) than REC-U or REC-P. With temephos, REC-R, REC-M and REC-P were more resistant (>2×) than REC-U (Table S1).

This study investigated the impact of insecticide selection regimes on four main physiological aspects of mosquito fitness: life-history traits, energy reserves, reproductive fitness and flight capability. The effect of different larval rearing densities and mosquito age were also considered. Figure 1 shows the study design and experimental pathway for each cohort of mosquitoes.

Mosquito life traits

Immature development time. Mosquitoes from each of the four strains, at both rearing densities (standard rearing trays: Rec-R $n = 3$, REC-U $n = 3$, REC-M $n = 2$, REC-P $n = 3$; crowded rearing trays: REC-R $n = 2$, REC-U $n = 2$, REC-M $n = 2$, REC-P $n = 1$), were separated by sex upon pupation into individual male and female holding containers. The number pupating per day was recorded. Mosquito eclosion was recorded for each sex and strain, and adults were retained in separate containers prior to assays.

Longevity

Longevity was recorded for mosquitoes from each strain, at the standard rearing density of 200 larvae/tray. Four cups of females and four cups of males each containing 20 adults were maintained on 10% sugar solution and monitored until all mosquitoes had naturally died. Due to different eclosion dates, each strain had a staggered start date, with the longest experiment lasting for a total of 60 days. The temperature and humidity of the insectary remained constant (27 °C ± 2 °C, and 80% relative humidity) and cup placement rotated daily to ensure standardized conditions. Death was recorded daily.

Objective	Cohort	Outcome	Measured endpoints	Target sample size per strain
Life traits	Standard density	Immature development	Number successfully pupated and time to pupation and sex ratio	3 trays, $n = 200$
			Number successfully eclosed and time to eclosion and sex ratio	
		Adult longevity	Day of death	80 females, 80 males
	Crowded density	Immature development	Time to pupation and sex ratio	2 trays, $n = 500$
			Time to eclosion and sex ratio	
Energy reserves	Standard density	Bloodmeal volume	Haemoglobin content *	10 females
		Reserves (day 2)	Lipid content (ug/mL) *	16 females
			Glycogen content (ug/mL) *	
		Reserves (day 8)	Lipid content (ug/mL) *	16 females
			Glycogen content (ug/mL) *	
	Crowded density	Reserves (day 2)	Lipid content (ug/mL) *	16 females
			Glycogen content (ug/mL) *	
		Reserves (day 8)	Lipid content (ug/mL) *	16 females
			Glycogen content (ug/mL) *	
Reproductive fitness	Male	Fertility	Total sperm count per male *	15 males
			Sperm number per mm of wing length	
		Individual mating success	Number of females inseminated per male	22 males
		Cross mating success	Number of females inseminated per male	10 males
	Female	Female fecundity	Total egg number per female fed to repletion	20 females
			Total L1 per female fed to repletion	
Flight capability	Female	Flight distance	Total distance (m)	33 females
			Average speed (m/s)	
		Flight bursts	Number of bursts over test period	

Fig 1. Study objectives, measured endpoints and target sample sizes. *Wing length measurements were taken for each of the mosquitoes in this assay. The sample size calculation for each primary outcome was based on a pilot study. Statistical modelling of the relationship between measured endpoint and strain indicated that differences between strains explained approximately 10% of variation in the data. Thus, on the assumption of an effect size of 0.1, the R package 'pwr' was used to calculate the minimum sample size under the following assumptions: degrees of freedom for numerator: 5; type I error prop: 0.05; type II error prob: 0.20; effect size: 0.1.

Quantification of energy resources

Bloodmeal volume. Bloodmeal volume was evaluated by quantifying haemoglobin amount (Briegel *et al.*, 1979), using Drabkin's reagent method. Midguts of blood-fed female mosquitoes were dissected 1 h post bloodmeal and the carcass was stored at −20 °C for subsequent wing measurements. Individual midguts were placed into 1.5-mL Eppendorf tubes containing 500 µL Drabkin's reagent and one metal ball bearing on ice. Samples were agitated in a tissue lyser for 1 min at 15 Hz and another 500 µL Drabkin's reagent was added. Samples were centrifuged at 12770 g for 15 min, before 200 µL of each sample was loaded onto a flat bottomed 96-well plate and read at 540 nm using Gen5 Epoch plate reader. Triplicate readings were recorded for each sample and an average was taken.

Wing length. Wing length was used as an estimate for body size. The right-wing from each female was removed from the thorax and an image was taken using a GXCAM ECLIPSE Wi-Fi microscope camera attached to a GX Stereo microscope. The length of the wing from the axial vein to the distal end of the R1 vein (not including the hairs on the edges of wings) was measured using GXCAM software (GXCAM Ver6.7).

Lipid and glycogen. We determined the lipid and glycogen content of mosquitoes using a standard protocol (*Methods in Anopheles Research*, 2015) with vanillin and anthrone reagents. Mosquitoes from all four strains, at both rearing densities, were split into two separate cohorts to allow energy analysis at two different time points; reserves measured at two days post-emergence (DPE) and reserves measured at eight DPE.

Reproductive fitness

Sperm number

Male and female mosquitoes were separated upon pupation and allowed to emerge in separate holding containers. Fifteen 1-day-old males were removed and individually knocked down on ice before dissection of the testes and seminal vesicles into 50 μL of phosphate-buffered saline (PBS). Samples were torn gently with dissecting pins and pins washed with 150 μL of PBS to obtain a final stock volume of 200 μL. Samples were mixed and 10 μL transferred into multi-well slides (20 individual wells per mosquito). Slides were air-dried, fixed with 70% ethanol and stained with Giemsa dye. Mosquito sperm heads were counted under ×40 magnification. One wing from each male was measured using the method described earlier.

Individual mating success

To determine individual mating success, 22 virgin male mosquitoes of each strain were housed individually in holding cups with three virgin females of the same strain. Males were given four days to mate. On the fourth day, female mosquitoes were knocked down briefly on ice and all three spermatheca were scanned for spermatozoa. Mosquitoes were recorded as either 'positive' or 'negative' for insemination.

Cross mating success. Following the results of strain-specific differences in mating success, REC-M and REC-R strains were further evaluated through a cross mating experiment to determine whether mating success was a male or female trait. The same method was repeated, with 10 virgin males individually housed with three virgin females from either the same strain or the alternate strain, resulting in four different crosses.

Female fecundity

Three mosquito rearing cages (28.5 × 29.5 × 28 cm) for REC-R, REC-U and REC-M, and two rearing cages for REC-P, were prepared with 30 female and 30 male mosquitoes introduced at the same time. Females were given four days to mate and then offered a human bloodmeal using a Hemotek membrane feeding system. All non-fed females were removed from the cage, and an oviposition pot containing damp cottonwool and filter paper was placed into the cage three days later, left overnight and then removed the following day. Multiple parameters were recorded: number of females fed to repletion, number of eggs laid and L1 hatch rate.

Quantification of flight ability

To investigate the effects of IR on mosquito flight ability, we used a tethered insect flight mill (provided by Dr. Jason Lim of Rothamsted Research), housed under standard insectary conditions. Due to low numbers of REC-M at the time of this assay, we only compared females from three strains: REC-R ($n = 33$), REC-U ($n = 66$) and REC-P ($n = 33$). REC-U females were flown at the same time as either REC-R or REC-P females to serve as a comparator.

Then, 2–5-day-old, non-blood-fed, virgin mosquitoes were knocked down briefly on ice before attachment to the tethered flight mill as follows. The rotor arm of the flight mill (radius 4 cm) was dipped into non-solvent glue and held gently onto the upper thorax of the mosquito, avoiding the wings. Mosquitoes on the rotor arm were then placed into one of the eight tethered flight mills, held in place between two opposing magnets to minimize friction, and briefly observed to check flight capability (Fig. S1). After a 30-minute recovery period, mosquitoes could fly freely for one h. The distance covered every five second (to the nearest 10 cm) was recorded using the flight mill software (Flight Mill Version 2).

Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics (Version 24) or in RStudio (R version 3.6.0). To evaluate differences between strains in number of mosquitoes successfully pupating and eclosing, *t* tests were performed in SPSS, with differences in sex ratio for both pupae and adults analysed using chi-square test. Differences in the longevity of female and male mosquitoes from each strain were investigated using Kaplan–Meier survival curves and compared using Logrank (Mantel–Cox).

To determine if bloodmeal volume, wing length or energy content differed between strains, we used generalized linear mixed models (GLMMs) using the 'lme4' package in R. GLMMs for energy resources were fit with a Gaussian distribution. To account for variation in body size between individual mosquitoes, wing length was included in the GLMM as a random effect. Stepwise regression was used for model selection. All explanatory variables and two-way interactions were fit, and their significance was tested using log-likelihood ratio

tests by comparison to a null model with only an intercept. Pairwise comparisons between categories were conducted using Tukey range tests ('lsmeans' package Version 2.30-0), with the p value significance threshold adjusted using the Bonferroni correction method. To investigate male fecundity, we analysed sperm number per mm of wing length for each strain. For individual mating and cross mating, we investigated the associations between the proportion of females successfully inseminated and strain using GLMMs fit with a binomial distribution, following the same method as previously described. Statistical significance of female fecundity was investigated using t tests.

Flight ability parameters (average speed, maximum speed, number of flight bursts and flight burst length) were analysed using RStudio prior to further analysis using SPSS. Individuals, which flew less than 50 m, were not included in analysis to rule out the possibility that attachment to the flight mill may have compromised their flight. Then, t tests were carried out using SPSS.

Results

Mosquito life traits

Immature development time. At standard rearing density, REC-R and REC-U had the highest pupation and eclosion rates, and at the crowded rearing density, REC-R had the highest pupation and eclosion rate (Table 1). Female-to-male ratios also differed between strains for both pupae and adult mosquitoes (Table 1). For all strains, the time to 50% pupation and eclosion was slower in the higher density trays.

Longevity. With a mean female survival of 28.07 days [95% confidence intervals (95% CI) 25.23–30.91], REC-R had greater longevity than REC-U (20.49 days, 95% CI 18.74–22.25, $p < 0.001$), REC-M (22.68 days, 95% CI 20.99–24.37, $p < 0.001$) and REC-P (21.45 days, 95% CI 20.24–22.67, $p < 0.001$).

With a mean male survival of 35.13 days (95% CI 32.52–37.73), REC-R had greater longevity than REC-U (25.86 days, 95% CI 22.81–28.91, $p < 0.001$) and REC-M (27.09 days, 95% CI 24.67–29.52, $p < 0.001$). REC-P had a mean survival of 36.80 days (95% CI 34.51–39.09), also surviving significantly longer than REC-U ($p < 0.001$) and REC-M ($p < 0.001$) (Fig. 2).

Energy resources. To determine whether energetic resources differed between strains, we first explored adult body size, followed by the relationship between body size and blood volume consumed.

At the standard rearing density REC-R, REC-U and REC-P female mosquitoes were all significantly larger than REC-M

Table 1. Mosquito pupation, eclosion and sex ratios by strain and rearing density.

		Mean number pupated and time to 50% pupation				Mean number eclosed and time to 50% eclosed			
Density	Strain	Female	Male	% Pupated	Pupae sex ratio (F:M)	Female	Male	% Eclosed	Adult sex ratio (F:M)
200 larvae/tray	REC-R	96.0 (SD ± 2.4) 4 days	110.3 (SD ± 6.3) 3 days	100.0	1:1.15	80.3 (SD ± 9.2) 7 days	98.7 (SD ± 1.7) 5 days	89.5	1:1.23
	REC-U	92.0 (SD ± 7.8) 4 days	115.0 (SD ± 0) 3 days	100.0	1:1.23	87.0 (SD ± 7.9) 7 days	98.7 (SD ± 2.9) 5 days	92.8	1:1.13
	REC-M	75.5 (SD ± 13.5) 4 days	75.5 (SD ± 11.5) 2 days	75.5*	1:1	54.0 (SD ± 10) 5 days	54.5 (SD ± 2.5) 5 days	54.25*	1:1
	REC-P	76.7 (SD ± 10.2) 3 days	83.3 (SD ± 18.4) 2 days	80.0*	1:1.09	59.7 (SD ± 8.3) 6 days	63 (SD ± 13.4) 5 days	61.0*	1:1.07
500 larvae/tray	REC-R	213.0 (SD ± 6.0) 8 days	256.5 (SD ± 2.5) 4 days	93.9*	1:1.20	155.0 (SD ± 4) 10 days	217.4 (SD ± 1.5) 6 days	74.5*	1:1.40
	REC-U	118.5 (SD ± 6.5) 6 days	149.5 (SD ± 3.5) 4 days	53.6	1:1.26	88.5 (SD ± 1.5) 8 days	117 (SD ± 5) 6 days	41.1	1:1.32
	REC-M	111.5 (SD ± 2.5) 5 days	195.0 (SD ± 19.0) 3 days	61.3	1:1.75	79.5 (SD ± 0.5) 8 days	145.5 (SD ± 19.5) 6 days	45.0	1:1.83
	REC-P	217.0 (SD ± 0) 6 days	260.0 (SD ± 0) 4 days	47.6	1:1.19	160 (SD ± 0) 8 days	214 (SD ± 0) 7 days	37.4	1:1.34

*Significant difference when compared to REC-U ($p < 0.05$).

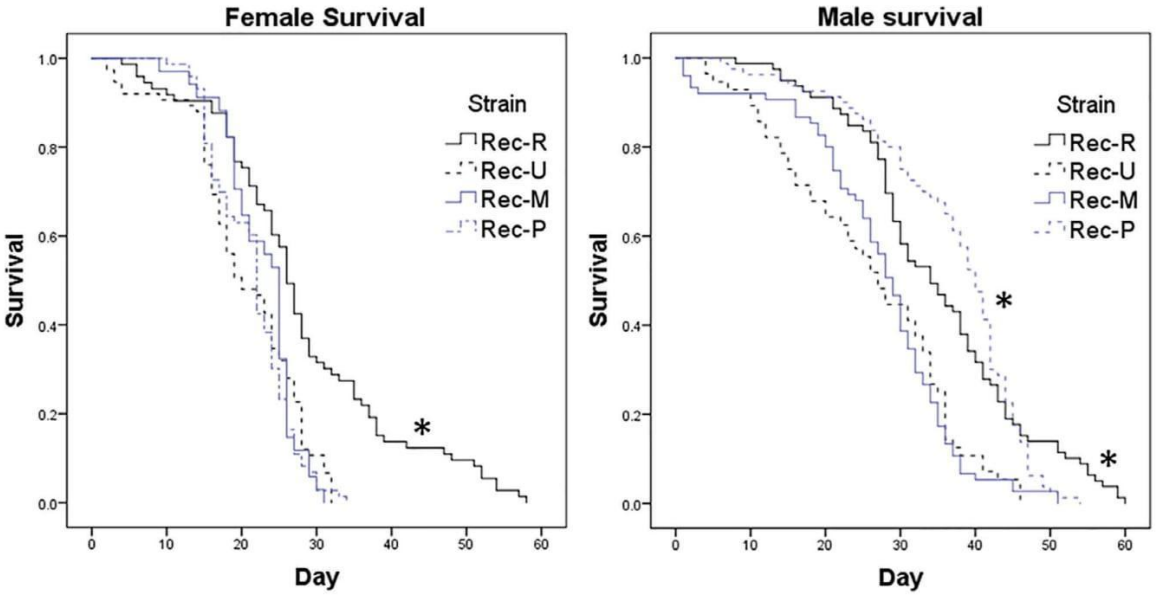

Fig 2. (A) Kaplan–Meier survival curves of REC-R ($n = 71$), REC-U ($n = 73$), REC-M ($n = 34$) and REC-P ($n = 76$) female mosquitoes and (B) Kaplan–Meier survival curves of REC-R ($n = 77$), REC-U ($n = 54$), REC-M ($n = 74$) and REC-P (n = 77) male mosquitoes. *$p < 0.05$.

(Fig. 3) (Table S2 and Fig. S2). At the crowded rearing density, there was a significant difference in size between all strains of mosquito.

There was a positive correlation ($R^2 = 0.27$) between bloodmeal volume and wing length ($\chi^2 = 15.599$, df = 1, $p < 0.001$), with no difference in this relationship between strains ($\chi^2 = 1.111$, df = 3, $p = 0.57$).

Lipid. The fixed effects of 'strain', 'density' and 'age' each contributed significantly to the explanatory power of the best fit model of lipid content (Table S3).

There was a significant interaction between 'strain' and 'density' ($\chi^2 = 34.138$, df = 3, $p < 0.001$). When reared at standard density there were no differences between any combinations of strains, however, at high-density lipid content for both REC-R and REC-U was significantly higher than REC-P [REC-P – REC-R ($p = < 0.001$, 95% CI −49.24 to −16.42), REC-P – REC-U ($p = 0.008$, 95% CI −51.27 to 12.347); Table S4].

The best fit model for lipid content also reported a significant interaction between 'strain' and 'age' ($\chi^2 = 50.503$, df = 3, $p < 0.001$; Fig. S3). At two DPE lipid content for REC-R was significantly higher than REC-M and REC-P [REC-M – REC-R ($p = < 0.001$, 95% CI −55.78 to −21.79), REC-P – REC-R ($p = < 0.001$, 95% CI −57.01 to −25.07)]. All other pairwise comparisons at two DPE were not significantly different. At eight DPE, REC-M lipids were significantly higher than REC-P with no difference between all other pairwise comparisons [REC-M – REC-P ($p = < 0.001$, 95% CI 17.73–54.70); Table S5].

Glycogen. The fixed effects of 'strain', 'density' and 'age' each contributed significantly to the explanatory power of the best fit model for glycogen content (Table S6).

There was a significant interaction between 'strain' and 'density', indicating that the relationship between strain and glycogen content was dependent on density at the larval stage ($\chi^2 = 22.241$, df = 3, $p < 0.001$). Pairwise comparisons showed that at standard density the mean glycogen content for REC-R was higher than both REC-P and REC-U, all other combinations were not significantly different [Rec-R – REC-P ($p = 0.003$, 95% CI 7.35 – 25.85), REC-R – REC-U ($p = < 0.001$, 95% CI 8.83–26.71); Table S7]. However, when reared at high density there was no difference in glycogen contents between any combinations of strains.

The interaction between 'strain' and 'age' also contributed to the model of glycogen content, indicating that the relationship between strain and glycogen content varied depending on the DPE ($\chi^2 = 24.985$, df = 3, $p < 0.001$). At two DPE, glycogen content for REC-R was significantly higher than REC-M, REC-P and REC-U, with no significant difference between any combination of these other strains [REC-M – REC-R ($p = 0.005$, 95% CI −26.74 to −7.02), REC-P – REC-R ($p = < 0.001$, 95% CI −29.25 to − 10.47), REC-R – REC-U ($p = < 0.001$, 95% CI 12.56–31.73); Table S8 and Fig. S3]. At eight DPE, there was no difference between any combinations of strains.

Reproductive fitness

Sperm number. REC-R contained a significantly higher number of sperm per mm of wing length than all other strains

Table 2. Mean sperm number, wing length and sperm number per mm of wing length for each of the four strains.

Strain	*N*	Sperm number (95% CI)	Wing length (mm) (95% CI)	Sperm number/mm wing length (95% CI)
REC-R	14	3806.14 (2222.24–5390.05)	2.60 (2.55–2.66)	1475.22* (851.17–2099.28)
REC-U	15	1779.07 (1033.09–2525.04)	2.62 (2.57–2.68)	681 (394.14–969.01)
REC-M	15	1318.27 (629.16–2007.37)	2.57 (2.53–2.61)	511.20 (244.88–777.53)
REC-P	14	1719.86 (1182.61–2257.10)	2.61 (2.56–2.65)	657.12 (448.64–865.60)

*Significant difference compared to all other strains $p < 0.05$.

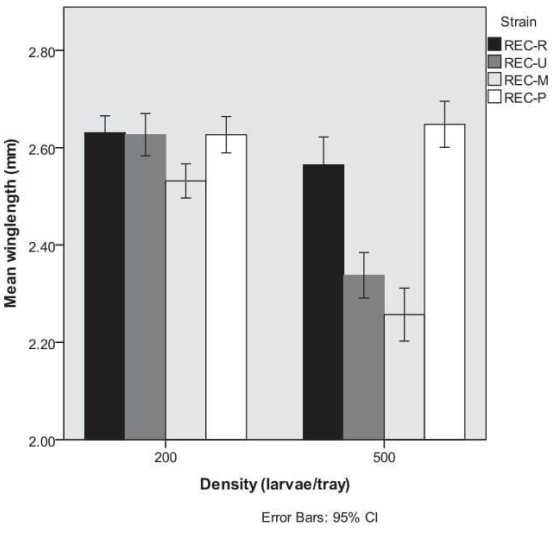

Fig 3. Wing length of four strains of *Aedes aegypti*, reared at standard 200/tray (REC-R $n = 36$, REC-U $n = 38$, REC-M $n = 35$, REC-P $n = 32$) and crowded 500/tray (REC-R $n = 32$, REC-U $n = 32$, REC-M $n = 35$, REC-P $n = 32$) larval densities. Different letters indicate statistically significant differences between strains ($p < 0.05$) per density, with 95% confidence intervals.

[Correction added on 19 November 2021, after first online publication: Figure 3 has been replaced with correct figure.]

[REC-U $t(27) = 2.5487$, $p = 0.017$; REC-M $t(27) = 3.1404$, $p = 0.004$; REC-P $t(26) = 2.6862$, $p = 0.012$] (Table 2).

Individual mating success. Binomial regression analysis showed that overall strain was a statistically significant factor for individual mating success over the 3-day period ($\chi^2 = 14.675$, df = 3, $p = 0.002$).

A significant difference in mating success was observed between REC-M and REC-R ($p = 0.002$, 95% CI 0.188) (Fig. 4 and Table S9). All other pairwise comparisons were not significantly different.

Cross mating. Mating success was explored further through cross mating of the poorest performing strain (REC-R) and the highest performing strain (REC-M). Results show that mating success is a male trait and again that strain is a significant factor

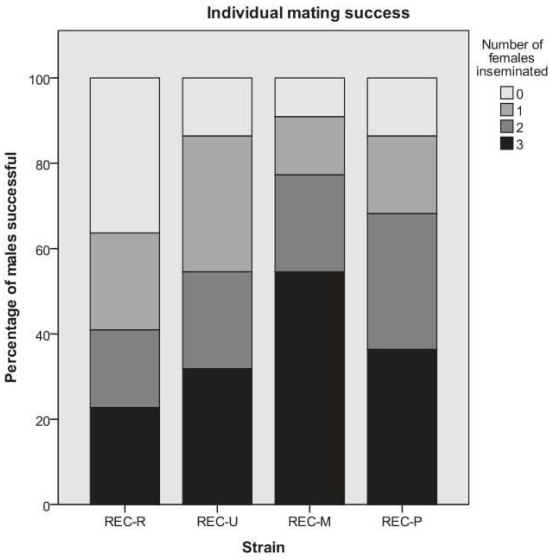

Fig 4. Individual mating success of one male mosquito ($n = 22$ per strain) with three female mosquitoes ($n = 66$ per strain).

($\chi^2 = 15.372$, df = 3, $p = 0.002$). REC-M males were more successful at inseminating both REC-M females ($p = 0.033$, 95% CI 11.976) and REC-R females ($p = 0.066$, 95% CI 6.345), than REC-R males were (Table S10).

Female fecundity. REC-U females produced a larger mean egg batch per female (35.02 eggs/female) than REC-R (18.03 eggs/female) and REC-M (22.60 eggs/female); however, neither comparison was statistically significant (REC-R $p = 0.122$, 95% CI −40.137 to 6.964; REC-M $p = 0.289$, 95% CI −40.176 to 15.642; Table 3). REC-U also had a higher larval hatch rate per female (26.6 larvae/female) than REC-R (13.2 larvae/female), REC-M (9.9 larvae/female) and REC-P (16.1 larvae/female); however, no comparisons were significantly different (REC-R $p = 0.205$, 95% CI −847.97 to249.97; REC-M $p = 0.143$, 95% CI −952.18 to 198.84; REC-P $p = 0.353$, 95% CI −1147.32 to 559.65).

Quantification of flight ability

A total of 99 mosquitoes were flown on the tethered insect flight mill. REC-P flew a longer distance within an hour than

Table 3. Fecundity of females fed to repletion.

Strain	*N*	Mean eggs	Mean L1	% Hatch
REC-R	63	18.03	13.2	73.0
REC-U	65	35.02	26.6	75.8
REC-M	60	22.6	9.9	44.0
REC-P	35	41.9	16.1	38.4

REC-R; however, neither strain was statistically significant compared to REC-U (Table 4) [REC-P $t(69) = 0.2792$, $p = 0.7809$; REC-R $t(71) = 0.8975$, $p = 0.3725$]. REC-P also showed more sustained flight when compared to REC-U, with less than half of the number of flight bursts of REC-R [REC-P $t(69) = 1.2982$, $p = 0.1985$; REC-R $t(71) = 0.5759$, $p = 0.5665$]; however, this was not statistically significant.

These results show that insecticide selection does have an impact on the life-history traits of both female and male mosquitoes. Compared to all other strains, REC-R had the highest pupation and eclosion rates at both rearing densities, female and male adults survived longer, females were larger at the crowded rearing density and males produced more sperm per mm of wing length. However, REC-R males and females had the poorest reproductive fitness with males inseminating the fewest females and females laying the fewest eggs. In comparison, REC-M had the smallest females at both rearing densities, but the highest individual female insemination success rate.

Discussion

Throughout this study, the temephos exposed REC-R strain has shown the most noticeable differences in fitness and fecundity when compared to the other exposed and unexposed. With higher pupation numbers at both rearing densities, males and females surviving longer, increased energy resources under certain conditions and highest sperm number, our results suggest a fitness advantage due to sustained temephos selection pressure. However, despite the increased sperm number seen in REC-R, there appears to be a net fecundity cost due to poor male mating success and lower mean egg numbers.

One possible explanation for why REC-R males had the highest sperm count but lowest insemination success is that this strain produces a larger ejaculate but at less frequent intervals. This result is mirrored in work by Belinato *et al.* (2012) who saw that mating efficacy was inversely proportional to temephos resistance ratio, and in work by Diniz *et al.* (2015) who showed that resistance status impacts male mating success. Body size is a well-documented factor in male mating success, with previous studies (Ponlawat & Harrington, 2007, 2009) reporting that *A. aegypti* body size was correlated with sperm number. However, our study confirmed that the significant differences in sperm number between strains were not attributable to differences in body size.

Our results on female fecundity are again similar to Belinato *et al.* (2012), who showed females from a highly resistant temephos field strain laid fewer eggs than the susceptible counterpart. One limitation of our study is we were unable to measure fecundity throughout the female's lifespan due to an unavoidable change in blood source after the first gonotrophic cycle.

While reduced fecundity in resistant strains could lead to lower mosquito densities, adult female longevity is a crucial factor in the vectorial capacity of wild mosquito populations. REC-R female and male mosquitoes survived for significantly longer than other strains in this study; however, previous work using a different *A. albopictus* reported that temephos resistant field strains had a shorter lifespan than their susceptible counterpart (Rahim *et al.*, 2017). There are important differences between our study design and the one followed by Rahim *et al.* (2017), most notably, we tested laboratory mosquitoes with an extended history of insecticide pressure, in contrast to a progeny originating from only one round of larval temephos exposure. We also did not offer a bloodmeal to females during the longevity assay and instead provided continued access to sucrose solution.

Results from energy content analysis show that teneral energy reserves do not explain the stark differences in fitness traits for REC-R. There was no significant difference in lipid or glycogen content observed between strains, instead differences were only observed between the two larval rearing densities and mosquito age. Energy content cannot, therefore, explain reductions in egg batch size, improved immature development or increased longevity. With lipids and glycogen being important for use in flight, we were not surprised to observe no difference in flight duration or flight burst number between strains.

It is important to note that while the strains used all originated from the same parental colony, these fitness experiments were carried out under laboratory-controlled conditions. The Recife colony used for selection had a background of previous temephos exposure and each strain underwent differential selection with exposure to insecticides using concentrations at 50% lethal dose (LD) over a period of 12 months. The physiological costs of resistance are often underestimated within a laboratory setting due to a lack of stress factors that are experienced in the field. In this study, however, we took the stress of larval crowding into consideration when assessing life-history traits.

Interestingly, our data suggest that continued selection to the organophosphate temephos at larval stages leads to shorter developmental time and increased longevity but reduced fecundity in the unexposed offspring. However, switching to selection with the organophosphate malathion in adult stage leads to better reproductive fitness but at the cost of longevity. With spermatogenesis thought to peak at the pupal stage, one explanation is that exposure during larval development can only lead to resource allocation that benefits longevity rather than reproduction. Conversely, improved fecundity in strains historically exposed during the adult life stage suggests that resources are diverted to offspring production rather than adult survival. These results have worrying implications for vector control programmes that target larval stages with insecticides, as longevity of the vector population is a key determinant of disease transmission potential.

Table 4. Mean flight distance and number of flight bursts over 1 h.

Strain	*N*	Distance (m) (95% CI)	Ratio*	Number flight bursts (95% CI)	Ratio*
REC-R	23	751.93 (387.39–1116.47)	0.80	21.22 (12.63–29.80)	1.20
REC-P	21	1012.57 (508.92–1519.22)	1.07	9.81 (2.87–16.75)	0.55
REC-U	50	944.64 (701.27–1188.01)	–	17.70 (10.32–25.08)	–

*Ratio compared to REC-U mosquitoes flown at the same time.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. The set-up of the tethered insect flight mill used to assess the flight capability of mosquitoes. Mosquitoes fly around a radius measuring 4 cm, causing the light encoder to periodically break a laser beam, which measures distance. One full rotation of the flight mill rotor arm = 25.13 cm. Image taken from (Somerville *et al.*, 2019).

Fig. S2. Bloodmeal volume relationship. Relationship between wing length and bloodmeal volume is not statistically distinguishable between strains. Shaded areas show upper and lower CIs for the line of best fit as predicted by the model. CIs overlap at all points in range, so all strains follow the same linear relationship.

Fig. S3. Predicted mean energy content for each *Aedes aegypti* strain reared at two different larval densities; lipid content at two days post-emergence (DPE) (A), lipid content at eight DPE (B), glycogen content at two DPE (C) and glycogen content at eight DPE (D).

Table S1. Lethal concentrations and resistance ratios of Recife strains for three insecticides (i.e. permethrin, malathion and temephos). Taken from Thornton *et al.* (2020).

Table S2. Mean wing length comparisons of four strains of *Aedes aegypti* reared at two different larval densities.

Table S3. GLMM lipid model statistics.

Table S4. The effects of strain and density on lipid content.

Table S5. The effects of strain and age on lipid content.

Table S6. GLMM glycogen model statistics.

Table S7. The effects of strain and density on glycogen content.

Table S8. The effects of strain and age on glycogen content.

Table S9. Differences in individual mating success between all four strains of *Aedes aegypti*.

Table S10. Cross mating success between REC-M and REC-R males when given the opportunity to mate with REC-M and REC-R females.

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All other authors declare no conflict of interest.

Author contributions

LJR and KG conceived and designed the study, KG collected the data, KG and FM analysed the data, KG wrote the first draft of the manuscript. All authors contributed to the final draft of the manuscript.

Date availability statement

The data that support the findings of this study are openly available in Open Science Framework at https://osf.io/crsmu/ (DOI 10.17605/OSF.IO/CRSMU)

References

Adlakha, V. & Pillai, M.K.K. (1976) Role of male accessory gland substance in the regulation of blood intake by mosquitoes. *Journal of Insect Physiology*, **22**, 1441–1442. https://doi.org/10.1016/0022-1910(76)90206-7.

Arnaud, L., Haubruge, E. & Gage, M.J.G. (2005) The malathion-specific resistance gene confers a sperm competition advantage in *Tribolium castaneum*. *Functional Ecology*, **19**, 1032–1039. https://doi.org/10.1111/j.1365-2435.2005.01055.x.

Beenakkers, A.M.T., Horst, D.J.V.D. & Marrewijk, W.J.A.V. (1981) Role of lipids in energy metabolism. *Energy Metabolism in Insects* (ed. by R.G.H. Downer), pp. 53–100. Springer: New York.

Belinato, T.A. & Martins, A.J. (2016) Insecticide resistance and fitness cost. *IntechOpen*, **32**, 243–261. https://doi.org/10.5772/61826.

Belinato, T.A., Martins, A.J. & Valle, D. (2012) Fitness evaluation of two Brazilian *Aedes aegypti* field populations with distinct levels of resistance to the organophosphate temephos. *Memorias Do Instituto Oswaldo Cruz*, **107**, 916–922. https://doi.org/10.1590/S0074-02762012000700013.

Bhatt, S., Weiss, D.J., Cameron, E., Bisanzio, D., Mappin, B. & Dalrymple, U. (2016) The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature*, **526**, 207–211. https://doi.org/10.1038/nature15535.The.

Briegel, H., Lea, A.O. & Klowden, M.J. (1979) Hemoglobinometry as a method for measuring blood meal sizes of mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology*, **15**, 235–238. https://doi.org/10.1093/jmedent/15.3.235.

Brito, L.P., Linss, J.G.B., Lima-Camara, T.N. *et al.* (2013) Assessing the effects of *Aedes aegypti* kdr mutations on pyrethroid resistance and its fitness cost. *PLoS One*, **8**, e60878. https://doi.org/10.1371/journal.pone.0060878.

Chan, H.H. & Zairi, J. (2013) Permethrin resistance in *Aedes albopictus* (Diptera: Culicidae) and associated fitness costs. *Journal of Medical Entomology*, **50**, 362–370. https://doi.org/10.1603/ME12182.

Coustau, C., Chevillon, C. & Ffrench-Constant, R. (2000) Resistance to xenobiotics and parasites: Can we count the cost? *Trends in Ecology and Evolution*, **15**, 378–383. https://doi.org/10.1016/S0169-5347(00)01929-7.

David, M.R., Garcia, G.A., Valle, D. & Maciel-De-Freitas, R. (2018) Insecticide resistance and fitness: The case of four *Aedes aegypti* populations from different Brazilian Regions. *BioMed Research International*, **2018**, 1–12. https://doi.org/10.1155/2018/6257860.

Diniz, D.F.A., Melo-Santos, M.A.V.D., Santos, E.M.D.M. *et al.* (2015) Fitness cost in field and laboratory *Aedes aegypti* populations associated with resistance to the insecticide temephos. *Parasites and Vectors*, **8**, 1–15. https://doi.org/10.1186/s13071-015-1276-5.

Downe, A.E.R. (1975) Internal regulation of rate of digestion of blood meals in the mosquito, *Aedes aegypti*. *Journal of Insect Physiology*, **21**, 1835–1839. https://doi.org/10.1016/0022-1910(75)90250-4.

Hiss, E.A. & Fuchs, M.S. (1972) The effect of matrone on oviposition in the mosquito, *Aedes aegypti*. *Journal of Insect Physiology*, **18**, 2217–2227. https://doi.org/10.1016/0022-1910(72)90250-8.

Klowden, M.J. (1993) Mating and nutritional state affect the reproduction of *Aedes albopictus* mosquitoes. *Reproduction*, **9**, 169–173.

Kramer, L.D. & Ebel, G.D. (2003) Dynamics of flavivirus infection in mosquitoes. *Advances in Virus Research*, Amsterdam, Netherlands: Elsevier, pp. 187–232.

Martins, A.J., Ribeiro, C.D.e.M., Bellinato, D.F., Peixoto, A.A., Valle, D. & Lima, J.B.P. (2012) Effect of insecticide resistance on development, longevity and reproduction of field or laboratory selected *Aedes aegypti* populations. *PLoS One*, **7**, 1–9. https://doi.org/10.1371/journal.pone.0031889.

Methods in Anopheles Research. 3rd edn. (2015). http://www.mr4.org.

Montella, I.R., Martins, A.J., Viana-Medeiros, P.F., Lima, J.B.P., Braga, I.A. & Valle, D. (2007) Insecticide resistance mechanisms of Brazilian *Aedes aegypti* populations from 2001 to 2004. *American Journal of Tropical Medicine and Hygiene*, **77**, 467–477. https://doi.org/10.4269/ajtmh.2007.77.467.

Moyes, C.L., Vontas, J., Martins, A.J. *et al.* (2017) Contemporary status of insecticide resistance in the major *Aedes* vectors of arboviruses infecting humans. *PLoS Neglected Tropical Diseases*, **11**, 1–20. https://doi.org/10.1371/journal.pntd.0005625.

Ponlawat, A. & Harrington, L.C. (2007) Age and body size influence male sperm capacity of the dengue vector *Aedes aegypti* (Diptera: Culicidae). *Journal of Medical Entomology*, **44**, 422–426. https://doi.org/10.1603/0022-2585(2007)44[422:aabsim]2.0.co;2.

Ponlawat, A. & Harrington, L.C. (2009) Factors associated with male mating success of the dengue vector mosquito, *Aedes aegypti*. *The American Journal of Tropical Medicine and Hygiene*, **80**, 395–400.

Rahim, J., Ahmad, A.H. & Maimusa, A.H. (2017) Effects of temephos resistance on life history traits of *Aedes albopictus* (Skuse) (Diptera: Culicidae), a vector of arboviruses. *Revista Brasileira de Entomologia*, **61**, 312–317. https://doi.org/10.1016/j.rbe.2017.08.001.

Ramos, R.S., Araújo, V.C.R.D., Pereira, R.R. *et al.* (2018) Investigation of the lethal and behavioral effects of commercial insecticides on the parasitoid wasp *Copidosoma truncatellum*. *Chemosphere*, **191**, 770–778. https://doi.org/10.1016/j.chemosphere.2017.10.113.

Ranson, H., Burhani, J., Lumjuan, N. & Black, W.C. IV (eds.) (2010) Insecticide resistance in dengue vectors. *Tropika*, **1**, 307–316.

Ranson, H. & Lissenden, N. (2016) Insecticide resistance in African anopheles mosquitoes: A worsening situation that needs urgent action to maintain malaria control. *Trends in Parasitology*, **32**, 187–196. https://doi.org/10.1016/j.pt.2015.11.010.

Rivero, A., Vézilier, J., Weill, M., Read, A.F. & Gandon, S. (2010) Insecticide control of vector-borne diseases: When is insecticide resistance a problem? *PLoS Pathogens*, **6**, 5–6. https://doi.org/10.1371/journal.ppat.1001000.

Saingamsook, J., Yanola, J., Lumjuan, N., Walton, C. & Somboon, P. (2019) Investigation of relative development and reproductivity fitness cost in three insecticide-resistant strains of *Aedes aegypti* from Thailand. *Insects*, **10**, 1–16. https://doi.org/10.3390/insects10090265.

Somerville, A.G.T., Gleave, K., Jones, C.M. & Reimer, L.J. (2019) The consequences of *Brugia malayi* infection on the flight and energy resources of *Aedes aegyptiI* mosquitoes. *Sci Rep*, **9**, 18449. https://doi.org/10.1038/s41598-019-54819-2.

Steele, J.E. (1981) The role of carbohydrate metabolism in physiological function. *Energy Metabolism in Insects*. Plenum, New York.

Thornton, J., Gomes, B., Ayres, C. & Reimer, L. (2020) *Insecticide resistance selection and reversal in two strains of Aedes aegypti*. Wellcome Open Research.

Viana-Medeiros, P.F., Bellinato, D.F., Martins, A.J. & Valle, D. (2017) Insecticide resistance, associated mechanisms and fitness aspects in two Brazilian *Stegomyia aegypti* (= *Aedes aegypti*) populations. *Medical and Veterinary Entomology*, **31**, 340–350. https://doi.org/10.1111/mve.12241.

Villarreal, S.M., Pitcher, S., Helinski, M.E.H., Johnson, L., Wolfner, M.F. & Harrington, L.C. (2018) Male contributions during mating increase female survival in the disease vector mosquito *Aedes aegypti*. *Journal of Insect Physiology*, **108**, 1–9. https://doi.org/10.1016/j.jinsphys.2018.05.001.

Vontas, J., Blass, C., Koutsos, A.C. *et al.* (2005) Gene expression in insecticide resistant and susceptible *Anopheles gambiae* strains constitutively or after insecticide exposure. *Insect Molecular Biology*, **14**, 509–521. https://doi.org/10.1111/j.1365-2583.2005.00582.x.

Vontas, J., Kioulos, E., Pavlidi, N., Morou, E., Della Torre, A. & Ranson, H. (2012) Insecticide resistance in the major dengue vectors *Aedes albopictus* and *Aedes aegypti*. *Pesticide Biochemistry and Physiology*, **104**, 126–131. https://doi.org/10.1016/j.pestbp.2012.05.008.

WHO (2018) *Global report on insecticide resistance in malaria vectors*, Geneva: World Health Organisation, pp. 2010–2016.

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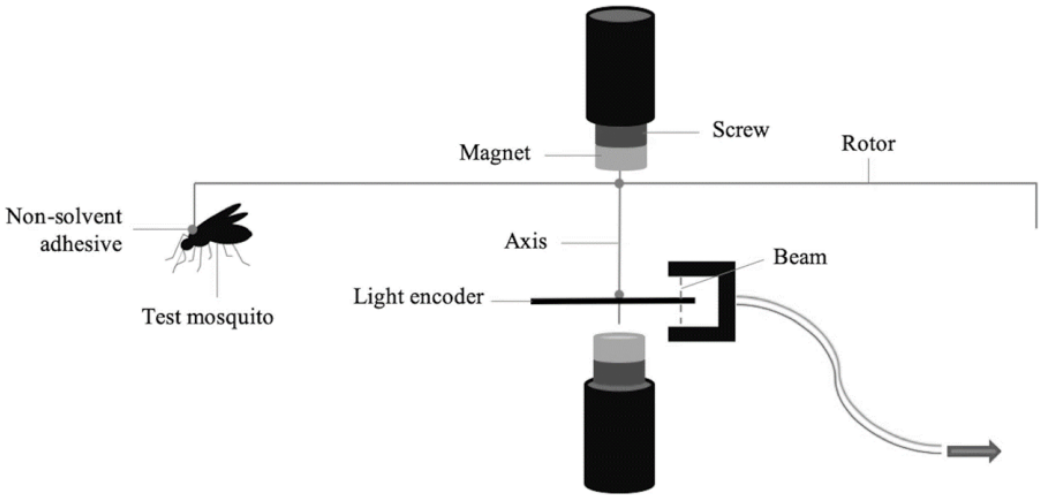
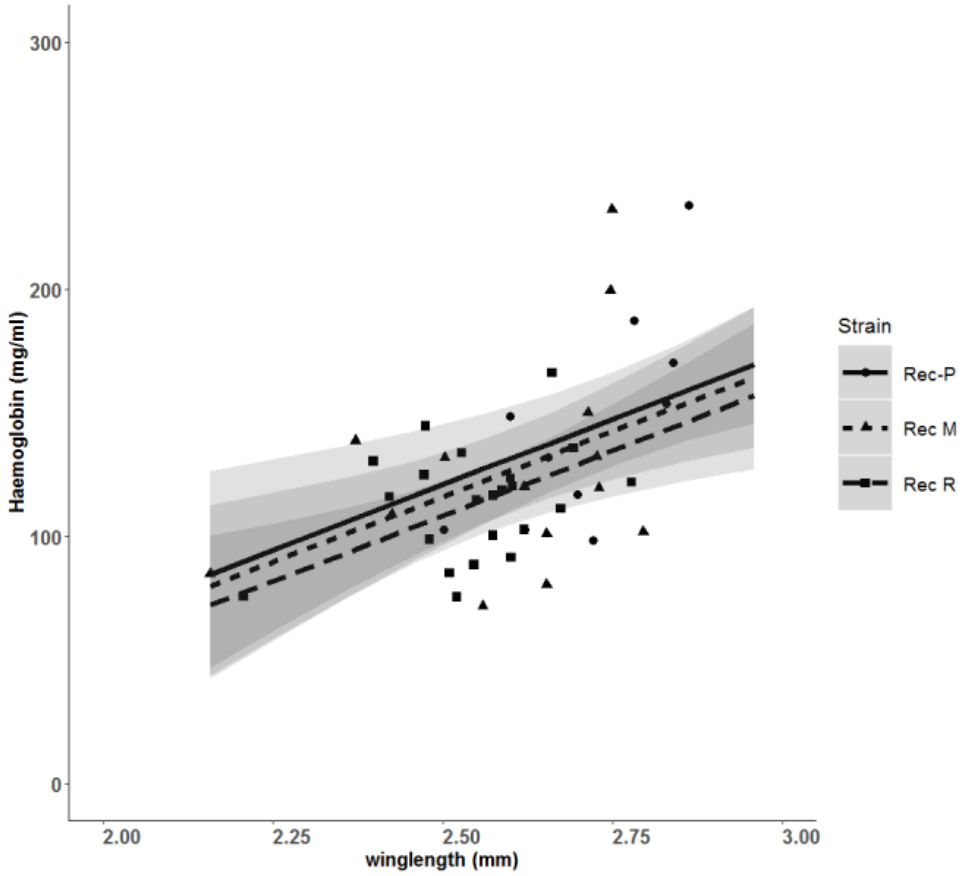
Supplementary files

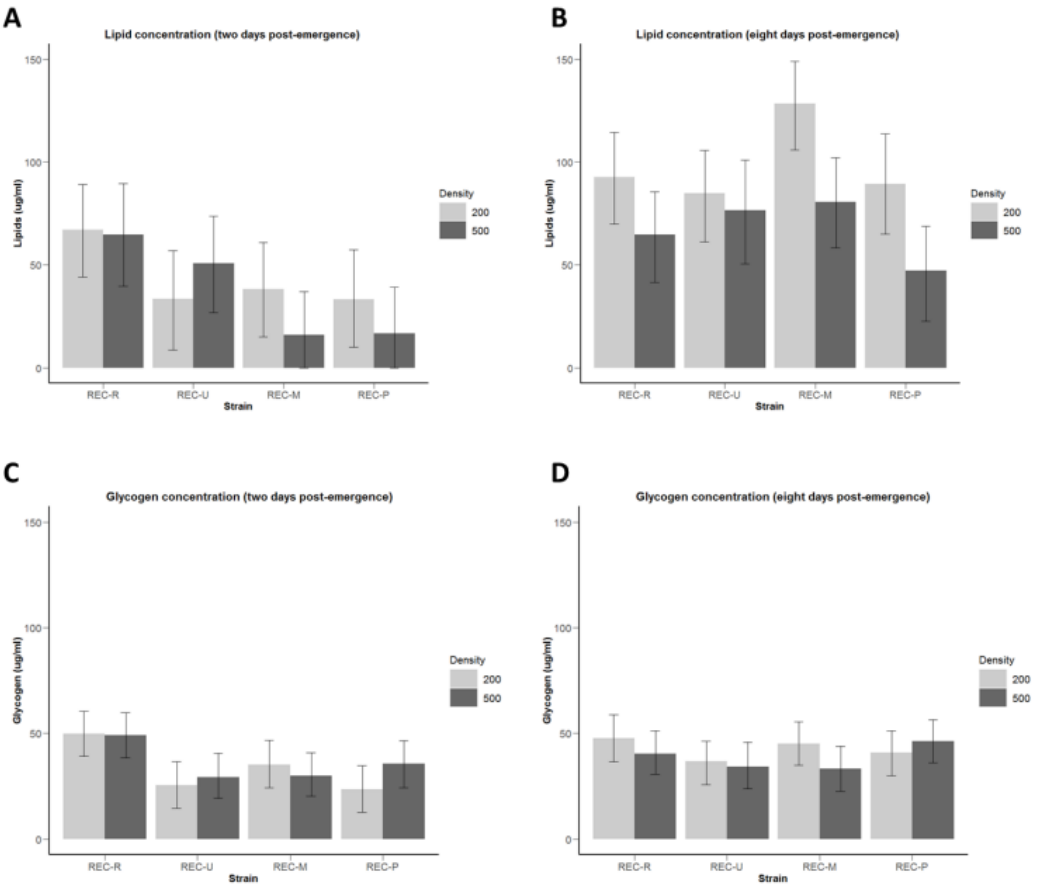


Supplementary Figure S1. The set-up of the tethered insect flight mill used to assess the flight capability of mosquitoes. Mosquitoes fly around a radius measuring 4cm, causing the light encoder to periodically break a laser beam which measures distance. One full rotation of the flight mill rotor arm = 25.13cm. Image taken from (Somerville, Gleave, Jones, & Reimer, 2019).



Supplementary Figure S2. Blood meal volume relationship.

Relationship between wing length and blood meal volume is not statistically distinguishable between strains. Shaded areas show upper and lower CIs for the line of best fit as predicted by the model. CIs overlap at all points in range, so all strains follow the same liner relationship.



Supplementary Figure S3. Predicted mean energy content for each *Aedes aegypti* strain reared at two different larval densities; lipid content at two days post-emergence (DPE) (panel A), lipid content at eight DPE (panel B), glycogen content at two DPE (panel C) and glycogen content at eight DPE (panel D).

Supplementary Table S1. Lethal concentrations and resistance ratios of Recife strains for three insecticides (i.e. permethrin, malathion and temephos) Taken from Thornton *et al*, 2020.

Insecticide	Strain	LC_{50}	RR_{50}	LC_{95}	RR_{95}
Permethrin	New Orleans	0.066 (0.053-0.083)	*N/A*	0.327 (0.211-0.507)	*N/A*
	REC-U	0.131 (0.106-0.162)	1.98	0.404 (0.259-0.631)	1.24
	REC-R	0.155 (0.121-0.198)	2.35	0.629 (0.390-1.014)	1.92
	REC-M	0.111 (0.0922-0.134)	1.68	0.451 (0.283-0.717)	1.38
	REC-P	0.657 (0.585-0.738)	9.94	2.876 (2.292-3.608)	8.80

Malathion	New Orleans	0.329 (0.274-0.394)	*N/A*	1.423 (1.109-1.825)	*N/A*
	REC-U	0.566 (0.490-0.654)	1.72	2.093 (1.694-2.586)	1.47
	REC-R	0.898 (0.741-1.087)	2.73	7.709 (4.302-13.812)	5.42
	REC-M	1.006 (0.876-1.155)	3.06	4.583 (3.430-6.124)	3.22
	REC-P	0.614 (0.566-0.666)	1.87	1.091 (0.957-1.245)	0.77
Temephos	New Orleans	0.011 (0.010-0.011)	*N/A*	0.032 (0.028-0.036)	*N/A*
	REC-U	0.145 (0.141-0.149)	13.81	0.304 (0.284-0.326)	9.53
	REC-R	0.342 (0.328-0.356)	32.57	1.163 (1.065-1.269)	36.46
	REC-M	0.376 (0.357-0.396)	35.81	0.845 (0.798-0.938)	26.49
	REC-P	0.355 (0.339-0.372)	33.81	0.810 (0.750-0.873)	25.39

LC_{50}: Lethal concentration for 50% mortality; RR_{50}: resistance ratio for LC_{50}; LC_{95}: Lethal concentration for 95% mortality; RR_{95}: resistance ratio for LC_{95}. REC-U: strain without insecticide exposure; REC-R: strain with temephos exposure; REC-M: strain selected for malathion; REC-P: strain selected for permethrin.

Supplementary Table S2. Mean wing length comparisons of four strains of *Aedes aegypti* reared at two different larval densities.

Density	Strain comparison	Summary statistics
200	REC-R v REC-U	t(72) =0.1444, p=0.8856
	REC-R v REC-M *	t(69) =4.0683, p<0.0001
	REC-R v REC-P	t(66) =0.1584, p=0.8746
	REC-U v REC-M *	t(71) =3.4131, p=0.0011
	REC-U v REC-P	t(68) =00006, p=0.9995
	REC-M v REC-P	t(65) =3.7722, p=0.0004
500	REC-R v REC-U *	t(62) =6.2511, p<0.0001
	REC-R v REC-M *	t(65) =7.9279, p<0.0001
	REC-R v REC-P *	t(62) =2.2860, p=0.0257
	REC-U v REC-M *	t(65) =2.2748, p=0.0262
	REC-U v REC-P *	t(62) =9.5108, p<0.0001
	REC-M v REC-P *	t(65) =109510, p<0.0001

***denotes significant difference p=0.05**

Supplementary Table S3. GLMM Lipid model statistics.

Variable	χ^2	Degrees of freedom	p
Strain	27.037	3	5.78×10^{-6}
Age	79.446	1	2.20×10^{-16}
Density	34.023	1	5.45×10^{-9}

Supplementary Table S4. The effects of strain and density on lipid content

Density: 200 larvae per tray

Comparison	Estimate	95% CI	p-value
REC-M – REC-P	22.07	5.86 – 38.28	0.040
REC-M – REC-R	3.47	-12.24 – 19.28	0.973
REC-M – REC-U	24.27	8.66 – 39.87	0.013
REC-P – REC-R	-18.59	-34.31 – -2.87	0.096
REC-P – REC-U	2.20	-13.55 – 17.95	0.993
REC-R – REC-U	20.79	5.56 – 36.02	0.040

Density: 500 larvae per tray

Comparison	Estimate	95% CI	p-value
REC-M – REC-P	16.41	-4.58 – 37.40	0.042
REC-M – REC-R	-16.42	-36.14 – 3.29	0.363
REC-M – REC-U	-15.40	-32.04 – 1.24	0.269
REC-P – REC-R	-32.83	-49.24 – -16.42	<0.001
REC-P – REC-U	-31.81	-51.27 – -12.347	0.008
REC-R – REC-U	1.02	-17.23 – 19.26	0.999

Supplementary Table S5. The effects of strain and age on lipid content.

Age: 2 days

Comparison	Estimate	95% CI	p-value
REC-M – REC-P	2.26	-15.28 – 19.80	0.994
REC-M – REC-R	-38.79	-55.78 – -21.79	<0.001
REC-M – REC-U	-14.93	-31.35 – 1.49	0.284
REC-P – REC-R	-41.04	-57.01 – -25.07	<0.001
REC-P – REC-U	-17.19	-34.01 – -0.37	0.189
REC-R – REC-U	23.85	7.48 – 40.22	0.024

Age: 8 days

Comparison	Estimate	95% CI	p-value
REC-M – REC-P	36.22	17.73 – 54.70	<0.001
REC-M – REC-R	25.85	7.94 – 43.76	0.026
REC-M – REC-U	23.80	8.18 – 39.42	0.016
REC-P – REC-R	-10.37	-26.72 – 5.97	0.599
REC-P – REC-U	-12.42	-29.82 – 4.98	0.501
REC-R – REC-U	-2.04	-18.81 – 14.73	0.995

Supplementary Table S6. GLMM Glycogen model statistics.

Variable	χ^2	Degrees of freedom	p
Strain	30.729	3	9.69×10^{-7}
Age	10.621	1	0.001
Density	4.030	1	0.045

Supplementary Table S7. The effects of strain and density on glycogen content.

Density: 200 larvae per tray

Comparison	Estimate	95% CI	p-value
REC-M – REC-P	7.84	-1.66 – 17.35	0.370
REC-M – REC-R	-8.75	-18.04 – 0.53	0.253
Rec-M – REC-U	9.01	-0.12 – 18.14	0.217
REC-P – REC-R	-16.60	7.35 – 25.85	0.003*
REC-P – REC-U	1.16	-8.09 – 10.41	0.99
REC-R – REC-U	17.77	8.83 – 26.71	0.001*

Density: 500 larvae per tray

Comparison	Estimate	95% CI	p-value
REC-M – REC-P	-9.30	-20.73 – 2.11	0.384
REC-M – REC-R	-13.09	-23.98 – -2.19	0.090
REC-M – REC-U	-0.17	-9.87 – 9.52	1.000
REC-P – REC-R	-3.78	-13.38 – 5.82	0.867
REC-P – REC-U	9.14	-1.88 – 20.15	0.366
REC-R – REC-U	12.92	2.44 – 23.38	0.077

Supplementary Table S8. The effects of strain and age on glycogen content.

Age: 2 days

Comparison	Estimate	95% CI	p-value
REC-M – REC-P	2.98	-7.16 – 13.13	0.939
REC-M – REC-R	-16.88	-26.74 – -7.02	0.005*
REC-M – REC-U	5.27	-4.31 – 14.86	0.703
REC-P – REC-R	-19.86	-29.25 – -10.47	<0.001*
REC-P – REC-U	2.29	-7.55 – 12.12	0.969
REC-R – REC-U	22.15	12.56 – 31.73	<0.001*

Age: 8 days

Comparison	Estimate	95% CI	p-value
REC-M – REC-P	-9.307	-20.73 – 2.12	0.384
REC-M – REC-R	-13.089	-23.98 – -2.19	0.090
REC-M – REC-U	-0.173	-9.77 – 9.43	1.000
REC-P – REC-R	-3.782	-13.38 – 5.82	0.867
REC-P – REC-U	9.135	-1.88 – 20.15	0.366
REC-R – REC-U	12.916	2.45 – 23.38	0.076

Supplementary Table S9. Differences in individual mating success between all four strains of *Aedes aegypti*.

Comparison	Odds ratio	95% CI	P value
REC-R – REC-U	0.543	0.375	0.306
REC-R – REC-M	0.256	0.188	0.002
REC-R – REC-P	0.421	0.295	0.073
REC-U – REC-M	0.471	0.347	0.186
REC-U – REC-P	0.776	0.543	0.892
REC-M – REC-P	1.647	1.228	0.555

Supplementary Table S10. Cross mating success between REC-M and REC-R males when given the opportunity to mate with REC-M and REC-R females.

Male strain	Female strain	Number of females inseminated
REC-R	REC-R	17
REC-R	REC-M	4
REC-M	REC-M	24
REC-M	REC-R	26

SCIENTIFIC REPORTS

Filarial infection influences mosquito behaviour and fecundity

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Understanding vector-parasite interactions is increasingly important as we move towards the endpoint goals set by the Global Programme for the Elimination of Lymphatic Filariasis (GPELF), as interaction dynamics may change with reduced transmission pressure. Elimination models used to predict programmatic endpoints include parameters for vector-specific transmission dynamics, despite the fact that our knowledge of the host-seeking behaviour of filariasis infected mosquitoes is lacking. We observed a dynamic, stage-specific and density dependent change in *Aedes aegypti* behaviour towards host cues when exposed to *Brugia malayi* filarial parasites. Infected mosquitoes exhibited reduced activation and flight towards a host during the period of larval development (L1/L2), transitioning to a 5 fold increase in activation and flight towards a host when infective stage larvae (L3) were present ($p < 0.001$). In uninfected control mosquitoes, we observed a reduction in convergence towards a host during the same period. Furthermore, this behaviour was density dependent with non-activated mosquitoes harbouring a greater burden of L1 and L2 larvae while activated mosquitoes harboured a greater number of L3 ($p < 0.001$). Reductions in fecundity were also density-dependent, and extended to mosquitoes that were exposed to microfilariae but did not support larval development.

Lymphatic filariasis (LF) is one of the neglected tropical diseases (NTD) and the second largest cause of permanent and long term disability worldwide[1]. An estimated 120 million human cases, across 55 countries, lead to a loss of 5.9 million disability-adjusted life-years (DALYs)[2–4].

Three species of filarial nematodes are responsible for causing LF; *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*[4]. Transmission of filarial worms is indirect, with parasites developing within a mosquito vector before being passed to definitive vertebrate hosts. Larval development occurs within the short lived mosquito and once transmitted to a vertebrate host, parasites carry out their longer life stages and reproduction[5]. Several different mosquito species from the *Culex*, *Anopheles*, *Aedes* and *Mansonia* genera can transmit LF[4] but susceptibility to parasite infection varies between species[6–8].

An understanding of LF transmission dynamics is crucial for the implementation and monitoring of elimination programmes[9–14]. Mathematical models are being used to guide decision making on the best strategies to eliminate lymphatic filariasis[15]. Slight changes in vector specific parameters can alter the likelihood of elimination and the most suitable approach to reach transmission breakpoints[13]. Important parameters of the vectorial capacity equation, such as the host encounter rate, gonotrophic cycle length, host preference and vector death rate, are based on the parasite-naive vector population. However, previous research in other disease systems has shown that infection status can influence vector physiology and behaviour, and alter these important determinants of transmission.

Studies on malaria have shown that sporozoite positive mosquitoes probed more frequently and for longer periods of time[16]. These results were complemented by field studies showing that naturally infected, sporozoite-positive mosquitoes fed on a significantly greater number of hosts[17] than uninfected mosquitoes. Anderson *et al.*[18] further showed that host-seeking behaviours differed between mosquitoes infected with developing oocysts and the infective stage sporozoites. In this study sporozoite-infected mosquitoes were more persistent and experienced greater host contact. While it is well known that parasite infection is associated with changes in host behaviour in ways that favour onward transmission, few studies are able to definitively show that such changes are adaptive[19]. Such changes may be due to the pathological response to infection, they may be parasite-mediated or host-mediated.

Results from Cator *et al.*[20] demonstrated that exposed mosquitoes were more responsive to host cues when the infective sporozoite stage of the parasite was present, as opposed to the developmental oocyst stage. However a

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Mf density	Mean mf ingested (0.5 DPE)	Mean developing L1/L2 (4–6 DPE)	Mean infective L3 (11–13 DPE)
Low	14.9 ± 3.3 (n = 7)	3.0 ± 0.7 (n = 17)	1.4 ± 0.2 (n = 18)
High	23.7 ± 1.4 (n = 29)	2.6 ± 0.3 (n = 135)	3.0 ± 0.3 (n = 77)

Table 1. Mean intensity of ingested microfilariae, developing larvae (L1/L2) and infective larvae (L3).

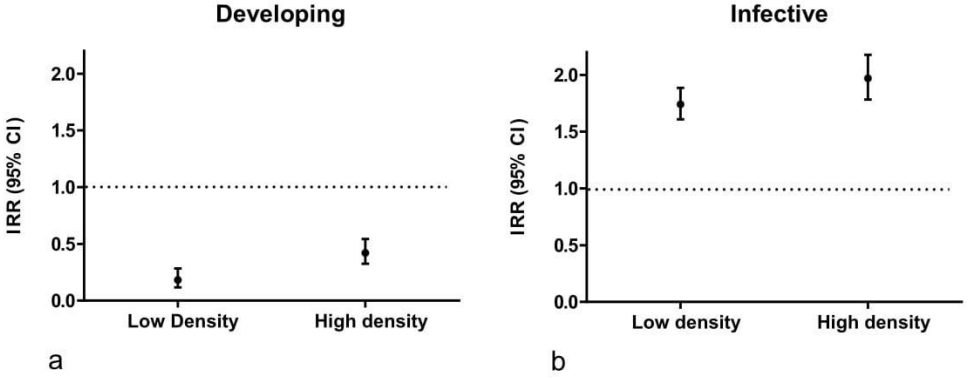

Figure 1. Incidence rate ratio (IRR) of mosquito convergence in the presence of host cues. (**a**) Converging mosquitoes at the developing stage (4–6 DPE) compared to the control cohort. (**b**) Converging mosquitoes at the infective stage (11–13 DPE) compared to the control cohort. All observed behaviours were significantly different than the control un-infected mosquitoes at both time points ($p < 0.0001$). Control (n = 790), Low Density (n = 250), High density (n = 930).

similar response was observed in mosquitoes inoculated with heat-killed *Escherichia coli*, suggesting the response is a generic infection response rather than parasite mediation. Further work by this group showed that the behavioural changes were linked to changes in insulin signalling and resource-based constraints of immunity, blood feeding and reproduction. Regardless of the mechanism, reducing risky host-seeking and feeding activities during parasite developmental stages will increase the chance of mosquito survival, and hence successful parasite transmission[21].

Further research on the behaviour and physiology of filariasis infected mosquitoes is needed to determine the capacity of vectors to sustain transmission though elimination efforts. The aim of this study was to determine how infection with *Brugia malayi* influences mosquito behaviour in the presence of host cues and the fecundity of mosquitoes. We evaluated convergence towards a human host in mosquitoes exposed to low and high densities of *B. malayi* microfilariae. We correlated the intensity of infection at the developing (L1/L2) and infective (L3) stages with the outcome in the assay and the number of mature eggs produced. We also compared the outcome between uninfected control mosquitoes and exposed mosquitoes that failed to establish an infection.

Results

Mean intensity and parasite yield. Mosquito infection was summarised using the mean intensity for microfilariae ingested, developing larvae (L1/L2) and infective larvae (L3) (Table 1). L1 were first detected after one day post exposure (DPE), L2 detected from five DPE and by 11 DPE 100% of all recovered filarial worms were infective L3 stage (Supplementary Fig. S1). Midgut removal confirmed that mf were escaping from the midgut and into the haemocoel within the first eight hours.

Short range host assay. The short-range host assay measures orientation and flight towards a human volunteer. The outcome measure is the proportion of mosquitoes in the cage where host cues were present out of the total number released, here termed 'host convergence'.

Results from the short-range host assay showed that mosquitoes exposed to *B. malayi* exhibited significantly different behaviour in the presence of a host than those fed uninfected blood (Fig. 1). During the developing time period, mosquitoes were less likely to converge on the host (low density: 14.5% [95% CI ± 5.9] converging; high density: 29.3% [95% CI ± 3.7] converging) compared to the controls (67.7% [95% CI ± 4.4]). However during the infective time period, exposed mosquitoes were more likely to converge on the host (low density: 69% [95% CI ± 9.1] converging; high density: 78% [95% CI ± 4.3] converging) than controls (43.1% [95% CI ± 5.2]) (Supplementary Fig. S2). While exposed mosquitoes exhibited significantly increased host convergence behaviour ($p < 0.0001$) during this period, control mosquitoes exhibited a significant decrease ($p < 0.0001$).

Next, we determined whether behaviours differed in mosquitoes exposed to *Brugia* but where infection failed to establish. The behaviour of mf-exposed but uninfected mosquitoes at 4–6 DPE was comparable to the control cohort with 71% converging towards host cues (n = 28) compared to 68% (n = 440) ($p = 0.84$). However, the

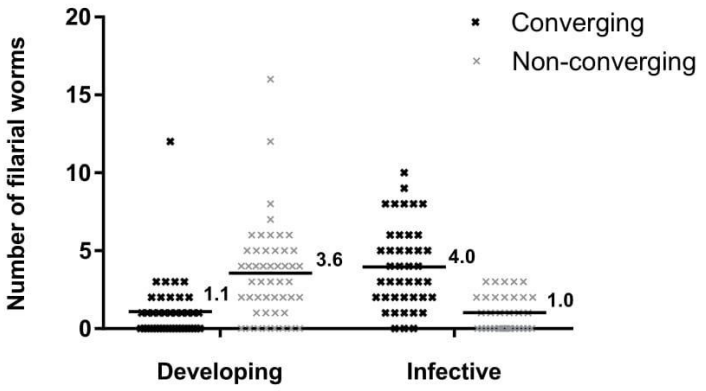

Figure 2. Worm burden in mosquitoes that converged on a host and those that remained in the holding cage at developing (4–6 DPE) and infective (11–13 DPE) life stages. Dark lines show mean worm burden of converging and non-converging mosquitoes.

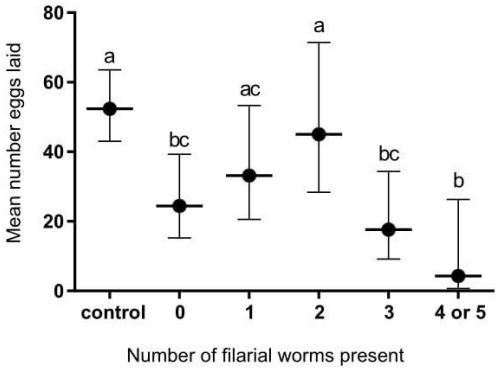

Figure 3. Mean number of mature eggs, including those laid or mature but free inside the abdomen, in unexposed mosquitoes (n = 20) and *B. malayi* exposed mosquitoes (n = 52) with 0–5 larvae present. Only 2 mosquitoes carried 5 larvae and neither of these had developed eggs therefore categories 4 and 5 where combined for statistical analysis. All mosquitoes had mated. Results with the same letter above the bar are not statistically significant from each other.

behaviour of the exposed but uninfected mosquitoes at 11–13 DPE was lower with 16% converging towards host cues (n = 19) compared to 43% in the control group (n = 350) ($p = 0.029$).

Following the behavioural assay, 166 mosquitoes were randomly sampled (83 from the host attraction cage and 83 from the release cage across both time points) for assessment of infection prevalence and intensity. Infection prevalence was 69% during the developing stage and 74% during the infective stage, showing no significant difference. Mean worm burden was compared between converging mosquitoes from the host cage and non-converging mosquitoes from the release cage, at both the developing and infective time points (Fig. 2). During the developing time period, non-converging mosquitoes had a significantly higher worm burden than converging mosquitoes (4.0 [95% CI 3.2: 4.8] and 1.1 [95% CI 0.6: 1.9] respectively, $p < 0.001$). However during the infective time period, converging mosquitoes contained significantly more worms than non-converging (3.6 [95% CI 2.8: 4.5] and 1.0 [95% CI 0.7: 1.5] respectively, $p < 0.001$).

Fecundity. The mean number of eggs laid per mosquito was compared to the number of filarial larvae present in 20 control females and 52 exposed females (Fig. 3), with a significant difference overall between groups ($p < 0.001$). There was a significant difference between the number of eggs laid between the control mosquitoes and those that were exposed but had no worms present (mean egg number: control 52.4 [95% CI 43.1: 63.6]; exposed but no worms 24.4 [95% CI 15.2: 39.3] $p < 0.05$). The difference between the control cohort and mosquitoes harbouring one or two filarial worms was not statistically significant (mean egg number: one worm present 33.2 [95% CI 20.6: 53.3]; two worms present 45 [95% CI 28.4: 71.4]) There was a significant difference when three, four or five worms were present within the mosquito (mean egg number: three worms present 17.6 [95% CI 9.1: 34.3], four or five worms present 4.3 [95% CI 16.1: 32.5], $p < 0.05$).

Survival. There were no significant differences in mosquito survival between the control and exposed groups. Total survival ranged from 80–93% at 16 days post-exposure, with no notable differences in worm burden between dead or moribund mosquitoes (mean worm burden = 2.9) or mosquitoes surviving through the assay (mean worm burden = 22, $p = 0.4$).

Blood meal volume. There was no statistical difference in blood meal volume, measured as concentration of haemoglobin (mg/ml), between mosquitoes fed on either uninfected control blood, or those fed on blood containing microfilariae (mean haemoglobin concentration 267.6 [95% CI 237.5: 297.6]; 274.5 [95% CI 248: 301] respectively, $p = 0.7015$).

Discussion

Parasite transmission is not only reliant on vector survival, but also on the ability of the vector to locate a host and feed[22]. Previous work on the effect of a *Plasmodium* infection on mosquito behaviour has shown a difference in behavioural characteristics such as host-seeking and probing[16,18,21]. These alterations in host-seeking behaviour appear to be stage-specific, with mosquitoes positive for infective sporozoites being more likely to initiate probing, probe for longer and feed to repletion. These results suggest that mosquito behaviour may be altered in order to reduce risky behaviour, such as host seeking foraging and blood feeding, when parasites are still developing, while promoting or increasing these behaviours when infective parasites are present. However subsequent work from Cator and colleagues[20,23] suggested that the change in receptivity and host-seeking behaviour was a generic response to exposure, corresponding with *Plasmodium* developmental stages. Hence we investigated whether analogous behavioural change occurred in filarial-infected mosquitoes and whether this was related to the development stage of the parasite.

Our study demonstrated that female mosquitoes fed on infected blood were up to 5 times less likely to converge on a host hand when the developing stages of *B. malayi* were present. Conversely, mosquitoes harbouring infective third-stage (L3) larvae showed a significantly greater convergence in the presence of a human host compared to controls. Control mosquitoes exhibited reduced convergence between the two time points which may have been due to mosquito senescence. Previous studies have shown that senescence can influence a variety of activities such as mortality, blood feeding and flight ability, all of which affect host-seeking behaviour[24–26]. This suggests that the enhanced behaviour in the presence of host cues in infective mosquitoes and absence of behaviour in infected mosquitoes might be separate processes that overcome the age-related changes in behaviour. Among exposed mosquitoes we observed significant differences in the mean worm burden of converging and non-converging mosquitoes at both time points, with heavy infections associated with non-convergence during the developing period and convergence during the infective period. This suggests that both suppressive and enhanced behavioural changes are density dependent.

The mechanistic cause of the change in behaviour could be a direct parasite mediated and stage specific dependent mechanism that could drive a suppression of mosquito behaviour during the development phase of the infection to protect the parasites from the risks associated with blood feeding, but that is reversed by infective stage parasites to facilitate their transmission to the definitive host. Alternatively, it could be due to an indirect infection response (e.g. immunological or physiological), that temporally coincides with infection-related processes that vary according to the developmental stage of the parasite. Another indirect mechanism may be related to tissue damage caused by the parasite, including mf damage to the gut wall, or developing or infective larval damage to thoracic musculature or the mouthparts. Up to 30% of mf-exposed mosquitoes were negative for filarial larvae at the time of the host assay, although over 95% of mosquitoes examined one day post-exposure were found with microfilariae in the haemocoel. These mf-exposed but free from developing or infective larvae mosquitoes suffered a fitness cost with significantly fewer mature eggs, though there was no difference in host convergence behaviour compared to the uninfected cohort during the developing time period. During the infective time period, this uninfected cohort showed significantly less convergence in the presence of a host than the controls, which was the opposite pattern observed in mosquitoes with L3 present. This suggests that inoculation with microfilariae, without further viable parasite development, was not sufficient to influence this aspect of host-seeking behaviour. Cator *et al.*[23] showed that immune challenge was sufficient to elicit the behavioural response seen in infected/infective mosquitoes, only when immediately following a blood meal. Further work is needed to confirm whether the presence of microfilariae in the haemocoel following a blood meal, rather than the presence of developing larvae and infective larvae in the thorax or other body parts, is causing the differences in convergence behaviour observed in our study.

A number of limitations exist when conducting behavioural assays in a laboratory environment with colonized mosquitoes. In this study we observed significant differences in convergence when mating and egg-laying were enabled (Supplementary Fig. S2). Previous studies on the behaviour of infected mosquitoes have not confirmed mating or enabled egg-laying, and the magnitude of differences observed in other studies may have been influenced by possible atypical physiological states of the females mosquitoes used.

We monitored mosquito survival throughout the assay in order to determine whether mosquitoes used in the L1/L2 time point may have had a higher worm burden than those used at the L3 time point due to reduced survival in heavily infected mosquitoes. In nature, filariasis vector survival is known to be affected by parasite density[8], however the strain of *Aedes aegypti* used in our study has been selected for susceptibility to *Brugia malayi*. With no significant differences in mortality between the various cohorts, and no significant differences in mean worm burden of dead and moribund mosquitoes, we conclude that density dependent mortality had no impact on our assay results.

Fecundity experiments carried out within a laboratory setting also have limitations, as mosquito oviposition is influenced by many external and environmental factors including light levels, substrate choice and the presence of conspecific eggs or larvae[27]. For this reason we measured egg maturation, and included mature and free eggs

within the abdomen as well as eggs laid in our analysis. Host fecundity reduction is a common outcome of parasite infection in insects[28–32]. This could be due to direct nutrient competition by developing parasites or indirect competition by draining energy reserves required for an immune response[27]. In this study the mf-exposed and infective as well as mf-exposed but without larval development cohorts both showed a decrease in the mean number of eggs produced. The reduction in fecundity was independent of variations in blood meal intake, which was similar in both groups. This suggests that exposure to infection, possibility due to immunity, may have additional fitness costs, which might influence vector physiology parameters in models.

Conclusions

Filariasis transmission models are based on parameters that describe host-seeking behaviour, physiology and vector-parasite interactions. However, as these traits can differ between uninfected, mf-exposed, developing infections and infective populations, in a density-dependent manner, the variability in the parameter estimates could have an impact on the validity of model predictions for elimination endpoints. This study shows that stage-specific behaviour change occurs in mosquitoes infected with *B. malayi*, with increased convergence towards a host when the infective L3 stage is present and decreased convergence when the developing stage is present. Changes in fecundity among exposed and infective mosquitoes demonstrates that there are additional fitness costs related to mf-exposure as well as high density infections. While mass drug administration is reducing community-wide microfilariae prevalence and intensity, the success of the elimination programme will also depend on the ability of mosquitoes to survive and transmit filariasis under these changing conditions. Further work is needed to determine to what extent these patterns of behaviour change in host-seeking, extend to wild vectors of filariasis and how the dynamic complexity of these behaviour changes contribute to transmission dynamics as the endpoint of elimination targets are reached.

Materials and Methods

Mosquito rearing and maintenance. *Aedes aegypti* Liverpool strain (LVP strain) mosquitoes were reared from eggs to adults in the insectary at the Liverpool School of Tropical Medicine (LSTM) under standard conditions (27 °C and 80% relative humidity). Eggs were transferred into plastic rearing trays (23.5 × 34.5 × 7.5 cm) filled with distilled water. Second instar larvae were split between fresh trays to achieve a lower larval density, and then maintained on Chinchilla pellets. Pupae were collected, transferred into cages (28.5 × 29.5 × 28 cm) and allowed to emerge as adults. All adult males were removed one day prior to exposure, and remaining females maintained on 10% sugar solution. This rearing ensured all mosquitoes were of the same strain and age when tested.

Our preliminary studies showed that the opportunity to lay eggs significantly influenced the outcome in the short range host assay (Supplementary Fig. S3) with a fivefold increase in host convergence behaviour in females that were allowed to lay eggs. Therefore, for all of the experiments in this study we confirmed that females had mated, by examining the spermathecae of 10 females per cohort for the presence of motile sperm, and all were given the opportunity to lay an egg batch after the blood meal. A flowchart that summarises the experimental design of the below assays is included (Supplementary Fig. S4).

Aedes aegypti exposure to Brugia malayi. Mosquitoes were split into different treatment cohorts and allowed to feed on either uninfected human blood to be used as controls or with human blood containing *B. malayi* parasites at different densities. Mf viability was confirmed through the preparation of a wet blood slide to observe motility. Microfilariae (mf) were recovered by intraperitoneal lavage from gerbils[33]. All experiments were performed in accordance with Home Office (UK) requirements. Approval was obtained for all animal experiments from the ethical committees of the University of Liverpool and LSTM. The recovered mf were diluted 1/100 in RPMI media (Sigma-Aldrich), 10 μl was added to a slide (in triplicate) and mf were counted to determine their concentration in the peritoneal lavage solution. Mf were added to human blood (obtained from the Royal Liverpool Hospital blood bank) to try and achieve the target concentrations of 7,500 mf/ml (low density) or 15,000 mf/ml (high density). Control blood had the relevant amount of RPMI media added to match that added to the infected blood when adding the mf. The actual microfilaremia in the blood samples fed to mosquitoes was calculated at the time of feeding and confirmed in triplicate. 2 drops of 2% formaldehyde solution was added to 20 μl of blood to lyse red blood cells. Using phase microscopy (x10 objective) the entire slide was scanned and all mf counted. In this way different mf densities of low (5,450–7,750 mf/ml) and high (10,550–15,400 mf/ml) ranges could be compared.

4–6 day old female mosquitoes were starved of sucrose for 18 hours prior to blood feeding with infected or non-infected blood. 3 ml of blood was offered using a Hemotek membrane feeding system. Each cohort of mosquitoes only received a single blood meal for the entirety of the study. Mosquitoes were allowed to feed for half an hour after which only fully engorged individuals were selected for all future experiments. Control mosquitoes were from the same rearing cycle and received uninfected blood to feed on at the same time as those receiving infected blood.

Mosquito dissections to recover and observe filarial parasites. Dissections and assays were based on the average *B. malayi* development times within the mosquito (Supplementary Fig. S1). All filarial worms recovered were included in the study and recorded as mf, L1, L2 or L3 along with the body region in which they were recovered. Developing worms (L1 and L2) are present from 3 DPE, and from 11 DPE infective L3 are present.

Midgut dissection. To estimate the number of mf ingested at different blood microfilaremic densities, 3–5 mosquitoes were removed 4 hours after exposure and knocked down briefly on ice, to avoid damage or death to filarial

worms. Intact midguts were removed and lysed in two drops of 2% formaldehyde. The number of mf present within the midgut and body was counted under x10 magnification and recorded as the number of mf ingested.

Abdomen and thorax dissection. Performed from <24 hours post exposure to 10 DPE. Following standard protocol, the thorax and abdomen were removed, teased apart and cover slipped to allow for the examination of mf, L1 and L2 life stages of the parasites by phase contrast microscopy.

Full body dissection. Full body dissections were performed 11 DPE to examine L3 stage larvae, as they were often found in the head and mouthparts of the mosquito but also observed in the haemocoel of the thorax and abdomen. Each body region was teased apart into 3–4 sections and left for 1 minute to observe motile L3 under a dissecting microscope. The mosquito was further teased apart and scanned to check for ay tissue-bound L3. Finally, the tissue was covered slipped and scanned under a phase microscope at x10 objective to look for developing larvae.

Mosquito survival. Unexposed (n = 240 across five replicates) and exposed (n = 239 across five replicates) mosquitoes were held in separate cages and the day of death recorded. Moribund mosquitoes were removed from the cage for dissection and the day of death was classed as the subsequent day. Otherwise mosquitoes were dissected on the day they died, with dissection type according to DPE, and the number of parasites present and developmental stage noted. This experiment was concluded for each exposure at 16 DPE.

Short range host assay. To assess the impact of infection on mosquito behaviour in the presence of host cues, a short range host assay, based on a behavioural assay used by Cator and colleagues[20], was carried out on mosquitoes that had been exposed to *B. malayi* at 4–6 DPE (developing larvae are present within the mosquito, Supplementary Fig. S1) and 11–13 DPE (infective larvae present, Supplementary Fig. S1), followed by dissections. Control mosquitoes (n = 790), mosquitoes exposed to low density microfilariae (n = 250) and high density microfilariae (n = 930) were starved of sucrose for 18 hours prior to assay. Ten mosquitoes per replicate were released into a mesh holding cage that was 17 cm × 17 cm × 17 cm. A tube 12 cm in diameter and 48 cm in length connected this cage to another one of the same dimensions where the experimenter placed their hand 2 cm from the mesh wall (Supplementary Fig. S5). Mosquitoes were allowed to settle in the holding cage for 15 seconds. A barrier between the holding cage and the tunnel was opened for 240 seconds before closing. Mosquitoes that remained in the holding cage were labelled non-converging while mosquitoes that had landed in the second odour cage were labelled converging. There were no instances of mosquitoes returning to the holding cage after entering the odour cage, however, we did observe a few mosquitoes briefly enter the tunnel and return to the holding cage, but all mosquitoes were found in one of the 2 cages at the end of the experiment. Either five or ten batches were assayed per day at each time point, with an equal number of control and infected mosquitoes assayed. Infected and uninfected cohorts were rotated to take into consideration any changes in the time of day while testing was taking place. Mosquitoes from both the converging and non-converging group were removed for dissection as described above (n = 83 from each group) to determine whether parasites were present. A subset of mosquitoes (Control blood n = 5, Infected blood n = 5), where held individually after blood feeding to ensure that oviposition would occur before the short range host assay that started 4–6 DPE.

Fecundity assay. Control (n = 20) and *B. malayi* exposed (n = 52) females were isolated 3 days after blood feeding for the fecundity assay. RPMI media was added to control blood in the same volume as the infected blood source, with all mosquitoes feeding at the same time. Females were placed into individual holding cups (9 cm diameter × 8 cm), covered with fine netting and provided with an oviposition cup. Mosquitoes were maintained on 10% sucrose. The cotton wool in the oviposition cups was regularly moistened with water so that it remained favourably wet for egg laying. Maintenance remained the same until death, when they were removed and the number of eggs that had been laid, if any, were counted. Ovary dissections were performed to view any eggs remaining inside the body cavity. Eggs were classified as mature if they were laid, or if they were free within the body, while immature eggs were not included in the analysis. We chose to include fully mature but retained eggs because numerous environmental and social factors may influence the deposition of eggs onto a substrate[34].

Blood meal volume. Blood meal volume ingested by mosquitoes fed on infected and non-infected blood (Control n = 10, Infected n = 10) was determined by quantification of total haemoglobin content in the abdomen using a colorimetric assay and Drabkin's Reagent (Sigma-Aldrich). Blood was prepared as described above with control blood containing RPMI media and both cohorts were fed at the same time. Abdomens of these mosquitoes were immediately homogenised in 1 ml Drabkin's reagent supplemented with Brij 23 solution (Sigma-Aldrich) and then cleared in a centrifuge for 15 minutes at 13,400 × g. Samples were loaded onto a 96 well plate and absorbance read at 540 nm using Epoch plate reader and Gen5 software. A standard curve was prepared using known concentrations of haem and haem content of experimental samples was calculated by applying the formula obtained from the standard curve.

Statistical analysis. Statistical analysis was carried out using STATA statistical package and graphs were prepared using GraphPad Prism 6. The numbers of exposed mosquitoes that converged in the presence of host cues was compared to the control group using a Poisson regression model with robust standard errors. Adjustment for clustering between days and with total numbers of mosquitoes in each assay included as an offset variable. The robust standard error accounts for the over dispersion in this dataset. The clustered analysis by day was chosen to

remove other sources of variance due to ontogeny or experimental conditions on the day the assay was conducted. The incidence rate ratio with 95% confidence intervals is presented for each time period, in relation to control mosquitoes.

The effect of worm burden on mosquito convergence towards a host, and mean number of mature eggs was analysed using Poisson regression models with robust standard errors. Blood meal volume was analysed using a paired t-test with 95% confidence intervals. Survival curves were compared using Log-rank (Mantel-Cox) comparison.

References

1. World Health Organization. Global Programme to Eliminate Lymphatic Filariasis: Progress Report 2000–2009 and Strategic Plan 2010–2020. Available at http://apps.who.int/iris/bitstream/10665/44473/1/9789241500722_eng.pdf (2010).
2. Gyapong, J. O., Kumaraswami, V., Biswas, G. & Ottensen, E. A. Treatment strategies underpinning the global programme to eliminate lymphatic filariasis. *Expert Opinion of Pharmacotherapeutics*. **6,** 179–200 (2005).
3. World Health Organization. The Global Burden of Disease: 2004 update. *Update*, *2010* 146 http://doi.org/10.1038/npp.2011.85.
4. World Health Organization. Lymphatic Filariasis factsheet (2016). Available at www.who.int/mediacentre/factsheets/fs102/en/ (Accessed: 21st April 2016).
5. Paily, K. P., Hoti, S. L. & Das, P. K. A review of the complexity of biology of lymphatic filarial parasites. *J. Paras. Dis*. **33(1),** 3–12 (2009).
6. Wattam, A. R. & Christensen, B. M. Induced polypeptides associated with filarial worm refractoriness in *Aedes aegypti*. *Proc. Natl. Acad. Sci. USA* **89(14),** 6502–6505 (1992).
7. Christensen, B. M. & Severson, D. W. Biochemical & molecular basis of mosquito susceptibilty to Plasmodium and filarial nematodes. In *Parasites & Pathogens of Insects 1st edn* (eds Beckage, N., Thompson, S. N. & Federici, B. A.) 245–265 (California Academic Press Inc. 1993).
8. Erickson, S. M. *et al.* Mosquito-parasite interactions can shape filariasis transmission dynamics and impact elimination programs. *PLoS Negl. Trop. Dis*. **7(9),** 1–7 (2013).
9. Dye, C. Does facilitation imply a threshold for the eradication of lymphatic filariasis? *Parasitology Today*, **8,** 109–110 (1992).
10. Bryan, J. H. & Southgate, B. A. Some observations on filariasis in Western Samoa after mass administration of diethylcarbamazine. *Trans. R. Soc. Trop. Med. Hyg*. **70(1),** 39–48 (1976).
11. Southgate, B. A. & Bryan, J. H. Factors affecting transmission of *Wuchereria bancrofti* by anopheline mosquitoes. 4. Facilitation, limitation, proportionality and their epidemiological significance. *Trans. R. Soc. Trop. Med. Hyg*. **86,** 523–530 (1992).
12. Michael, E. The epidemiology of lymphatic filariasis control. In *World Class Parasites: The Filaraie* 1st edn. (eds Rajan, T. V. & Klei, T.) pp 41–48 (Klumer Academic Publishers, 2002).
13. Irvine, M. A. *et al.* Modelling strategies to break transmission of lymphatic filariasis -aggregation, adherence and vector competence greatly alter elimination. *Parasit. Vectors*. **8,** 547 (2015).
14. Michael, E. & Singh, B. K. Heterogenous dynamics, robustness/fragility trade-offs, and the eradication of the macroparasitic disease, lymphatic filariasis. *BMC Medicine*. **14(1)** (2015)
15. Hollingsworth, T. D. *et al.* Quantitative analyses and modelling to support achievement of the 2020 goals for nine neglected tropical diseases. *Parasit. Vectors*. **8(1),** 630 (2015).
16. Wekesa, J. W., Robert, S. C. & Mwangi, R. W. Effect of *Plasmodium falciparum* on blood feeding behaviour of naturally infected *Anopheles* mosquitoes in West Kenya. *Am. J. Trop. Med. Hyg*. **47(4),** 484–488 (1992).
17. Koella, J. C., Sorensen, F. L. & Anderson, R. The malaria parasite, *Plasmodium falciparum*, increases the frequency of multiple feeding of its mosquito vector, *Anopheles gambiae*. *Proc Biol Sci*. **265(1398),** 763–768 (1998).
18. Anderson, R. A., Koella, J. C. & Hurd, H. The effect of *Plasmodium yoelii nigeriensis* infection on the feeding persistence of *Anopheles stephensi Liston* throughout the sporogonic cycle. *Proc. Biol. Sci*. **266(1430),** 1729–1733 (1999)
19. Poulin, R. "Adaptive" changes in the behaviour of parasitized animals: A critical review. *Int. J. Parasitol*. **25(12),** 1371–1383 (1995).
20. Cator, L. J. *et al.* "Manipulation" without the parasite: altered feeding behaviour of mosquitoes is not dependent on infection with malaria parasites. *Proc. Biol. Sci*. **280(1763)** (2013).
21. Koella, J. C. Stage-specific manipulation of a mosquito's host-seeking behaviour by the malaria parasite *Plasmodium gallinaceum*. *Behav. Ecol*. **13(6),** 816–820 (2002).
22. Takken, W. & Knols, B. G. J. Odour-mediated behaviour of Afrotropical malaria mosquitoes. *Annu. Rev. Entomol*. **44,** 131–137 (1999).
23. Cator, L. J. *et al.* Immune response and insulin signalling alter mosquito feeding behaviour to enhance malaria transmission potential. *Sci. Rep*. **5,** 11947 (2015).
24. Styer, L. M., Carey, J. R., Wang, J. L. & Scott, T. W. Mosquitoes do senesce: departure from the paradigm of constant mortality. *Am. J. Trop. Med. Hyg*. **76(1),** 111–117 (2007).
25. Rowley, W. A. & Graham, C. L. The effect of age on the flight performance of female *Aedes aegypti* mosquitoes. *J. Insect. Physiol*. **14(5),** 719–728 (1968).
26. Ariani, C. V., Juneja, P., Smith, S., Tinsley, M. C. & Jiggins, F. M. Vector competence of *Aedes aegypti* mosquitoes for filarial nematodes is affected by age and nutrient limitation. *Exp Gerontol*. **61,** 47–53 (2015).
27. Wong, J., Stoddard, S. T., Astete, H., Morrison, A. C. & Scott, T. W. Oviposition site selection by the dengue vector *Aedes aegypti* and its implications for dengue control. *PLoS Negl. Trop. Dis*. **5(4)** (2011).
28. Hurd, H. Host fecundity reduction: A strategy for damage limitation? *Trends Parasitol*. **17(8),** 363–368 (2001).
29. Sylvestre, G., Gandini, M. & Maciel-de-Freitas, R. Age-dependent effects of oral infection with dengue virus on *Aedes aegypti* (Diptera: Culicidae) feeding behaviour, survival, oviposition success and fecundity. *PLoS ONE* **8(3)** 1–8 (2013).
30. Ferdig, M., Beerntsen, B., Spray, F. J., Li, J. & Christensen, B. M. Reproductive costs associated with resistance in a mosquito-filarial worm system. *Am. J. Trop. Med. Hyg*. **49(6),** 756–762 (1993).
31. Gaaboub, I. A. Observations on the basal follicle numbers developed per female of two strains of *Aedes aegypti* after being fed on hosts with different levels of microfilariae of *Brugia pahangi*. *J. Invertebr. Pathol*. **28,** 203–207 (1976).
32. Hacker, C. The differential effect of *Plasmodium gallinacium* on the fecundity of several strains of *Aedes aegypti*. *J. Invertebr. Pathol*. **18,** 373–377 (1971).
33. Griffiths, K. G., Alworth, L. C., Harvey, S. B. & Michalski, M. L. Using an intravenous catheter to carry out abdominal lavage in the gerbil. *Lab Animal*. **39(5),** 143–148 (2010).
34. Bentley, M. D. & Day, J. F. Chemical Ecology and Behavioural Aspects of Mosquito Oviposition. *Ann. Rev. Entomol*. **34,** 401–421 (1989).

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K.G. and L.R. wrote the manuscript, K.G. and D.C. conducted the experiments. K.G., D.C., M.T. and L.R. conceived and designed the study. All authors contributed to and approved the final version of the manuscript.

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S1. Filarial worm development.

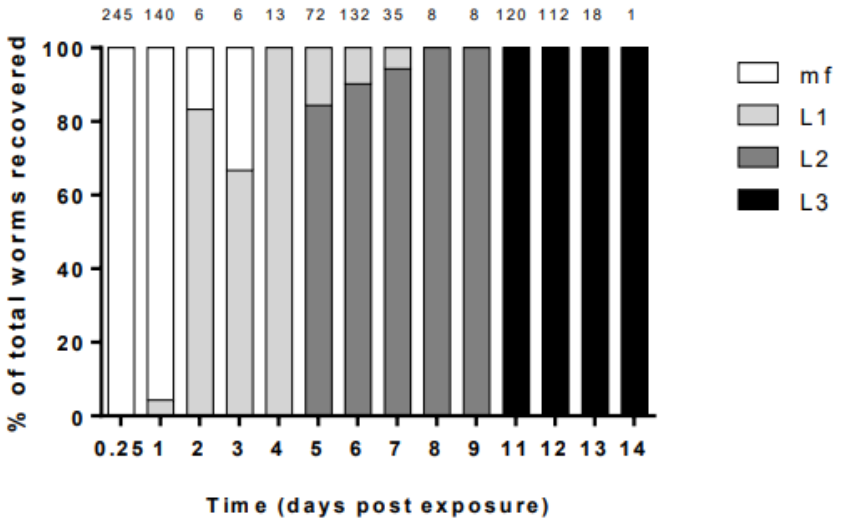

Figure S1. The development of *Brugia malayi* from microfilaria to infective stage larvae in *Aedes aegypti*. The total number of worms recovered is listed above each bar. (0.25 DPE n= 11, 1 DPE n= 12, 2 DPE n= 4, 3 DPE n= 8, 4 DPE n= 10, 5 DPE = 46, 6 DPE n= 82. 7 DPE n= 28, 8 DPE n= 3, 9 DPE n= 2, 11 DPE n= 50, 12 DPE n= 38, 13 DPE n= 4, 14 DPE n= 1).

S2. Mosquito convergence on a host when infected with developing (L1/L2) and infective (L3) stage filarial worms

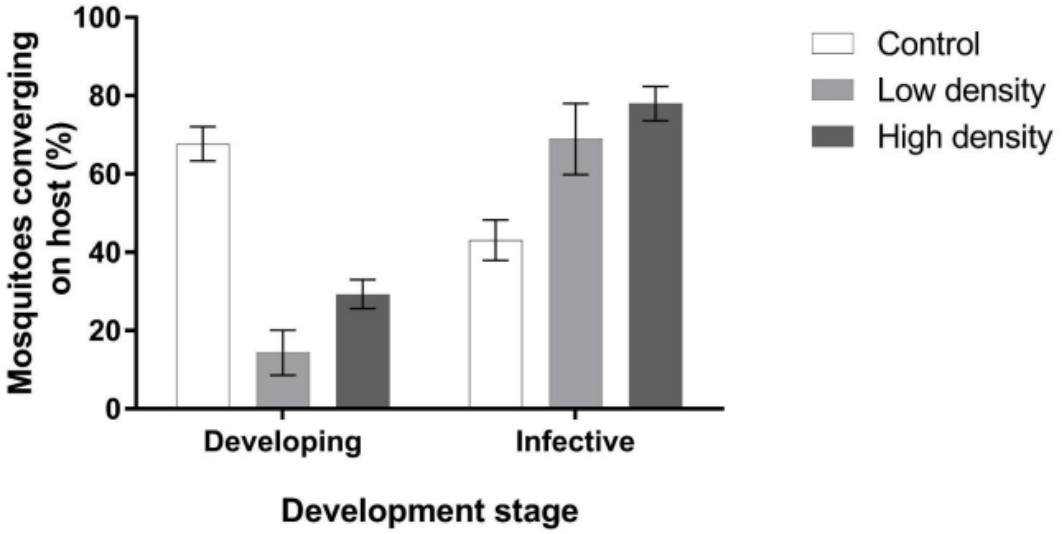

Figure S2. The percentage of mosquitoes converging on a host when exposed to uninfected and infected blood during two time points. A short range host assay was performed at developing (4-6 DPE) and infective (11-13 DPE) time points to assess the activation and orientation towards a human hand. Control (n=790), Low density (n=250), High density (n=930).

S3. Short range assay to assess the effect of oviposition.

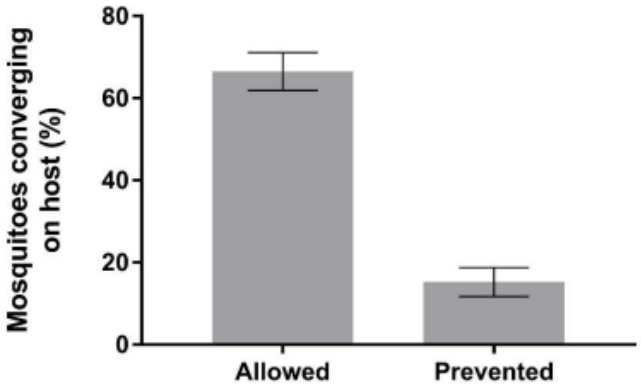

Figure S3. The percentage of mosquitoes converging on a host when oviposition was allowed or prevented. The short range assay was carried out on 2 groups of mosquitoes: one where oviposition was allowed by the addition of an oviposition substrate (n= 400), another where no substrate was provided (n= 400), to determine whether standard husbandry practices influenced activation and orientation towards a host. 6 day old females were offered a blood meal and after 3 days an oviposition cup was placed into one of the cohort cages, while the other did not receive one. Mosquitoes were sugar starved for 12 hours prior to the short range assay. This study involved 40 replicates of 10 females each per cohort. Assays were performed over 4 days, with cohorts assayed in alternate succession. We observed significantly greater activation and orientation in mosquitoes that were allowed to lay a batch of eggs after consuming their first blood meal ($P < 0.0001$ n = 400 per cohort). (Odds ratio 11.03 95% CI [7.831, 15.54]). Dissections from a subset of mosquitoes (10 per cohort) showed that 100% of females had mated.

S4. Flowchart of methodology

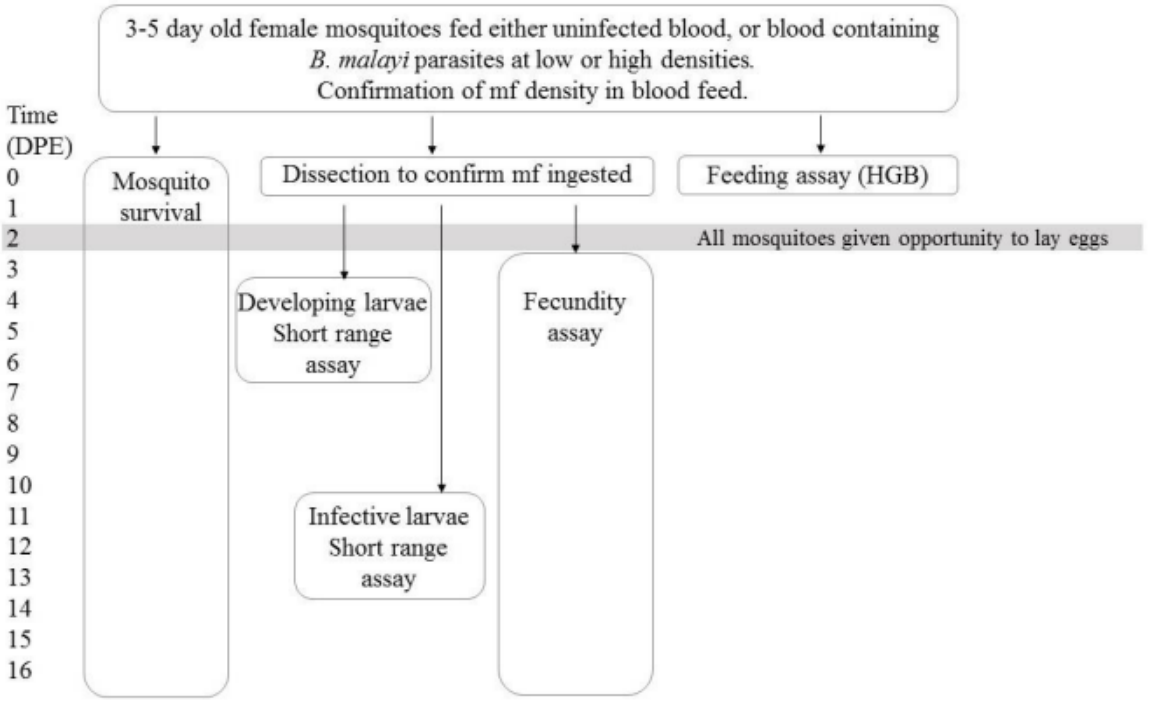

Figure S4. Flowchart of methodology. Flowchart showing structure of methodology and mosquito designation for each assay.

S5. Diagram of behavioural assay

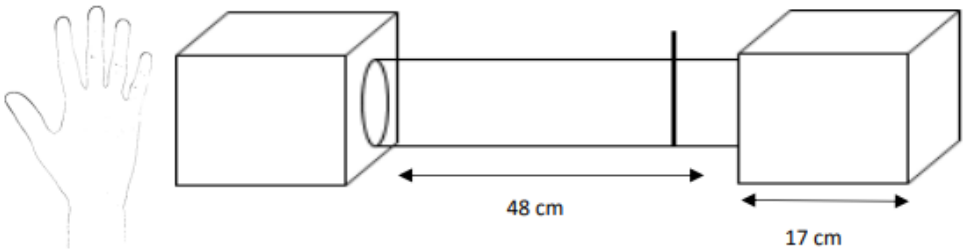

Figure S5. Experimental set-up for behavioural bioassay. A schematic of short range behavioural assay. Mosquitoes were released into a mesh holding cage that was 17cm x 17cm x 17cm. A tube 12cm in diameter and 48cm in length connected this cage to another one of the same dimensions. A gate prevented movement from one cage through to the other until the assay was ready to begin.

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The consequences of *Brugia malayi* infection on the flight and energy resources of *Aedes aegypti* mosquitoes

Alastair G. T. Somerville, Katherine Gleave, Christopher M. Jones & Lisa J. Reimer*

Evidence from experimental infection studies has shown that infected mosquitoes exhibit altered host-seeking behaviours, with suppression and activation of behaviours dependent on the parasite's development stage. The mechanisms are poorly characterised; however, infections can impact mosquito energy reserves, thereby influencing key life-history traits and behaviours. In addition, filarial infection is likely detrimental to flight due to damage caused by developing worms. This study aimed to evaluate the impacts of *Brugia malayi* infection on *Aedes aegypti* flight parameters: distance, average speed, maximum speed and number of flight bursts, using a tethered flight mill. In addition, we explored whether differences in flight capacity may be due to the effect of infection on glycogen and lipid reserves. Infection with filarial worms significantly reduced flight distance but increased the number of flight bursts. Exposure to microfilaermic blood led to a significant decrease in average and maximum flight speeds even in the absence of an established infection. Mosquitoes fed on microfilaraemic blood showed reduced levels of glycogen (−37.9%) and lipids (−49.7%) compared to controls at nine days post-exposure. However, a one-hour period of flight activity caused an increase in lipid content for both infected and control mosquitoes. Consequential flight incapacitation may serve in explaining the heterogeneous distribution of lymphatic filariasis.

Lymphatic filariasis (LF) is a parasitic disease caused by three nematode species: *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*[1,2]. Regarded as a Neglected Tropical Disease (NTD), recent estimates suggest that 67 million people suffer from LF[3] across 73 tropical and sub-tropical countries[4]. Morbidity, largely in the form of elephantiasis and hydrocele, causes substantial physical, social and psychological damage[5–7]. The resulting loss of 2.8 million disability-adjusted life years (DALYs)[8] places LF as a significant global health problem. Microfilariae (mf) released by gravid female worms are ingested by mosquitoes during bloodfeeding. Approximately 10 days later, following successive developmental stages in the flight muscles, the infective larvae (L3) migrate to the mouthparts of the mosquito, ready to be deposited onto vertebrate host skin in subsequent feeds. This mode of development causes substantial histological and physiological damage within the vector[9–13]. As such, filarial infection is likely to be detrimental to mosquito flight.

Evidence of the changes in mosquito flight following filarial infection are equivocal. Previous work suggests that flight activity, when defined as the number of minutes within an hour containing at least a single flight attempt, increases three-fold during filarial infection prior to the development of L3 worms[14]. Conversely, some studies suggest that filarial infection produces significant declines in continuous measures of flight ability, namely distance and time[15–18]. Comparing study outcomes is therefore difficult due to differences in measured flight variables; an issue which is further complicated considering that there are a range of tools with which to quantify flight. Therefore, while infection may indeed lead to an increased number of flight attempts but overall reduced flight outputs, no study to date has explored both aspects of flight ability and activity.

Lipids and glycogen are important sources of energy in mosquitoes, being utilised in a number of life history traits and behaviours, such as flight[19,20], vitellogenesis[20] and immune responses[21,22]. The relative availability of these resources may therefore influence flight ability or activity. Blood-fed and sugar-fed mosquitoes have been shown to differ in flight speed and distance[20], perhaps due to the differential utilisation and availability of energy resources. Research which has shown reduced flight distance and time as a result of filarial infection[18]

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Flight Parameter	Unit	Definition	Rationale
Flight Distance	Meters (m)	The total distance covered over one hour.	Damage caused to thoracic flight muscles by developing filarial worms is likely to affect flight distance. Previous studies indicate reduced distance from filarial infection[18].
Average Speed	Meters per second (ms^{-1})	The average (harmonic mean) distance covered per second across one hour.	Damage caused to thoracic flight muscles by developing filarial worms is likely to affect measures of flight speed.
Maximum Speed	Meters per second (ms^{-1})	The highest speed reached within flight testing	Damage caused to thoracic flight muscles by developing filarial worms is likely to affect measures of flight speed.
Number of Flight Bursts	—	Any flight attempt that lasts more than 5 seconds and covered a distance of at least 0.25 m*	Previous studies indicate reduced flight attempts following filarial infection[14].

Table 1. The definition and rationale for the flight responses measured and analysed using the tethered flight mill system. *Definition of a flight burst was based on pre-existing definitions of a flight burst[65].

hypothesised this was a result of depleted energy reserves, however no study to date has explored energy in infected mosquitoes.

Mosquitoes infected with *Plasmodium* show alterations in host-seeking and host-feeding strategies depending on the stage of infection[23–28]. Similar changes in host-seeking behaviour have been observed in mosquito-filarial systems, including *Aedes aegypti* mosquitoes infected with *B. malayi*[29]. When harbouring the non-infective L2 stage, mosquitoes show a five-fold reduction in host-seeking following exposure to human host cues compared to uninfected controls. Once developed into L3s, this pattern is reversed, and infected mosquitoes are significantly more likely to respond to host cues than uninfected mosquitoes[29]. These behavioural changes offer clear advantages to the parasite in minimising mortality risk during development and increasing the chances of host-contact once transmissible. The cause of this altered host seeking behaviour remains unclear, however damage to the flight muscles caused by the developing filarial worm may inhibit or deter flight in mosquitoes prior to the L3 stage.

Behavioural and physiological changes in vectors following infection can have significant implications for disease transmission[30,31], and modelling frameworks are likely to benefit from an increased understanding of the interaction between vector and parasite, particularly during infection[32]. However, the absolute fitness costs associated with infection in mosquitoes remain ambiguous[33]. Coevolution between obligate parasites and vectors may lead to neutral relationships to maintain effective transmission, but immune responses are costly, and parasites can cause direct physical damage to the insect[9–13].

The primary aim of our study was to determine the influence of filarial infection on a range of mosquito flight parameters: distance (m), speed (ms^{-1}), maximum speed (ms^{-1}) and number of flight bursts (justifications for which can be seen in Table 1). Our secondary aim was to determine whether infection also led to a depletion of glycogen and lipid resources, and if this depletion was associated with flight performance.

Results

A total of 217 female *Ae. aegypti* mosquitoes (including 123 fed *Brugia malayi* and 94 fed uninfected blood) were flown on tethered flight mills across three replicate experiments. Midgut dissections conducted 3 hours post-bloodfeeding confirmed substantial uptake of microfilariae (mf) in mosquitoes which fed on *B. malayi* infected blood ($n = 3, \bar{x} = 109.7 \pm 10.7$). Post-flight dissections of mosquitoes which fed on *B. malayi* infected blood found that 41/65 (63.1%) were infected with L1s and/or L2s at 4 to 6 days post-exposure (DPE), and 29/58 (50.0%) were infected with L3s at 11 to 13 DPE (Table 2). We categorised mosquitoes into three groups based on infection status: "Infected" (fed on infected blood and contained at least one worm at the time of dissection), "Exposed" (fed on infected blood but contained no worms at the time of dissection), and "Control" (fed on uninfected blood).

Effect of *B. malayi* infection on mosquito flight. Generalised Linear Mixed Models (GLMMs) using likelihood ratio testing indicated that infection status had a significant effect on flight distance ($\chi^2 = 10.5$, $P = 0.005$), average speed ($\chi^2 = 10.3$, $P = 0.006$), maximum speed ($\chi^2 = 20.5$, $P < 0.001$) and the number of flight bursts ($\chi^2 = 17.6$, $P < 0.001$). Pairwise comparisons based on least square means found that infection and exposure both lead to declines in the distance and average speed flown, as well as an increase in the number of flight bursts when compared to controls (see Table 3). The number of days post-exposure only had a significant impact on flight distance and the number of flight bursts, with flight distance decreasing significantly by 11–13 DPE ($\chi^2 = 6.4$, $P = 0.012$), whereas the number of flight bursts increased significantly ($\chi^2 = 16.2$, $P < 0.001$). A significant interaction between infection status and DPE was observed for the number of flight bursts ($\chi^2 = 120.3$, $P = < 0.001$). This indicates that the change in the number of flight bursts over time between infection groups was different. The unadjusted means for each measured parameter are shown in Fig. 1. Wing length measured from a total of 34 mosquitoes found the average wing length to be 3.101 ± 0.022 mm. Scatter-plots of wing length against measured parameters of flight found no correlation (Supplementary Fig. S1). Linear regression analysis also found that worm burden was not a significant predictor for any flight parameter, with the exception of maximum speed during the L3 stage ($P = 0.001$), which increased with increasing worm burden (Supplementary files S2 and S3).

Effect of *B. malayi* infection on lipid and glycogen content in mosquitoes. A total of 76 mosquitoes underwent glycogen and lipid content analysis using anthrone and vanillin reagent, respectively. Midgut dissections conducted 3 hours post-bloodfeeding confirmed uptake of mf in *B. malayi* exposed female

Days Post Exposure	Infection Status†	Sample Size	Total Number of Worms Recovered	Mean Intensity (95% CI)
4–6	Infected	41	185	5.08 (3.79–6.37)
	Exposed	24	0	0
	Control	48	—	—
11–13	Infected	29	108	4.10 (3.53–4.68)
	Exposed	29	0	0
	Control	46	—	—
Total	**217**	**293**	—	

Table 2. Numbers of mosquitoes assayed for flight and the intensity of *B. malayi* infection. †Exposed mosquitoes were fed the same bloodmeal as infected but were found not to contain larvae after flight testing.

Flight response	Independent variable	Pairwise comparison†	Estimate	Std. Error	p-value
Flight distance (m)	Infection status	Control - Exposed	0.109	0.180	0.817
		Control - Infected	0.497	0.151	0.003**
		Exposed - Infected	0.388	0.188	0.097
	DPE	—	−0.337	0.132	0.012*
Average speed (ms^{-1})	Infection status	Control - Exposed	0.544	0.165	0.003**
		Control - Infected	0.210	0.145	0.318
		Exposed - Infected	−0.334	0.170	0.120
	DPE	—	−0.236	0.128	0.067
Max speed (ms^{-1})	Infection status	Control - Exposed	0.618	0.134	<0.001***
		Control - Infected	0.230	0.109	0.087
		Exposed - Infected	−0.389	0.144	0.019*
	DPE	—	0.018	0.092	0.849
Number of flight bursts	Infection status	Control - Exposed	0.002	0.045	0.999
		Control - Infected	0.162	0.045	<0.001***
		Exposed - Infected	0.159	0.043	<0.001***
	DPE	—	0.138	0.034	<0.001***

Table 3. Results of Generalised Linear Mixed Models on the effect of *B. malayi* infection status on mosquito flight. DPE = Days Post Exposure. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. †Exposed mosquitoes were fed the same bloodmeal as infected but were found not to contain larvae after flight testing.

mosquitoes ($n = 6, \bar{x} = 32.3 \pm 9.12$). Feeding on *B. malayi* positive blood was found to have a significantly detrimental effect on glycogen ($\chi^2 = 6.0$, $P = 0.014$), decreasing it by 37.6%, and lipid ($\chi^2 = 28.2$, $P < 0.001$), decreasing it by 49.7%. Due to the nature of content extraction, infection could not be confirmed in these mosquitoes.

Effect of flight on lipid and glycogen content in mosquitoes. Flight activity had no significant effect on glycogen levels ($\chi^2 = 2.3$, $P = 0.132$), but did lead to significantly increased lipid content ($\chi^2 = 13.3$, $P < 0.001$) of 34.7% (Table 4). Changes in the average glycogen and lipid levels between groups are shown in Fig. 2.

Discussion

This study found that *B. malayi* infection has a detrimental impact on *Ae. aegypti* flight distance, average speed and maximum speed. Previous research has hypothesised that decreased flight capabilities were the result of depleted host energy reserves and/or functional incapacitation of flight muscles[18]. *Ae. aegypti* muscle fibres become devoid of glycogen granules when infected with developing filarial larvae due to consumption by the worm[10,11]. Immune responses in insects are also energetically costly[34,35], and infection can lead to significant declines in glycogen and lipid content in both *Drosophila*[36,37] and *Ae. aegypti*[38]. Upregulation of lipid transporter proteins during *Ae. aegypti* infection suggests an increased utilisation of lipids during systemic immune responses[21]. The declines in flight outputs observed in this study may therefore be a consequence of energy resource deprivation resulting from costly immune responses, and/or the direct consumption of intramuscular glycogen by the filarial worm. This is supported by the significant declines of glycogen and lipid content in *Brugia*-infected mosquitoes observed here.

Insect flight is one of the most energetically demanding exercises in the animal kingdom and requires highly efficient systems to transport energy reserves to flight muscles[39]. Glycogen and lipids are the primary sources of energy for insect flight, including mosquitoes[40,41], but their levels did not decrease following the one-hour

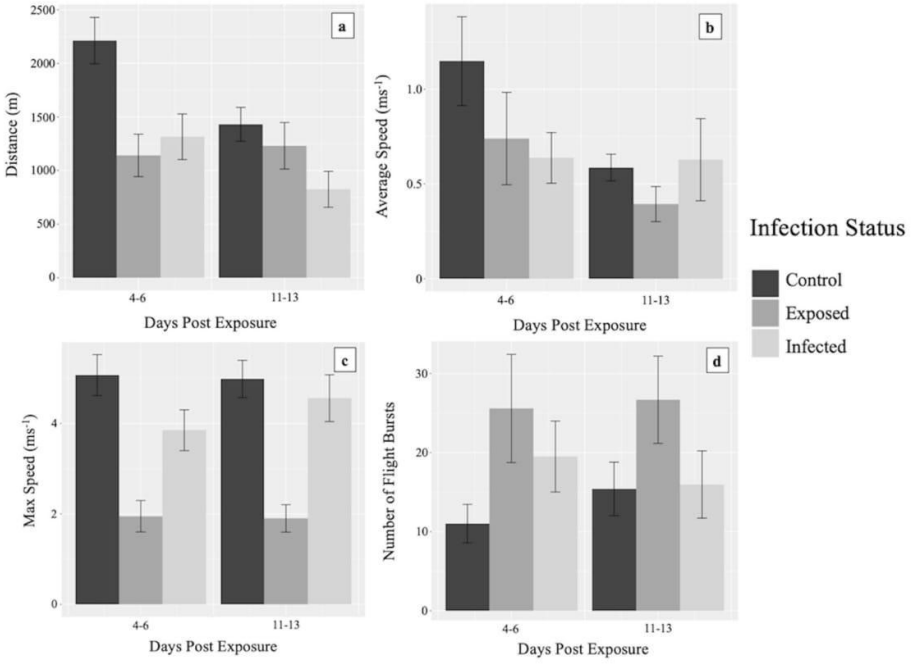

Figure 1. The relationship between *B. malayi* infection status and flight activity in *Ae. aegypti* mosquitoes post-exposure. (**a**) Distance (m), (**b**) average speed (ms^{-1}), (**c**) maximum speed (ms^{-1}), (**d**) number of flight bursts. All mosquitoes were flown for a total time of one hour. Standard error bars are shown. Exposed mosquitoes were fed the same bloodmeal as infected but were found not to contain larvae after flight testing.

Energy resource	Independent variable	Estimate	Std. Error	p-value
Glycogen	Infection status	−0.468	0.205	0.014*
	Flight status	0.287	0.205	0.132
Lipid	Infection status	−0.717	0.162	<0.001***
	Flight status	0.473	0.162	<0.001***

Table 4. Effect of *B. malayi* infection and flight on energy resources. *$P < 0.05$; ***$P < 0.001$.

flight period in this study. In the wild, mosquitoes derive their energy from the nectar of a range of available plant sources before it is converted to glycogen and storage lipids over a period of two days[20]. The cohorts of *Ae. aegypti* were provided with 10% glucose solution up until flight testing and therefore declines in glycogen levels following flight may not have been observed due to the preferential use of glucose. Conversely, why lipid content increased following flight is not immediately clear. Short-term stress, such as flight, can lead to liberation of lipids from the fat body[42–44], a phenomenon shown in a number of different insect species[45–48]. Lipids are stored as triacylgycerols in the fat body, before being converted to diacylglycerol during liberation and transportation in the haemolymph[49]. There is evidence that vanillin reagent, the choice of reagent for this study, fails to react with triacylglycerols[50], perhaps suggesting that lipids were only detectable once liberated from the fat body for flight.

Interestingly, infection was associated with an increase in the number of flight bursts, suggesting that infected vectors may make a larger number of slow, short, flight attempts. This finding lends its support to previous research which has found *Plasmodium* infection is associated with an incapacitation of flight[51,52], but increased nectar-feeding[53]. Reduced energetic reserves caused by harbouring an infection support this idea, although further research is needed. Conversely, it may simply be that the presence of parasitic worms agitates the invertebrate host, leading to increases in the number of flight bursts.

Physical damage from filarial worm development appeared to have little to no additive impact on the flight ability of mosquitoes. Mosquitoes undergo age-associated declines in flight activity[54], which was also observed in our study. However, the proportional decrease in distance and average speed over time was less in mosquitoes infected with L3 larvae compared to controls. The detrimental effect of infection on flight ability may therefore occur rapidly following exposure. Previous studies which identified significant declines in flight ability and activity following the development of L3s did so using unnaturally high burdens of infection (10+ worms)[14]. Thus,

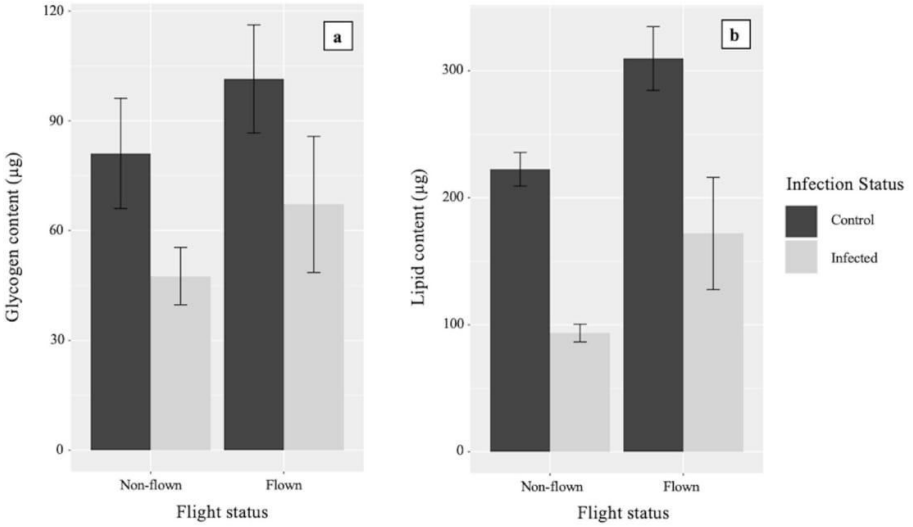

Figure 2. The glycogen and lipid content of *Ae. aegypti* mosquitoes based on *B. malayi* mf feeding status and flight status. (**a**) Glycogen, (**b**) Lipid. All mosquitoes were allowed to fly for a total time of one hour. Mosquitoes are categorised as either controls, or having fed on infected blood, as confirmation of infection intensity was not possible. Standard error bars are shown.

while high burdens of infection may influence flight at the infective stage, this has limited applicability to natural settings, where burdens of infection rarely exceed five L3 worms per host[55,56]. Flight muscle fibres can carry out post-trauma reparation if such damage is limited[10], further supporting this idea that L3s only cause noticeable detriment to host flight in unnaturally high intensity infections. Previously identified stage-specific switches in host-seeking behaviour[29] are unlikely to be due to the generic effects of flight capacity, however it could be partially attributed to filarial infection causing an apparent increase in the number of flight bursts. Control mosquitoes in this study otherwise saw a relative decline in the number of flight bursts over time.

The distinction between infection and exposure (the absence of developing or infective larvae) on flight outputs is not obvious. While exposure appeared to match infection in its effect on flight distance and average speed, it had a significantly greater detriment to maximum speed than infection. Regardless of the reason for this difference, these results clearly highlight that exposure and clearance of filarial worms may be sufficient to cause significant changes in vector flight.

Conclusion

This study highlights the impacts that filarial infection can bear on vector flight. Further investigations into the flight behaviour of infected mosquitoes is necessary to apply this to different ecological settings, however the interplay between infection, immunity and flight is clearly complex. Furthermore, understanding the impact of fitness costs on the ability of mosquitoes to transmit disease may help explain the heterogeneity of filariasis transmission[57]. The behavioural and physiological consequences of filarial infection on their invertebrate hosts, such as flight incapacitation, may contribute to the heterogeneous nature of LF, which can pose a challenge for elimination. Remarkable differences between mosquito species in their behavioural and physiological responses to infection warrants continued exploration with additional parasite-vector systems.

Methods

Study design. We tested the impact of filarial infection on various flight parameters in mosquitoes using a set of eight tethered flight mills. *Ae. aegypti* Liverpool (LVP) strain mosquitoes which had fed on either microfilaermic blood or uninfected blood were flown at 4 to 6 days post-exposure (DPE) or 11 to 13 DPE, corresponding to the L1/L2 and L3 stages of *B. malayi* respectively[58]. Those which fed on blood containing *B. malayi* mf underwent subsequent dissections to confirm the prevalence and intensity of infection. The wing length of 34 infected mosquitoes was measured to determine the correlation with flight activity. Mosquitoes were subject to either (i) a one-hour flight mill assay on day 4–6 DPE followed by dissection, (ii) a one-hour flight mill assay on day 11–13 DPE followed by dissection, (iii) a one-hour flight mill assay on day 9 DPE followed by glycogen and lipid analysis, or (iv) glycogen and lipid analysis on day 9 DPE with no flight. A single energy content analysis was conducted to observe the impact of infection and flight on glycogen and lipid reserves.

Mosquito rearing and husbandry. Rearing of the *Ae. aegypti* LVP mosquitoes took place at the Liverpool School of Tropical Medicine (LSTM) insectaries under controlled conditions (80% relative humidity,

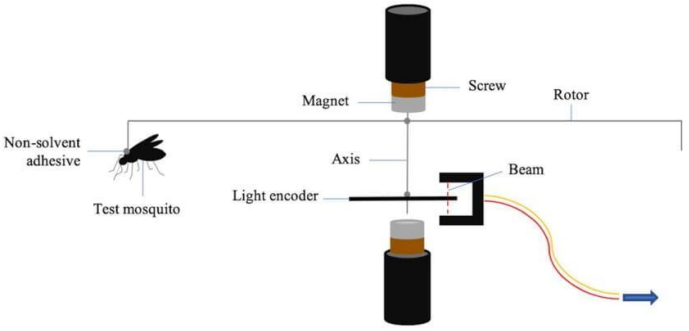

Figure 3. The set-up of a flight mill used during testing in this study, including rotor. Mosquitoes fly around a radius measuring 4 cm, causing the light encoder to periodically break a laser beam which measures distance. 1 rotation = 25.13 cm. Image provided by A. Somerville.

27 °C and 12:12 light/dark cycle). Internally sourced mosquito egg papers were floated out into plastic trays (235 × 345 × 75 mm) containing distilled water, with a larval density of approximately 200/larvae per tray. Larvae were fed on Brewers' yeast pellets before transfer to cages (285 × 295 × 280 mm) once pupated. We maintained adults on 10% sugar solution prior to bloodfeeding. All adult females were between 2 and 5 days old when bloodfed.

Mosquito exposure to blood sources. On the day of bloodfeeding, female mosquitoes were split into two separate cages. We then fed cohorts on either uninfected human blood (controls) or human blood containing *B. malayi* mf at a density of 20,000 mf/ml. Mf were obtained via intraperitoneal lavage of infected gerbils by a third party, in accordance with UK Home Office requirements and following LSTM approval, before suspension in RPMI media at a dilution of 1/100. Triplicate microscopic observations confirmed the presence and mobility of mf. Uninfected control blood was diluted with equal measures of non-infected RPMI media. Approximately 3 ml of blood was then offered to mosquitoes using a Hemotek® membrane feeding system kept at 37 °C. Following successful bloodfeeding, we removed all mosquitoes that had not fed to repletion. All research red cells and plasma were supplied by National Health Service Blood and Transfusion and were mixed in a 50:50 ratio before use. To verify mf uptake, three engorged females had their midgut contents homogenised on a microscope slide, which was then scanned with phase optics at 10x magnification.

Quantification of flight ability. We assessed the flight activity of *Ae. aegypti* using tethered flight mills (provided by Dr. Lim of Rothamsted Research), which were housed under standardised insectary conditions described above (Fig. 3). Females were briefly knocked down on ice and placed on a metal plate with their dorsal surface showing. The arm of a flight mill rotor (radius = 4 cm) was dipped in non-solvent fast-drying glue, before being placed onto the scutum and held for approximately one minute to allow the glue to dry. Mosquito orientation was kept horizontal and perpendicular to the rotor bar. Successfully adhered mosquitoes were allowed to rest with tarsal contact prior to placement into one of eight flight mill chambers, which holds the central steel axis of the rotor in place by two opposing magnets to minimise friction. Individuals were briefly observed to ensure initiation of flight before being left to fly freely for one hour. Mosquitoes which failed to fly when stimulated were removed and replaced. The distance covered every 5 seconds to the nearest 10 cm is automatically recorded on software (Flight Mill v1.2) according to flight mill design. All flight mill experiments were performed in LSTM insectaries under rearing conditions and occurred between 0900 and 1700. We chose to measure a total of four flight activity response variables to determine the impact of infection on flight performance. The definitions and justifications for these are provided in Table 1.

Dissections of mosquitoes to categorise infection. Dissections of mosquitoes corresponded to the developmental time points of *B. malayi*. At 4 to 6 DPE, mosquito thoraces were separated from the body in *Aedes* saline[59] on a microscope slide, and the number of worms present was counted on a stage microscope as described for mf uptake verification. At 11 to 13 DPE, the abdomen, thoraces and heads were all separated from individual mosquitoes and placed in *Aedes* saline. Each body part was then broken into large defined pieces and left for approximately two minutes. The number of L3 was counted under a dissection microscope. We considered mosquitoes which had fed on microfilaraemic blood but contained no worms as "Exposed" during analysis.

Energy content analysis. Nine days post-feeding, glycogen and lipid content were analysed. Glycogen and lipids were extracted using the standardised methods as described by Van Handel[60,61] and quantified using anthrone and vanillin reagent respectively. A 96 well plate held triplicate aliquots of 0.2 ml extract from each mosquito sample, and photospectrometry optical density (OD) readings at 625 nm quantified contents. Triplicate OD readings were carried out using a plate spectrophotometer and recorded on software (Gen5 Version 2.04) before averaging. Standard curves of lipid and glycogen content were created using olive oil suspended in chloroform

and anhydrous glucose suspended in de-ionized water, respectively. Both standard solutions included 100 mg of substrate dissolved in 100 ml of liquid. Using the standard curve equations, OD readings of glycogen and lipid content from the mosquito samples were converted to quantity readings (μg) prior to statistical analysis.

Statistical analysis. All statistical analysis was conducted in RStudio (version 1.1.1456)[62] using the lme4 (version 1.1–20) package[63] and visualised using ggplot2 (version 2.2.1)[64]. Statistical analysis of flight ability was conducted using GLMMs. Prior to analysis, Wald test assessments of the random variables (the flight mill number and replicate) found one of the flight mills to be faulty, so individuals flown on this flight mill ($n = 24$) were omitted from further analysis. Individuals which flew <50 m were also not included as it was assumed that attachment to the flight mill had compromised their flight. Linear regression analysis found that wing length did not correlate with any flight parameters, and so it was removed as a random variable in models. We assessed the distribution of each flight variable by plotting a histogram of individual mosquito flights and modelled them accordingly using GLMMs. Likelihood ratio testing (LRT) between individual GLMMs tested for an effect of either infection status or DPE on each flight variable. A least square means approach for multiple comparisons with a Tukey adjustment ($\alpha = 0.05$) tested for differences between infection status categories. All best fit models were assessed using residual-fitted plots and/or Normal Q-Q plots to ensure reliability. LRT between Generalised Linear Models (GLMs) tested for associations between energy reserves and flight and infection status.

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References

1. Bockarie, M. J., Pedersen, E. M., White, G. B. & Michael, E. Role of vector control in the Global Program to Eliminate Lymphatic Filariasis. *Annu. Rev. Entomol.* **54**, 469–487 (2008).
2. World Health Organisation. *Lymphatic filariasis*, http://www.who.int/news-room/fact-sheets/detail/lymphatic-filariasis (2018).
3. Uniting to Combat NTDs. *Lymphatic filariasis*, http://unitingtocombatntds.org/ntds/lymphatic-filariasis/ (2018).
4. Ramaiah, K. D. & Ottesen, E. A. Progress and impact of 13 years of the Global Programme to Eliminate Lymphatic Filariasis on reducing the burden of filarial disease. *PLoS Negl. Trop. Dis.* **8**, e3319, https://doi.org/10.1016/S1473-3099(16)30544-8 (2014).
5. Krishna, A. K., Harichandrakumar, K. T., Das, L. K. & Krishnamoorthy, K. Physical and psychosocial burden due to lymphatic filariasis as perceived by patients and medical experts. *Trop. Med. Int. Health.* **10**, 567–573 (2005).
6. Weiss, M. G. Stigma and the social burden of Neglected Tropical Diseases. *PLoS Negl. Trop. Dis.* **2**, e237, https://doi.org/10.1371/journal.pntd.0000237 (2008).
7. Person, B., Bartholomew, L. K., Gyapong, M., Addiss, D. G. & Van Den Borne, B. Health-related stigma among women with lymphatic filariasis from the Dominican Republic and Ghana. *Soc. Sci. Med.* **68**, 30–38 (2009).
8. World Health Organisation. *Lymphatic filariasis: Epidemiology*, https://www.who.int/lymphaticfilariasis/epidemiology/en/ (2019).
9. Beckett, E. B. Histological changes in mosquito flight muscle fibres associated with parasitization by filarial larvae. *Parasitology.* **63**, 365–372 (1971).
10. Kan, S. P. & Ho, B. C. Development of *Brugia pahangi* in the flight muscles of *Aedes togoi*. Ultrastructural changes in the infected muscles fibers and the infecting filarial larvae. *Am. J. Trop. Med. Hyg.* **22**, 179–88 (1973).
11. Lehane, M. J. & Laurence, B. R. Flight muscle ultrastructure of susceptible and refractory mosquitoes parasitized by *Brugia pahangi*. *Parasitology.* **74**, 87–92 (1977).
12. Beckett, E. B. Species variation in mosquito flight-muscle damage resulting from a single filarial infection and its repercussions on second infection. *Parasitol. Res.* **76**, 606–609 (1990).
13. Li, J., Bao, H. & Wang, J. Histochemical changes in *Brugia malayi* microfilariae-infected *Anopheles sinensis*. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi.* **13**, 123–125 (1995).
14. Berry, W. J., Rowley, W. A. & Christensen, B. M. Influence of developing *Brugia pahangi* on spontaneous flight activity of *Aedes aegypti* (Diptera: Culicidae). *J. Med. Entomol.* **23**, 441–445 (1986).
15. Husain, A. & Kershaw, W. E. The effect of the development of filariae on the ability of mosquitoes to fly and feed. *Trans. R. Soc. Trop. Med. Hyg.* **60**, 18 (1966).
16. Townson, H. The effect of infection with *Brugia pahangi* on the flight of *Aedes aegypti*. *Ann. Trop. Med. Parasitol.* **64**, 411–420 (1970).
17. Husain, A. & Kershaw, W. E. The effect of filariasis on the ability of a vector mosquito to fly and feed and to transmit the infection. *Trans. R. Soc. Trop. Med. Hyg.* **65**, 617–619 (1971).
18. Hockmeyer, W. T., Schiefer, B. A., Redington, B. C. & Elridge, B. F. *Brugia pahangi:* effects upon the flight capability of *Aedes aegypti*. *Exp. Parasitol.* **38**, 1–5 (1975).
19. Nayar, J. K. & Van Handel, E. The fuel for sustained mosquito flight. *J. Insect Physiol.* **17**, 471–481 (1971).
20. Kaufmann, C. & Briegel, H. Flight performance of the malaria vector *Anopheles gambiae* and *Anopheles atroparvus*. *J. Vector Ecol.* **29**, 140–153 (2004).
21. Cheon, H., Shin, S. W., Bian, G., Park, J. & Raikel, A. S. Regulation of lipid metabolism genes, lipid carrier protein lipophorin, and its receptor during immune challenge in the mosquito *Aedes aegypti*. *J. Parasitol.* **281**, 8426–8435 (2006).
22. Barletta, A. B. F. *et al.* Emerging role of lipid droplets in *Aedes aegypti* immune response against bacteria and dengue virus. *Sci. Rep.* **6**, 19928, https://doi.org/10.1038/srep19928 (2016).
23. Rossignol, P. A., Ribeiro, J. M. & Spielman, A. Increased intradermal probing time in sporozoite-infected mosquitoes. *Am. J. Trop. Med. Hyg.* **33**, 17–20 (1984).
24. Ponnudurai, T., Lensen, A. H., Van Gemert, G. J., Bolmer, M. G. & Meuwissen, J. H. Feeding behaviour and sporozoite ejection by infected *Anopheles stephensi*. *Trans. R. Soc. Trop. Med. Hyg.* **85**, 175–180 (1991).
25. Wekesa, J. W., Copeland, R. S. & Mwangi, R. W. Effect of *Plasmodium falciparum* on blood feeding behaviour of naturally infected *Anopheles* mosquitoes in Western Kenya. *Am. J. Trop. Med. Hyg.* **47**, 484–488 (1992).
26. Koella, J. C., Sørensen, F. L. & Anderson, R. A. The malaria parasite, *Plasmodium falciparum*, increases the frequency of multiple feeding of its mosquito vector, *Anopheles gambiae*. *Proc. R. Soc. Lond. B Biol. Sci.* **265**, 763–768 (1998).
27. Anderson, R. A., Koella, J. C. & Hurd, H. The effect of *Plasmodium yoelii nigeriensis* infection on the feeding persistence *of Anopheles stephensi* Liston throughout the sporogonic cycle. *Proc. R. Soc. Lond. B Biol. Sci.* **266**, 1729–1733 (1999).
28. Koella, J. C., Rieu, L. & Paul, R. E. L. Stage-specific manipulation of a mosquito's host-seeking behavior by the malaria parasite *Plasmodium gallinaceum*. *Behav. Evol.* **13**, 816–820 (2002).
29. Gleave, K., Cook, D., Taylor, M. J. & Reimer, L. J. Filarial infection influences mosquito behaviour and fecundity. *Sci. Rep.* **6**, 36319, https://doi.org/10.1038/srep36319 (2016).
30. Killeen, G. F. *et al.* Measuring, manipulating and exploiting behaviours of adult mosquitoes to optimise malaria vector control impact. *BMJ Glob. Health.* **2**, e000212, https://doi.org/10.1136/bmjgh-2016-000212 (2017).

31. Cator, L. J., Lynch, P. A., Thomas, M. B. & Read, A. F. Alterations in mosquito behaviour by malaria parasites: potential impact on force of infection. *Malaria J.* **13**, 164, https://doi.org/10.1186/1475-2875-13-164 (2014).
32. Irvine, M. A. *et al*. Modelling strategies to break transmission of lymphatic filariasis – aggregation, adherence and vector competence greatly alter elimination. *Parasit. Vectors.* **8**, 547, https://doi.org/10.1186/s13071-015-1152-3 (2015).
33. Ferguson, H. M. & Read, A. F. Why is the effect of malaria parasites on mosquito survival still unresolved? *Trends Parasitol.* **18**, 256–261 (2002).
34. Freitak, D., Ots, I., Vanatoa, A. & Hõrak, P. Immune response is energetically costly in white cabbage butterfly pupae. *Proc. R. Soc. Lond. B Biol. Sci.* **270**(Suppl. 2), 220–22 (2003).
35. Ardia, D. R., Gantz, J. E., Schneider, B. C. & Strebel, S. Costs of immunity in insects: an induced immune response increases metabolic rate and decreases antimicrobial activity. *Funct. Ecol.* **26**, 732–739 (2012).
36. Chambers, M. C., Song, K. H. & Schneider, D. S. *Listeria monocytogenes* infection causes metabolic shifts in *Drosophila melanogaster*. *PLoS One.* **7**, e50679, https://doi.org/10.1371/journal.pone.0050679 (2012).
37. Lee, K. & Lee, W. Immune-metabolic interactions during systemic and enteric infection in *Drosophila*. *Curr. Opin. Insect Sci.* **29**, 21–26 (2018).
38. Rivero, A., Agnew, P., Bedhomme, S., Sidobre, C. & Michalakis, Y. Resource depletion in *Aedes aegypti* mosquitoes infected by the microsporidia *Vavraia culicis*. *Parasitology.* **134**, 1355–1362 (2007).
39. Van der Horst, D. J. & Ryan, R. O. Comprehensive molecular insect science (4th ed.), Lipid transport, 225–246. Amsterdam: Elsevier, (2005).
40. Arrese, E. L. & Soulages, J. L. Insect fat body: energy, metabolism, and regulation. *Annu. Rev. Entomol.* **55**, 207–225 (2010).
41. Nayar, J. K. & Sauerman, D. M. Jr. A comparative study of flight performance and fuel utilization as a function of age in females of Florida mosquitoes. *J. Insect Physiol.* **19**, 1977–1988 (1973).
42. Even, N., Devaud, J. M. & Barron, A. B. General stress responses in the honey bee. *Insects.* **3**, 1271–1298 (2012).
43. Adamo, S. A. The effects of stress hormones on immune function may be vital for the adaptive reconfiguration of the immune system during fight-or-flight behavior. *Integr. Comp. Biol.* **54**, 419–426 (2014).
44. Woestmann, K., Kvist, J. & Saastamoinen, M. Fight or flight? – flight increases immune gene expression but does not help to fight an infection. *J. Evol. Biol.* **30**, 501–511 (2017).
45. Beenakkers, A. M. T. Influence of flight in lipid metabolism in *Locusta migratoria*. *Insect Biochem.* **3**, 303–308 (1973).
46. Weeda, E., de Kort, C. A. D. & Beenakkers, A. M. Fuels for energy metabolism in the Colorado potato beetle, *Leptinotarsa decemlineata* Say. *J. Insect Physiol.* **25**, 951–955 (1979).
47. Weers, P. M., Van Baal, J., Van Doorn, J. M., Ziegler, R. & Van der Horst, D. J. Biosynthetic route of locust apolipophorin III isoforms. *Biol. Chem.* **374**, 863–869 (1993).
48. Canavoso, L. E., Stariolo, R. & Rubiolo, E. R. Flight metabolism in *Panstrongylus megistus* (Hemiptera: Reduviidae): the role of carbohydrates and lipids. *Mem. Inst. Oswaldo Cruz.* **98**, 909–914 (2003).
49. Arrese, E. L., Gazard, J. L., Flowers, M. T., Soulages, J. L. & Wells, M. A. Diacylglycerol transport in the insect fat body: evidence of involvement of lipid droplets and the cytosolic fraction. *J. Lipid Res.* **42**, 225–234 (2001).
50. Knight, J. A., Anderson, S. & Rawle, J. M. Chemical basis of the sulfo-phospho-vanillin reaction for estimating total serum lipids. *Clin. Chem.* **18**, 199–202 (1972).
51. Schiefer, B. A., Ward, R. A. & Eldridge, B. F. *Plasmodium cynomolgi*: effects of malaria infection on laboratory flight performance on *Anopheles stephensi* mosquitoes. *Exp. Parasitol.* **41**, 397–404 (1977).
52. Rowland, M. W. & Boersma, E. Changes in the spontaneous flight activity of the mosquito *Anopheles stephensi* by parasitization with the rodent malaria *Plasmodium yoelii*. *Parasitology.* **97**, 221–227 (1988).
53. Nyasembe, V. O. *et al*. *Plasmodium falciparum*-infection increases *Anopheles gambiae* attraction to nectar sources and sugar uptake. *Curr. Biol.* **24**, 217–221 (2014).
54. Rowley, W. A. & Graham, C. L. Effect of age on flight performance of female *Aedes aegypti* mosquitoes. *J. Insect Physiol.* **14**, 719–728 (1968).
55. Ramaiah, K. D., Das, P. K., Vanamail, P. & Pani, S. P. The impact of six round of single-dose mass administration of diethylcarbamazine or ivermectin on the transmission of *Wuchereria bancrofti* by *Culex quinquefasciatus* and its implication for lymphatic filariasis elimination programmes. *Trop. Med. Int. Health.* **8**, 1082–1092 (2003).
56. Aboagye-Antwi, F. *et al*. Transmission indices and microfilariae prevalence in human population prior to mass drug administration with ivermectin and albendazole in the Gomoa District of Ghana. *Parasit. Vectors.* **8**, 562, https://doi.org/10.1186/s13071-015-1105-x (2015).
57. Irvine, M. A., Kazura, J. W., Hollingsworth, T. D. & Reimer, L. J. Understanding heterogeneities in mosquito bite exposure and infection distributions for the elimination of lymphatic filariasis. *Proc. R. Soc. B Biol. Sci.* **285**, 20172253, https://doi.org/10.1098/rspb.2017.2253 (2018).
58. World Health Organisation. *Lymphatic filariasis: Practical Entomology. A Handbook for National Elimination Programmes*, https://apps.who.int/iris/bitstream/handle/10665/87989/9789241505642_eng.pdf;jsessionid=0C945B00C7B6A8FCE85502D6F6F7B4DD?sequence=1 (2013).
59. Hayes, R. O. Determination of a physiological saline solution for *Aedes aegypti* (L.). *J. Econ. Entomol.* **46**, 624–627 (1953).
60. Van Handel, E. Rapid determination of glycogen and sugars in mosquitoes. *J. Am. Mosq. Control Assoc.* **1**, 299–301 (1985).
61. Van Handel, E. Rapid determination of total lipids in mosquitoes. *J. Am. Mosq. Control Assoc.* **1**, 302–304 (1985).
62. RStudio Team. *RStudio: Integrated Development for R*. RStudio, Inc., Boston, MA, http://www.rstufio.com/ (2015).
63. Bates, D., Maechler, M., Bolker, B. & Walker, S. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **67**, 1–48 (2015).
64. Wickham, H. ggplot2: Elegant graphics for data analysis. Spring-Verlag, New York (2016).
65. Kees, A. M., Hefty, A. R., Venette, R. C., Seybold, S. J. & Aukema, B. H. Flight capacity of the walnut twig beetle (Coleoptera: Scolytidae) in a laboratory flight mill. *Environ. Entomol.* **46**, 633–641, https://doi.org/10.1093/ee/nvx055 (2017).

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Author contributions

L.R. and A.S. conceived and designed the study, A.S. and K.G. collected the data, C.J. contributed analysis tools, A.S. analysed the data and wrote the first draft of the paper. All authors contributed to the final draft of the paper.

Competing interests

The authors declare no competing interests.

Additional information

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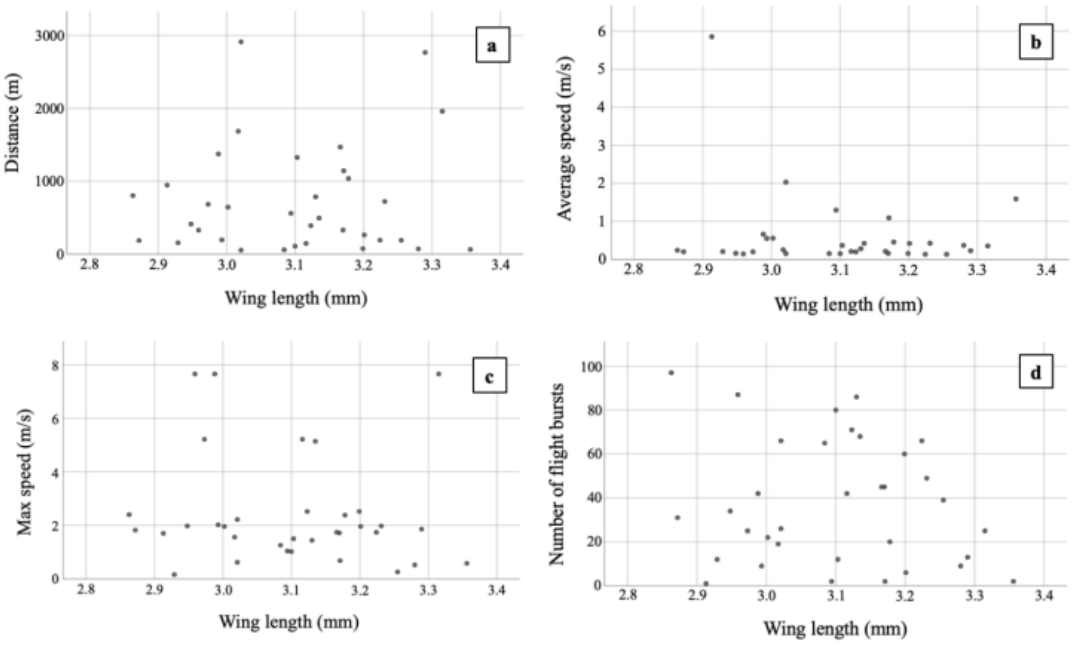
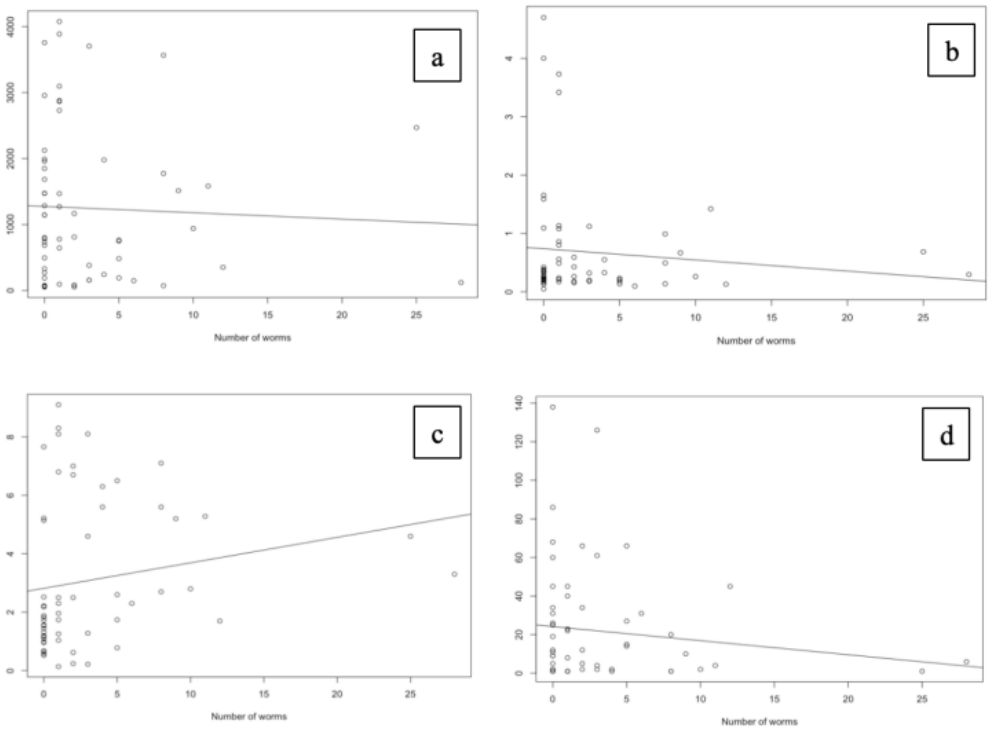
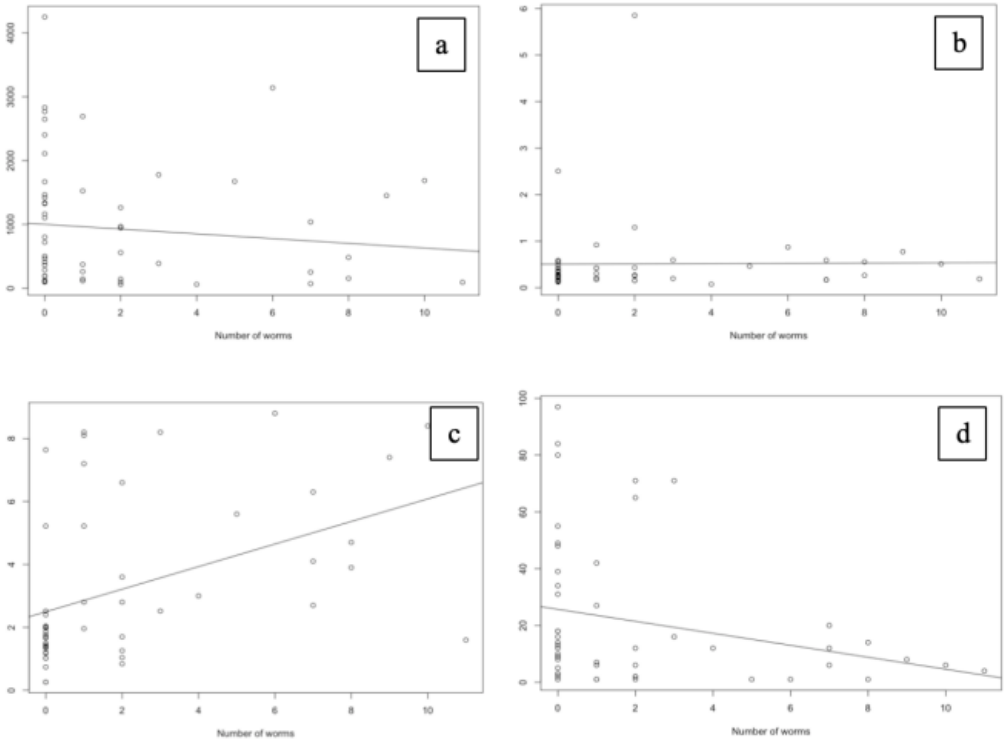


Supplementary figure 1. Scatter-plots of flight parameters against wing length. a) Distance, b) Average speed, c) Max speed, d) Number of flight bursts.



Supplementary figure 2. Linear regression plots of measured flight parameters against worm burden from 4 to 6 DPE. a) Distance, b) Average speed, c) Maximum speed, d) Number of flight bursts. For no variable was the linear regression result significant.



Supplementary figure 3. Linear regression plots of measured flight parameters against worm burden from 11 to 13 DPE. a) Distance, b) Average speed, c) Maximum speed, d) Number of flight bursts. Worm burden was found to be a significant predictor of maximum speed only (P=0.001).