Effects of pyrethroid exposure and insecticide resistance on the sporogonic development of *Plasmodium falciparum* in *Anopheles gambiae* s.l.

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Abstract

Pyrethroid resistance has spread in the *Anopheles gambiae* s.l. populations in most African countries, often at high frequency. As pyrethroids are still used in all insecticide treated nets, this poses a potentially major threat to the effectiveness of vector control strategies. However, even though insecticide resistance is widespread, malaria control has not yet failed outright, but detecting the effects of resistance on control measures and measuring how much the effectiveness of control has changed is problematic. A few laboratory-based studies carried out over two decades between the 1980s and early 2000s tried to test whether insecticide exposure affects parasite development, with little follow up. The aim of this project was therefore to investigate the possibility that pyrethroid exposure of *An. gambiae* s.l. might impair the sporogonic development of *Plasmodium falciparum* in field conditions, and to explore if insecticide resistance further affects sporogony.

The effects of sub-lethal doses of deltamethrin on sporogony in wild pyrethroid resistant *An. gambiae* s.s. in Uganda were studied, showing that exposure of *kdr* resistant mosquitoes to sub-lethal doses of pyrethroids significantly reduces both parasite prevalence and intensity of infection. Mean ambient temperature during the incubation period, and temperature range during the first 24 hours and on day 4 post-infectious feed also had a highly significant effect on risk of infection, where increases in mean temperature and temperature range were associated with lower infection.

Furthermore, deltamethrin significantly impaired survival of *kdr* homozygous mosquitoes, while mean temperature and relative humidity also had a significant effect on mosquito mortality.

Deltamethrin exposure significantly impaired both ookinete conversion and motility of *P. berghei* at doses that malaria parasites are likely to encounter when mosquitoes are exposed to insecticides in field conditions, while high performance liquid chromatography-photodiode array assay (HPLC-PDA) analysis showed that each mosquito picks up to approximately 10ng of deltamethrin following exposure to a long-lasting insecticidal net (LLIN) (PermaNet 2.0).

Potential interference of *kdr* resistance with the development of *P. falciparum* within the vector was also investigated. The effects of *kdr* genotype on *Plasmodium* infection rates in *An. gambiae* s.s. and *An. arabiensis* were explored in mid-western Uganda, together with

variations in phenotypic and genetic resistance against commonly used insecticides. Bioassay mortality was only weakly associated with *kdr* genotype in both sibling species, implying that other metabolic resistance mechanisms play a significant role in the study area. Oocyst prevalence rates and infection intensity were not significantly different between *kdr* genotypes, nor did they vary between the two species, while sporozoite rates in *An. gambiae* s.s. were not significantly different between *kdr* genotypes.

These results imply that even if resistant mosquitoes survive insecticide exposure, their vector competence is impaired as parasite development is affected, suggesting that pyrethroid-based interventions could still have a role in malaria control at least until alternative insecticides are available.

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List of abbreviations

AChE: acetylcholinesterase

C: vectorial capacity

CH_3CN: acetonitrile

CNS: central nervous system

CSP: circumsporozoite protein

CYP: cytochrome P450 monooxygenases

DDT: dichlorodiphenyltrichloroethane

DMSO: dimethyl sulfoxide

EIR: entomological inoculation rate

EST: carboxylesterases

EtOH: ethanol

GC: gas chromatography

GPIRM: Global Plan for Insecticide Resistance Management

GST: glutathione S-transferases

H_2O_2: hydrogen peroxide

HPLC: high performance liquid chromatography

HPLC-PDA: high performance liquid chromatography-photodiode array assay

IRS: indoor residual spraying

ITN: insecticide treated nets

Kdr: knockdown resistance

LC: lethal concentration

LLIN: long-lasting insecticidal nets

PABA: 4-aminobenzoic acid or para-aminobenzoic acid

PAMP: pathogen associated molecular pattern

PBO: piperonyl butoxide

PBS: phosphate-buffered saline

PFA: paraformaldehyde

PRR: pattern recognition receptors

R_0: basic reproduction number

RH: relative humidity

RNS: reactive nitrogen species

ROS: reactive oxygen species

RPMI: Roswell Park Memorial Institute medium

SNP: single nucleotide polymorphism

SSA: Sub-Saharan Africa

T: temperature

TBI: transmission blocking intervention

TEP-1: thioester-containing protein 1

VGCC: voltage-gated calcium channel

VGSC: voltage-gated sodium channel

WHO: World Health Organization

Chapter 1. Introduction

1.1. Background

Malaria is a vector-borne disease caused by six species of protozoan parasites of the genus *Plasmodium* (Apicomplexa: Haemosporidae), transmitted by mosquitoes of the genus *Anopheles.* Ninety-one countries still had indigenous malaria cases in 2016, with the greatest burden in the African region, and according to estimates 216 million cases of malaria occurred globally, leading to 445,000 deaths [1].

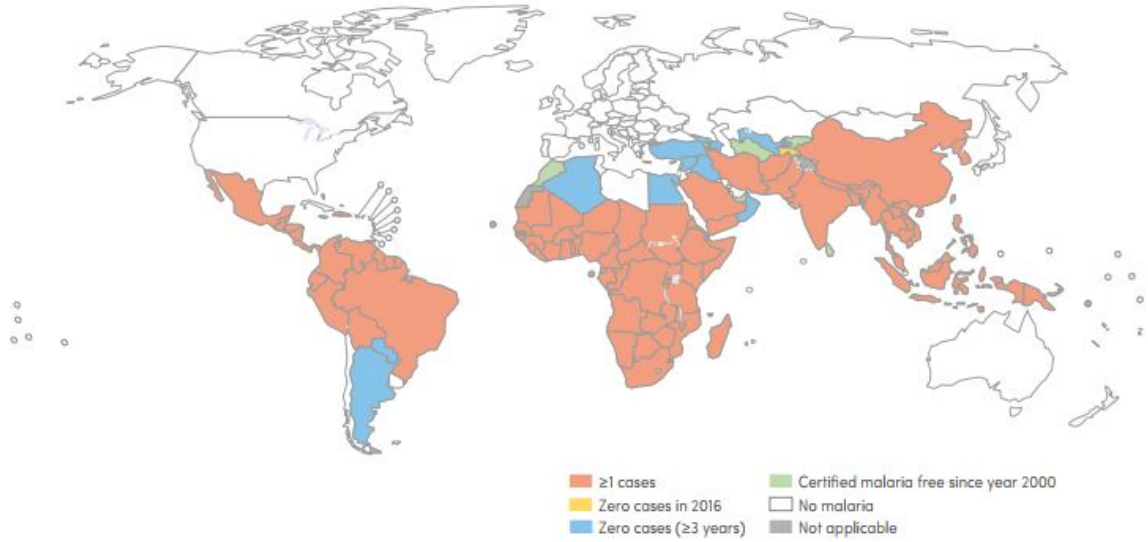

Fig. 1-1. Countries and territories with indigenous malaria cases in 2000 and in 2016. Countries in green have been certified malaria-free since 2000. Countries in blue have had zero indigenous cases over at least the past 3 consecutive years and are eligible to request certification of malaria free status from WHO. Reproduced from *World malaria report 2017* [1]
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Great progress in disease control has been made in recent years following large investments of funds, as an increasing proportion of the population - especially in sub-Saharan Africa - is protected by insecticide treated nets (ITNs) and indoor residual spraying (IRS) [2]. Concerns

about the development of insecticide resistance arose in the early 1950s, following the large IRS campaigns during the malaria eradication era [3-5]. Insecticide resistance has now been reported against all four insecticide classes used for public health purposes and is widespread around the world, especially in sub-Saharan Africa, posing a potentially serious threat to the control efforts [1]. Resistance to pyrethroids is especially worrisome as they are currently still used on all ITNs.

Control and prevention efforts have mainly targeted the vectors, or parasites in humans. However, it has been recognized that interrupting malaria transmission is of paramount importance if we are to successfully control and ultimately eradicate the disease [6]. Transmission-blocking interventions (TBIs) aim to stop the transmission by interrupting the sporogonic cycle or by making mosquitoes unable to transmit the parasites. There are indications that compounds which are not traditionally considered as TBIs – such as insecticides or endectocides - may have an additional effect on *Plasmodium* during the parasite's sporogonic development inside mosquitoes [7, 8]. Furthermore, the effects of different insecticide resistance mechanisms on mosquitoes and malaria transmission are still not completely understood, yet insecticide resistance mechanisms themselves have been shown to affect vectors [9-11], and parasites developing in them [12, 13]. Although insecticide resistance is now widespread, its entomological and especially epidemiological impact is not known [14, 15] and there is still no evidence that there is a definite association between pyrethroid resistance, LLIN effectiveness and malaria disease burden [16].

1.2. Overview of *Plasmodium* life cycle

Plasmodium parasites need two different hosts to complete their multi-stage life cycle - vertebrates and mosquito vectors (Fig. 1-2).

1.2.1. Human host stage

Malaria parasites were discovered by Laveran in 1880 and were the first protozoan parasites to be found inside human blood cells (erythrocytes) [17]. Upon infection, sporozoites infect hepatocytes during the exo-erythrocytic cycle, resulting in the release of merozoites into the blood stream, which then invade erythrocytes. During the erythrocytic cycle, each parasite develops through different stages, repeatedly producing merozoites, which re-invade new erythrocytes.

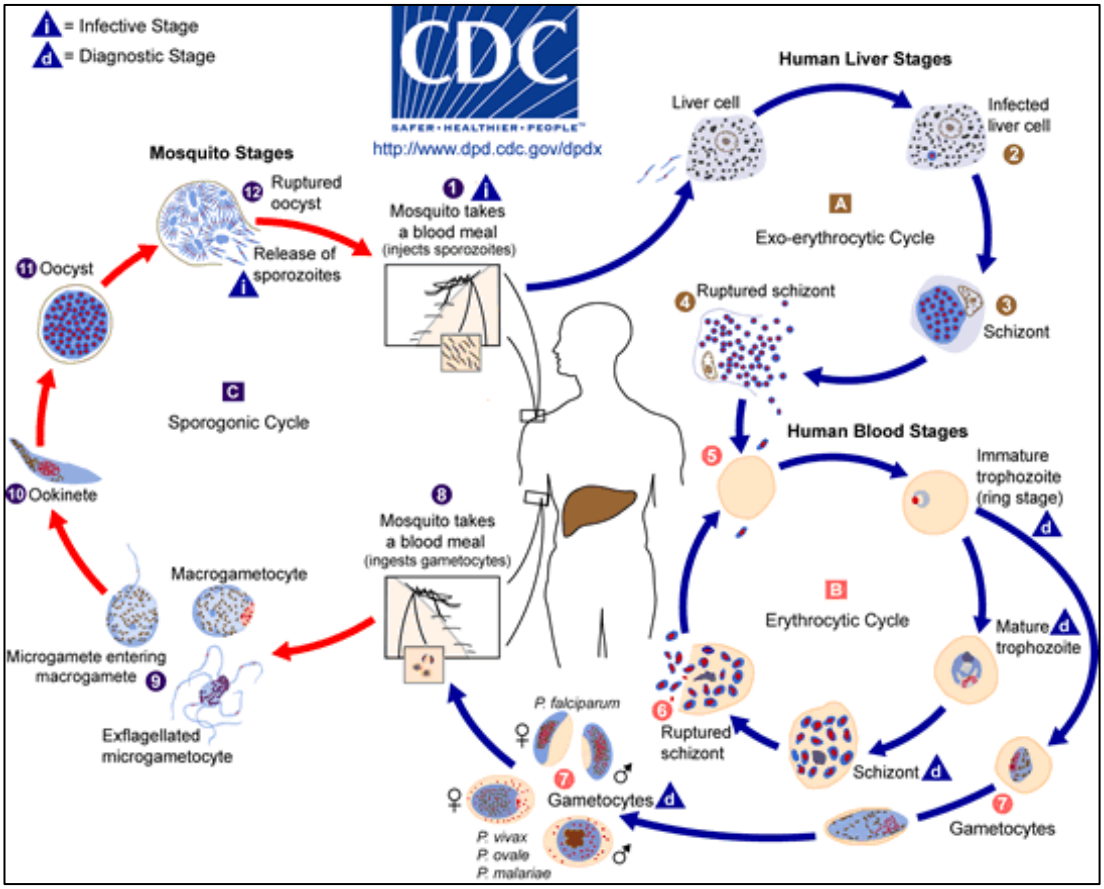

Fig. 1-2. *Plasmodium* parasites require a vertebrate host and a mosquito host to complete their life cycle. Whereas asexual multiplication takes place in vertebrates, the sexual phase (sporogony) is completed in mosquitoes. Reproduced from the CDC website on malaria lifecycle (https://www.cdc.gov/malaria/about/biology/index.html) [18].

In erythrocytes, a small fraction of parasites develop into sexual stages - gametocytes - which infect mosquitoes and are necessary for malaria transmission.

1.2.2. Sporogonic cycle

In 1897, Ronald Ross was the first to demonstrate that malaria parasites develop in *Anopheles* mosquitoes following ingestion of gametocytes from infected patients [19], and in 1898 he demonstrated that mosquitoes were intermediate hosts for *Plasmodium* parasites, and necessary for malaria transmission [20].

The sexual phase of the parasite's life cycle - sporogony - takes place in *Anopheles* mosquitoes (Figures 1-2 and 1-3), generally lasting 8 to >20 days in the tropics. The length

differs between *Plasmodium* species and is dependent upon external factors such as temperature and humidity.

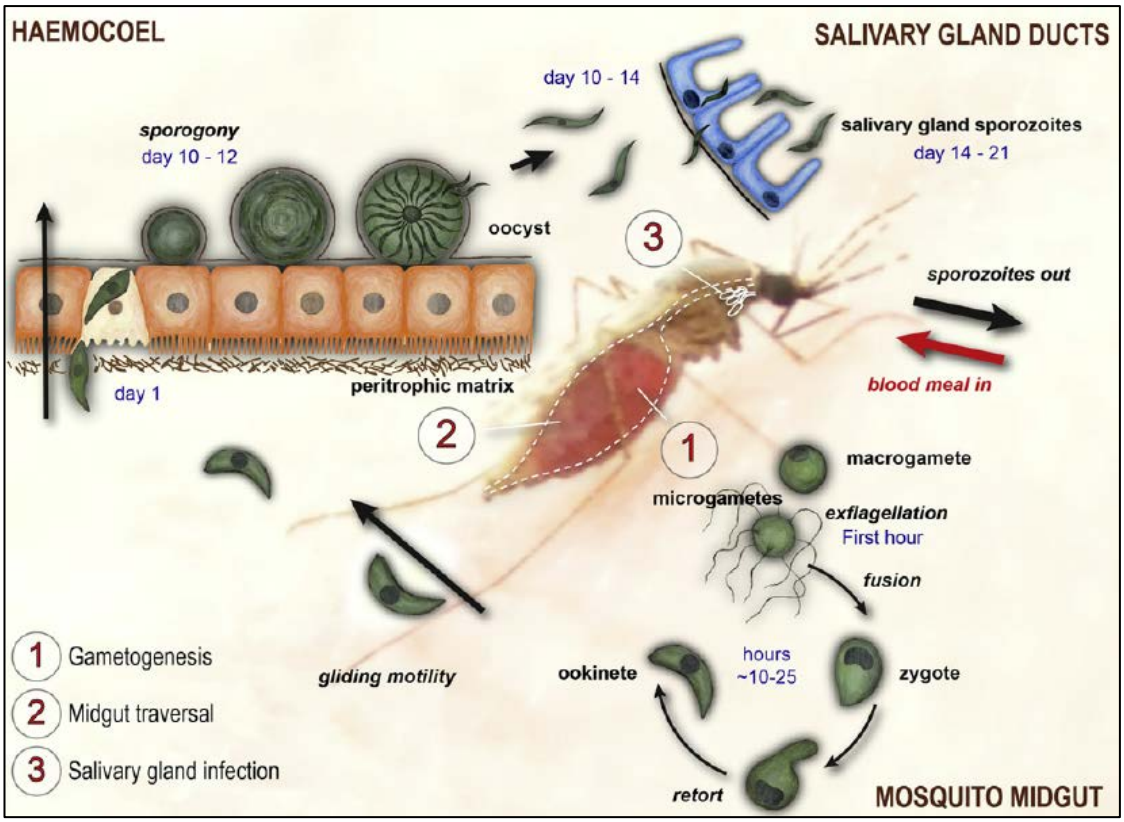

Fig. 1-3. *Plasmodium* sporogonic development in the mosquito starts when a female mosquito feeds on an infected vertebrate host. Three key stages taking place during sporogony are (1) gametogenesis, (2) ookinete midgut traversal followed by the establishment of oocysts, and (3) sporozoite salivary gland infection. Each of the stages is a bottleneck, with parasite numbers only increasing once sporozoites are produced in the oocysts. Reprinted from Angrisano *et al,* 2012 [21], with permission from Elsevier.

"Early sporogony" is relatively brief, lasting 1-2 days, and occurs during blood meal digestion. Gametogenesis is triggered by external factors including a drop in temperature, pH changes, and the presence of xanthurenic acid (XA), an exflagellation trigger [22, 23]. Following gametogenesis, about 24 hours post-infective blood meal, a zygote transforms into a motile, banana-shaped ookinete [24]. These forms actively migrate from the midgut lumen, pass through the midgut epithelium to the basal side, causing significant morphological changes and tissue damage of the midgut wall [25, 26], which triggers regenerative cell division and differentiation of midgut epithelium [27]. Surviving ookinetes transform into oocysts just beneath the midgut basal lamina approximately 24-48 hours post-blood meal.

"Mid sporogony", a process lasting 7 days or more, is a period of oocyst growth and multiplication, when numerous sporozoites are formed inside oocysts [28, 29].

"Late sporogony" begins with oocysts bursting, releasing sporozoites into the hemocoel, and ends with their invasion of the salivary glands, usually within 10-16 days after the mosquito took the infective blood meal [30]. Each oocyst can produce thousands of sporozoites [28, 29]. When midgut infections are light, as in the majority of cases, all oocysts appear to develop at the same rate, whereas in heavy infections the rate of development in different oocysts can vary significantly [31].

1.3. Parasite distribution

Plasmodium distribution, from gametocytes in humans and throughout sporogony in mosquitoes, is very heterogenous, over-dispersed, and highly variable at every level [30, 32].

The density of mature gametocytes in peripheral blood, from where they can infect mosquitoes, tends to be very low [33] and only a fraction of ingested female gametocytes will form oocysts [30]. Gametocytes mostly circulate at sub-microscopic levels (i.e. < 5,000 gametocytes/ml) and although often undetected by microscopy, they might be present in the majority of infections [34].

The relationship between gametocyte density and proportion of infected mosquitoes shows a high level of variability and remains largely unclear [35-37]. Mosquito infections resulting from sub-microscopic gametocytaemias have been recorded, whereas high gametocyte densities do not necessarily result in mosquito infections [28, 34, 38]. Moreover, the parasites are not randomly and evenly distributed among the mosquitoes, with majority of mosquitoes containing no or few oocysts. This type of aggregation fits the negative binomial (over-dispersed) distribution and can occur due to (a) heterogeneity in the density of infective gametocytes ingested by mosquitoes or (b) heterogeneity in individual mosquito susceptibility to the development of ingested gametocytes [30, 39, 40].

The relationship between gametocyte density and oocyst density is also highly variable, yet it does show a strong positive correlation [35, 41]. *Plasmodium falciparum* oocyst densities in naturally infected mosquitoes vary greatly but are typically low, with often fewer than five oocysts per mosquito [28].

The majority of oocyst-positive mosquitoes eventually become sporozoite-positive [42]. A mosquito is usually classified as “infectious” if sporozoites are detected in its salivary glands, regardless of their number. Data on whether there is any correlation between oocyst density, gland infection, and between gland load and inoculum size, are conflicting and poorly understood [43]. Rosenberg *et al* found a correlation and recorded a median *P. falciparum* oocyst density in *An. stephensi* of nine, while a median gland infection was < 9,000 sporozoites, and a median of 15 sporozoites were ejected [44]. Ponnudurai *et al* however found no correlation and observed an even lower median number of ejected sporozoites [45]. However, a recent study shows that not all mosquitoes are equally infectious as those with lower sporozoite burdens have a lower chance of successfully spreading the infection [43].

Malaria parasites undergo significant losses during sporogonic development, with reductions occurring at each developmental step. The two motile stages – ookinetes and sporozoites – play a pivotal role in sporogonic development, migrating within mosquitoes to allow further development and finally transmission to a new host, and are also the main bottleneck stages in the life cycle, as fewer than 20 % of sporozoites released from oocysts will ever reach the salivary glands [28, 46-49].

1.4. Malaria transmission

A range of vertebrate host, mosquito and parasite factors influence malaria transmission - from the likelihood that a mosquito will become infected and survive long enough for the parasites to complete sporogony, to the likelihood that the mosquito will transmit the sporozoites to a new host [50].

1.4.1. Measuring intensity of transmission

To measure the intensity of transmission, five inter-related measures of intensity of transmission are commonly used [51]:

- Incidence rate (number of new infections in a given population unit)
- Prevalence rate (fraction of a population infected at a given point in time)
- Entomological inoculation rate (*EIR*)
- Vectorial capacity (*C*)

- Basic reproduction number (also called the basic reproduction rate) (R_0).

The entomological inoculation rate, ***EIR***, gives the number of infective mosquito bites received per person per time unit. It is often used in field studies and can be defined mathematically as:

$$EIR = mas$$

where *m* = density of vectors relative to humans, *a* = number of blood meals taken on humans per vector per day (and together *ma* = human biting rate), *s* = sporozoite rate. *EIR* is considered a standard metric of malaria transmission and can be used to evaluate the impact of interventions but does have a number of limitations [52-54].

The vectorial capacity, ***C***, estimates the daily rate of potential transmission, i.e. the capacity of a local vector population to transmit malaria in terms of the potential number of secondary inoculations originating per day from an infective person. The formula for C was given by Garrett-Jones in 1964 [55] and was based on Macdonald's mathematical terminology and expression for the basic reproduction number of malaria [56]:

$$C = \frac{\mathrm{m}\, a^2 p^n}{-log_e p}$$

where *m* = density of vectors in relation to humans, *a* = number of blood meals taken on humans per vector per day or man-biting habit (and *ma* = human biting rate), *p* = daily survival probability (i.e. proportion of vectors surviving per day), and *n* = incubation period in the vector (i.e. length of the sporogonic cycle in days). Alternatively, vectorial capacity can include a term for vector competence, usually denoted as *V* or *b*, in the numerator [57].

The basic reproduction number, $\boldsymbol{R_0}$, is the potential total number of secondary cases originating from one primary case, assuming that a population is, and remains, fully susceptible. R_0 can be used as a threshold criterion for transmission: if $R_0 < 1$, the disease will eventually become extinct, and if $R_0 > 1$ it will spread. The R_0 for malaria is composed of two parts, to account for the transmission of parasites from mosquito to human, and then from human to mosquito.

Macdonald's expression for R_0 was published in 1957 [56]:

$$R_0 = \frac{m\, a^2 b\, p^n c}{r\, (-log_e p)}$$

where *m* = density of vectors in relation to humans, *a* = number of blood meals taken on humans per vector per day or man-biting habit (and *ma* = human biting rate), *p* = daily survival probability (i.e. proportion of vectors surviving per day), *n* = incubation period in the vector (i.e. length of the sporogonic cycle in days), *b* = the proportion of gland-positive mosquitoes that is actually infective (i.e. transmission probability from an infective mosquito to human, also called vector competence), *c* = transmission probability from an infectious human to mosquitoes, and *r* = recovery rate of man from infection.

While vectorial capacity describes the potential capacity of mosquitoes to transmit malaria from one vertebrate host to another, some of its components could potentially be determined, either directly or indirectly, by parasites or could be affected by different insecticide resistance mechanisms [58, 59].

1.4.2. Intensity and prevalence of infection in mosquitoes

Complete understanding of malaria epidemiology depends on determination of the prevalence and intensity of infection in mosquitoes. This is especially important when assessing effectiveness of control programmes and various transmission blocking interventions (TBIs). The relationship between prevalence and intensity can be described by the negative binomial distribution, with a high degree of heterogeneity due to parasite-related and mosquito-related factors [32].

When studying transmission of malaria parasites from humans to vectors, the following indices are used for reporting infection success:

- Oocyst prevalence or infection prevalence: the proportion of infected mosquitoes with oocysts
- Sporozoite rate: the proportion of infected mosquitoes with sporozoites
- Oocyst density or burden or intensity of infection: mean number of oocysts per midgut

As mosquito infectiousness was found to vary with the size of sporozoite load in salivary glands and the size of inoculum [43], mean numbers of sporozoites per salivary gland might be assessed more frequently in the future.

1.4.3. Concept of transmission blocking

The concept of transmission blocking focuses on interventions administered to infected (or potentially infected) people, in order to reduce the ability of those people to transmit parasites onward to other people, usually by means of factors in the blood that are transferred to the vector with the bloodmeal and disrupt the development of the parasite within the vector, with the ultimate goal to reduce the prevalence of malaria in affected populations [60].

Traditionally, only gametocytocidal (e.g. primaquine) and sporontocidal antimalarial drugs (e.g. pyrimethamine and proguanil [61]) and vaccines which prevent parasite development in the mosquito have been described as TBIs [62], while other novel interventions include the use of transgenic mosquitoes and exploitation of mosquito refractoriness to malaria infection [62, 63]. However, there are other factors with transmission blocking effects. Transmission blocking antibodies, such as Pfs48/45 and Pfs25, present in humans during *Plasmodium* infections can affect gamete fertilization, viability of zygotes, and their transformation into ookinetes when ingested in the blood meal [35, 64]. The presence of insecticide resistance mechanisms can significantly change mosquito physiology and make it less suitable for parasite development [12, 58]. Studies have also shown that pyrethroids [7, 65-67] and other compounds such as the anthelmintic ivermectin [68], and some HIV treatments [69], may have additional negative effects on *Plasmodium* sporogony, thus in effect, work like TBIs.

The most important measure of TBI efficacy is a reduction in the proportion of infective mosquitoes and a reduction of the intensity of infection, which can be detected at either oocyst or sporozoite level [70, 71]. This is most commonly done at the oocyst stage due to the following advantages:

- Earlier time-point, as oocysts can be detected six to eight days post-infection (it takes 12-14 days for sporozoites)
- Higher likelihood of mosquito survival (>80 % at oocyst level, <50 % at sporozoite level in the laboratory conditions)
- Less hazardous (mosquitoes carrying human-parasite sporozoites present a hazard for laboratory personnel) [42]

“Transmission blockade” is traditionally defined as the mean percentage reduction in oocyst intensity and prevalence caused by a transmission blocking intervention, in comparison with

mean values from an appropriate control replicate. Percentage reduction in oocyst intensity/prevalence is expressed as:

$$Efficacy = \frac{C - E}{C} \times 100$$

where *C* = the mean prevalence/intensity in control group, *E* = the mean prevalence/intensity in the intervention group [70, 71].

1.5. Factors affecting transmission of malaria by mosquitoes

Mosquito vectors are the key determinant of malaria transmission as the definitive hosts for the parasites. It is now known that *Plasmodium–Anopheles* interactions are a major factor influencing mosquito vector competence [72], but a long-held view was that they are mere "flying syringes", passive carriers of parasites, despite Huff proposing that the immune responses of some mosquitoes may affect parasite infectivity as early as 1927 [73].

Different abiotic (e.g. temperature, humidity, day length, light cycle, different compounds in the environment) and biotic factors (e.g. larval and adult nutrition, competition, gut microbes) affect mosquito distribution and abundance. Some of these factors (e.g. temperature) also directly affect parasite development in mosquitoes or can modulate parasite-vector interactions [74-76].

Vectors provide a very specific environment in which parasites complete their developmental cycle before infecting a new host; this environment can be changed drastically after exposure to insecticides or when vectors become resistant to insecticides. The presence of insecticide resistance mechanisms can additionally affect disease transmission in both a positive and a negative manner, by having an impact on vector longevity, vector competence and vector behaviour [58].

1.5.1. *Plasmodium – Anopheles* interactions

Susceptibility of *Anopheles* mosquitoes to *Plasmodium* parasites is genetically determined [77-84]. Several mechanisms have been described which prevent or limit parasite development [85-92].

The mosquito's innate immune system plays a key role in affecting parasite development and killing of parasites [93-97] and is *Anopheles*-species specific [98]. The three primary defence mechanisms are cell-mediated phagocytosis, melanisation, and lysis, and all are

initiated by pattern recognition receptors (PRRs) capable of specific binding to pathogen associated molecular patterns (PAMPs). The factors which then lead to elimination of parasites can be subdivided into two tightly interwoven parts: (a) a humoral response, and (b) a cell-mediated response, with additional defence systems such as oxidative and nitric oxide-mediated killing mechanisms [48].

Blood feeding is a significant immune system activator in *An. gambiae*, inducing hemocyte proliferation, upregulating production of factors with broad anti-pathogenic activity, such as thioester-containing protein 1 (TEP-1), and inducing oxidative stress [99, 100]. During blood meal digestion and nutrient absorption, expression of reactive oxygen species (ROS) detoxification enzymes is induced as a response to the systemic accumulation of ROS, such as hydrogen peroxide (H_2O_2) in hemolymph [101-106].

Plasmodium midgut infection takes place while the blood meal is being digested, and the parasites encounter mosquito's innate immune responses which peak when the largest parasite losses occur [93, 95, 96]. The presence of *Plasmodium* parasites exacerbates the feeding-related oxidative stress, and invasion of the midgut by ookinetes, which is at least partially mediated by P25 and/or P28 ookinete surface proteins, leads to major changes in ROS metabolism, producing active nitrogen and oxygen radicals. While traversing the midgut, the ookinetes trigger a series of reactions leading to apoptosis of the invaded cells, while cellular responses of invaded midgut cells limit ookinete survival [26, 107, 108]. A range of mosquito anti-*Plasmodium* responses are activated against ookinetes: generation of ROS and reactive nitrogen species (RNS), activation of immune signalling pathways, and the presence of components of the mosquito complement-like system [109]. In order to escape the immune response and form oocysts, ookinetes must exit the invaded midgut cells as quickly as possible to avoid being modified by nitration reactions and later recognised by the mosquito complement system [107, 110, 111].

As *Plasmodium* infection in mosquitoes progresses, the mosquito's detoxification gene expression is altered. Major changes occur during midgut invasion, one day post feeding during the ookinete stage, with down-regulation of many detoxification genes, such as several cytochrome P450 monooxygenases (CYPs) and glutathione S-transferases (GSTs). Gene expression levels are considerably different on day 11 post feed, when sporozoites are being released from oocysts. However, *CYP6M2* expression was actually up-regulated on day one, and down-regulated on day 11 post feed, in response to *P. berghei* infection [112]. This

gene is over-expressed in response to parasite infection [112, 113] and has also been implicated in pyrethroid resistance [114-116].

Some of the detoxification enzymes produced in response to oxidative stress and infection are also involved in detoxification of insecticides and have been implicated in insecticide resistance. CYPs play a vital role in the metabolism of a wide variety of compounds, have been implicated in a number of life processes, are a backbone of metabolic resistance mechanisms especially against pyrethroids, but also DDT and organophosphates, and are involved in mosquito response to microbial challenge and malaria infection [117]. GSTs are a major family of detoxification enzymes, involved in protection against oxidative damage and oxidative stress. They also play an important role in insecticide resistance against organophosphates and organochlorines (e.g. DDT), and have a secondary role in resistance against pyrethroids [112, 118, 119].

When mosquitoes are exposed to parasites and to insecticides, this might result in changes in expression of detoxification enzymes leading to a trade-off between the necessary control of ROS levels in response to infection and the elimination of insecticides, increasing sensitivity to insecticides [120-122].

1.5.2. Environmental factors

Environmental factors such as ambient temperature and relative humidity affect malaria transmission through their effect on mosquitoes and parasites.

Mosquitoes are small ectothermic insects, and their internal temperature is determined by and approximates air temperature. Changes in air temperature, therefore, have an immediate effect on mosquito's vital functions and affect mosquito biting rates, digestion, excretion, gonotrophic cycle duration, movement, reproduction and fecundity, development of larval stages, and survival of larvae and adults [123]. Insects have absolute limits of temperature outside which they cannot exist [123], however survival at different temperatures also depends on relative humidity, and only certain temperature/relative humidity combinations support longevity of vectors that is adequate in allowing them to live long enough to transmit malaria [31, 124-127]. Temperature also affects mosquito immune system [128-130] and consequently parasite development.

Air temperature also influences the rate of parasite development or duration of sporogony, which varies inversely with the temperature of mosquito's environment [31], within the permissive range which lies between 16°C and 35°C for *Plasmodium* sporogony [131].

Different mosquito-parasite species combinations have different temperature and humidity requirements which allow completion of sporogony [126]. Within a certain range, the higher the temperature, the shorter the incubation period (Fig. 1-4).

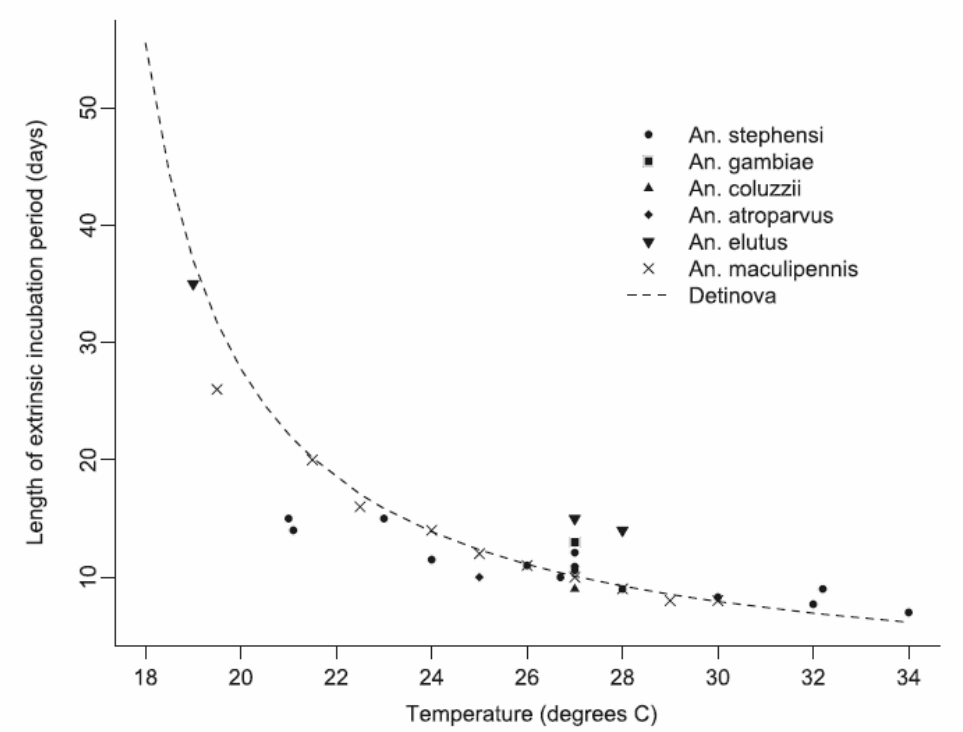

Fig. 1-4. Studies of *P. falciparum* infection in different vector species at different incubation temperatures until detection of sporozoites following an infectious feed. Extrinsic incubation period refers to parasite development in mosquitoes, i.e. sporogony. The fitted curve represents Detinova's standard degree-day model and data points included have been extracted from a number of studies. Reproduced from Ohm *et al,* 2018 [132] (CC BY 4.0; https://creativecommons.org/licenses/by/4.0/).

While high temperatures are lethal to parasites, sporogony at low temperatures is lengthened to an extent that mosquitoes may not survive long enough to be able to transmit the parasites [31, 133]. The early part of sporogony (exflagellation, fertilization, penetration of the midgut wall by ookinetes, up to oocyst formation) is thought to be extremely sensitive to temperature [31, 134-139].

The efficacy of insecticides against mosquitoes is, to some extent, also temperature-dependent due to the effect of temperature on biological processes such as uptake, elimination, and functioning of neurons, and the effect of temperature on biotransformation rates of insecticides [140-143].

1.5.3. Vector longevity

Vector longevity plays an essential role in malaria transmission and is the most important parameter of vectorial capacity. An infected mosquito has to survive until completion of sporogony in order to be able to infect a new vertebrate host. Vector longevity can be described by the longevity factor (as part of vectorial capacity, *C*)

$$\frac{p^n}{-log_e p}$$

where *p* = daily survival probability (i.e. proportion of vectors surviving per day), *n* = incubation period in the vector (i.e. length of the sporogonic cycle in days), and $1/log_e p$ = expectation of life.

After the first couple of days of life, the survival rate of female *Anopheles* mosquitoes (measured as the probability of surviving from one gonotrophic cycle to the next) appears to be remarkably independent of age. It is, however, dependent on the effects of climate and predators, and is partly associated with feeding and other external circumstances [56].

Some studies found that *Plasmodium* parasites can reduce vector longevity, mostly due to the cell damage they cause during passage through mosquito midgut and salivary gland epithelia, higher parasite burdens which can cause resource depletion, properties of an infected blood meal, such as anaemia and antibodies against the parasites, and mosquito immune responses to infection [144, 145].

Insecticidal interventions such as IRS and ITNs certainly do reduce mosquito longevity, thereby reducing the proportion of mosquitoes surviving long enough to be able to transmit disease, making LLINs and IRS successful interventions. Although in general insecticide resistance mechanisms increase survival when vectors are exposed to insecticides, they may also decrease vector longevity due to resource trade-offs necessary for production of additional enzymes in insects with metabolic resistance or because of increased activation of the immune system and oxidative stress [58].

1.5.4. Vector competence

Vector competence describes the capability of vectors – mechanical or biological - to transmit a pathogen. It combines both the vector's susceptibility to a parasite and mechanisms used to fight the infection (e.g. immune system), and the parasite's infectivity and the mechanisms used to overcome host's defences. Vector competence is a complex phenotypic trait determined by host and parasite genetic factors, non-genetic environmental factors and interactions between all these. For malaria, the competence varies between different *Anopheles* and *Plasmodium* species and strains [74, 82, 146, 147].

Vectors provide a very specific environment in which parasites can complete their developmental cycles before infecting a new host. Mosquito innate immunity has an important role in controlling the level of infection and in eliminating parasites [82, 148]. Different groups of genes appear to be responsible for controlling variations in infection intensity (TOLL pathway) and prevalence (IMD pathway) [82].

The internal vector environment can be drastically changed when vectors become resistant to insecticides (e.g. by changing potential redox reactions in tissues where parasites develop [12]). Different insecticide resistance mechanisms can have a wide range of pleiotropic effects, with both positive or negative effects on R_0 [58, 144].

1.6. Insecticides and insecticide resistance

Insecticide-based vector control interventions are the main tools for malaria prevention and control. Levels of intervention coverage have significantly increased across sub-Saharan Africa in the last two decades, and especially since 2010. ITNs are the most widely used vector control method with estimated 54 % of the population at risk in sub-Saharan Africa now sleeping under an ITN, compared with 30 % in 2010 [1]. Since year 2000, it is estimated that vector control interventions have averted 663 million clinical malaria cases in Africa, with about 68 % of cases averted due to the use of ITNs and additional 13 % due to IRS [2].

1.6.1. Pyrethroids

Four classes of insecticides are currently used for malaria vector control: organochlorines, organophosphates, carbamates, and pyrethroids. However, vector control interventions largely rely on pyrethroid insecticides, which are still the only insecticide class used on all ITNs, including LLINs, and are also used for IRS [149-152].

Natural pyrethrins (e.g. pyrethrum) are extracted from *Chrysanthemum* plants, and it is believed that they were first used by the Chinese more than 2,000 years ago. The first synthetic pyrethroids were synthesized by Schechter and colleagues in 1949 [153], while the first photostable pyrethroids – including permethrin – were synthesized by Elliot and colleagues in the 1970s [154].

Pyrethroids are neurotoxins that target the *para* voltage-gated sodium channels (VGSC) on the mosquito's neurons [155, 156]. They are biodegradable, do not accumulate in the environment, have low mammalian toxicity, and are relatively inexpensive. They work well on nets and when sprayed on surfaces because of their knock-down effect, fast killing properties, and long residual action [157], and currently still play a leading role in public health vector control interventions.

1.6.2. Insecticide resistance

Reports of insecticide resistance following the use of insecticides for vector control started emerging in the late 1940s and 1950s [5, 158-160]. Insecticide resistance was first defined in 1953 as "*the development of an ability in a strain of an insect to tolerate doses of toxicants which would prove harmful to the majority of individuals of a normal population of the same species. The term 'behaviouristic resistance' describes the ability to avoid a dose which would prove harmful*" [5]. The two main insecticide resistance mechanisms are target site resistance and metabolic resistance. Two other less well-known mechanisms are cuticular resistance, causing reduced uptake of insecticides due to cuticle modifications, and behavioural resistance, due to changes in insect behavior which help mosquitoes avoid contact with the insecticides [15].

Insecticide resistance has since become widespread. According to the WHO, globally 61 out of 76 malaria-endemic countries providing data for the period 2010 – 2016 reported resistance to at least one insecticide, with pyrethroid resistance the most commonly reported [1].

Widespread resistance of anopheline mosquitoes to pyrethroids has been reported, and presently no African country has fully pyrethroid-susceptible malaria vectors, posing a major threat to the effectiveness of vector control strategies [15, 149, 161, 162]. As a response, the Global Plan for Insecticide Resistance Management (GPIRM) was developed in 2012, incorporating a new global strategy consisting of five activities, or pillars. These include planning and implementation of insecticide resistance management strategies, design and

use of monitoring and data management plans, development of new and innovative vector control tools, gathering additional information and evidence on mechanisms of resistance and their impact on vector control and malaria transmission, and providing the advocacy and resources necessary for successful implementation of the GPIRM [149]. The distribution and strength of pyrethroid resistance has increased significantly in recent years, as has the number of reports of resistance to other insecticide classes [163, 164].

New active ingredients, with novel modes of action, are being developed by the Innovative Vector Control Consortium (IVCC) and its industrial partners [165]. For example, new ITNs treated with a combination of a pyrethroid alpha-cypermethrin and chlorfenapyr, an N-substituted halogenated pyrrole, are currently under review and have been given an "Interim" status of WHO recommendation [152, 166]. However, until novel compounds are available, a clear understanding of the impact of pyrethroid resistance on the effectiveness of pyrethroid-based control interventions is needed.

Pyrethroid resistance in malaria vectors has occurred in waves. It first appeared in Africa in the 1970s in Sudan and then in the late 1980s /early 1990s in West Africa and Western Kenya [167-170], in the form of two point mutations in the VGSC gene which confer knockdown resistance (*kdr*) to DDT and pyrethroids. These have now become widespread in *An. gambiae* s.l. [15, 149]. The second wave of resistance consisted of various metabolic resistance mechanisms in both *An. gambiae* s.l. and *An. funestus*, while the third wave started emerging in West African *An. gambiae* s.l. in 2013 and is based on cytochrome P450 monooxygenases [161]. This third wave, recorded in Burkina Faso and the Ivory Coast, produces much higher levels of resistance than previously widely spread metabolic - or metabolic combined with *kdr* - resistance, and can spread rapidly once selected [161, 171].

1.6.2.1. Target site resistance

Target site resistance is caused by changes in the target site of insecticides, due to mutations such as amino acid replacements that alter the target site, reducing binding and the action of chemicals.

The best-known mutation associated with pyrethroid and DDT resistance in *An. gambiae* s.l. is a substitution of the leucine residue found at codon 1014 with either phenylalanine (*L1014F* – also called *kdr West* or *Rw*) [172] or serine (*L1014S* – also called *kdr East* or *Re*) [173], causing knock-down resistance (*kdr*). This makes resistant insects able to withstand prolonged exposure to insecticides without being "knocked-down". *Kdr* locus is located in

domain II of the VGSC gene, on the left arm of chromosome two [174]. *L1014F* is thought to provide more protection against insecticide binding due to the presence of phenylalanine [175]. The degree of resistance caused by *kdr L1014F* or *L1014S* was found to vary with the insecticide treatment used, but *L1014S* is thought to provide greater protection against insecticides when paired with *L1014F* in heterozygote (*Re/Rw*) form [176].

Another substitution at position 1575 (an asparagine-to-tyrosine mutation at this position, *N1575Y*) has been identified more recently within the linker between domains III-IV of the VGSC. This has so far been observed only in a *L1014F* haplotype [177]. It is thought to have an additive effect and may compensate for deleterious fitness costs incurred by *L1014F* in the absence of insecticide exposure [177].

Mosquitoes carrying the *kdr* mutation have decreased neuronal and behavioural excitability, since *kdr* mutation enhances closed-state inactivation of nerves, and more stimulation is required to make the nerves fire impulses and release acetylcholine. This has been interpreted as implying that there could be a behavioural cost associated with the *kdr* allele [178].

It is important to note that comparable forms of knockdown-resistance, based on similar mutations in the sodium-channel target-site molecule, have been observed in a wide range of other insects, including houseflies and many agricultural pests. Within the genus *Anopheles*, so far, seven different *kdr* mutations have been recorded in at least 13 species from three continents [179].

1.6.2.2. Metabolic resistance

The three main enzyme families implicated in metabolic insecticide resistance mechanisms are carboxylesterases (ESTs), cytochrome P450 monooxygenases (CYPs), and glutathione S-transferases (GSTs) [180].

CYPs are the main group of enzymes responsible for pyrethroid metabolism in insects. They are involved in oxidative metabolism of numerous substances, generally detoxifying the substrate. CYP P450 enzymes are a complex family; 111 genes have been identified in *An. gambiae* s.s. but it is not known how many of these are capable of detoxifying insecticides [117, 181].

GSTs play a secondary role by protecting from insecticide-exposure-induced oxidative stress, detoxifying secondary products of P450-based metabolism, or by binding insecticides and lowering their concentration [15, 180].

1.7. Impact of pyrethroids on sporogony

1.7.1. Additional target sites of pyrethroids

Insecticides are thought to affect insect immunity and the susceptibility of insects to pathogens. They are known to affect both humoral and cellular immune responses, activate detoxification mechanisms, affect immunity via oxidative stress, and affect ROS production and regulation [182]. Insecticides can also alter the number of hemocytes and/or induce structural abnormalities in them [183].

Pyrethroids have been documented to affect serine proteases, lytic enzymes such as esterases, carboxylesterases, and lysozymes, and ROS production, all of which play vital roles in the insect's immune system [182]. Serine proteases are key components of mosquito innate immune system, involved in signal amplification cascades leading to anti-parasite responses, such as melanization, through the Toll pathway [96, 113, 184].

Pyrethoids also cause free-radical-mediated lipid peroxidation, glutathione depletion and protein oxidation, inducing strong oxidative damage [119]. Although ROS are normally produced by mitochondrial respiration, they are also a part of the immune response against bacteria and *Plasmodium* parasites in mosquitoes such as *An. gambiae* [110]. Furthermore, pyrethroids – especially type II with an α-cyano group (e.g. deltamethrin) - also seem to inhibit complex I (the proton-translocating NADH:ubiquinone oxidoreductase) of the mitochondrial respiratory chain [119, 185].

1.7.2. Effects of pyrethroids on *Plasmodium* sporogony

Insecticides primarily act directly upon mosquitoes, reducing their longevity and population density, and thereby their vectorial capacity. However, pyrethroids also appear to affect parasites developing within the vectors, with two possible modes of action. They may exert direct toxicity on *Plasmodium* parasites through an unknown target. Alternatively, they may have an indirect effect, changing vector physiology or triggering different immune pathways, thereby modulating vector-parasite interactions and/or making the environment inside vectors unsuitable for parasite development [7, 66].

Different classes of insecticides have been tested for their effect on *Plasmodium* parasites. Although organochlorines, carbamates and organophosphates were found to have no effect on infection rates in mosquitoes [7, 186, 187], a recent report [188] showed that exposure to DDT and bendiocarb inhibited development of *P. falciparum* in insecticide-resistant *An. gambiae* s.s..

Early studies by Carle *et al* [65] and Elissa *et al* [66, 189] indicated that sub-lethal doses of pyrethroids have a significant effect on *Plasmodium* parasites, although other studies reported contrasting findings [190]. *In vitro*, exposure of erythrocytic stages of *P. falciparum* to low concentrations of deltamethrin resulted in reduction of parasitaemia of up to 58 % [65]. *In vivo*, exposure of larvae and adult *An. stephensi* to sub-lethal doses of deltamethrin resulted in reduced *P. yoelii yoelii* infection prevalence in adult mosquitoes at both oocyst and sporozoite stage, and also reduced oocyst intensity [65, 66, 189]. Three other pyrethroids were tested: exposure to bioallethrin and fenvalerate caused a reduction in sporozoite rates but did not significantly affect oocyst rates, whereas exposure to cypermethrin had no effect on *P. y. yoelii* sporogony [66].

Hill (2002) carried out research on the effects of sub-lethal doses of pyrethroids on malaria vectors and vector-parasite interactions and showed that exposure to pyrethroids can inhibit development of *Plasmodium* in mosquitoes under laboratory conditions [7]. His findings showed that exposure of insecticide resistant *An. stephensi* to the synthetic pyrethroids permethrin, deltamethrin and lambda-cyhalothrin resulted in significant inhibition of the sporogonic development and infectivity of *P. yoelii nigeriensis* and *P. falciparum*. *In vitro*, pyrethroids did not affect *P. falciparum* blood stages at realistic concentrations that might naturally be encountered by parasites within the blood meal; they also had no significant effects on parasite exflagellation, nor on trypsin and chitinase activities, which play a major role in blood meal digestion and ookinete migration. Pyrethroids significantly affected parasite development only if mosquito exposure was within 24 hours or less prior to the infective feed, during the feed, and up to 18 hours after the feed; once oocysts were formed, the insecticides no longer had an impact on the parasites [7]. This coincides with the time period during which anti-*Plasmodium* responses such as human complement and transmission-blocking antibodies within the blood meal, and mosquito microbiota, can affect the sporogony [47]. Pyrethroids might change the internal mosquito environment to which the parasite is exposed during the sporogonic development by triggering the processes involved in detoxification. There are different possible routes of metabolism of pyrethroids

by CYPs, such as *CYP6M2*, resulting in several metabolites with unknown relative toxicity, which might have detrimental impact on sporogony [115].

Mosquito immune signaling pathways are activated following an infectious blood meal, resulting in production of anti-pathogen molecules that can help combat the infection and thus modulate malaria transmission [91, 148]. Insecticides too affect insect humoral and cellular immune responses, potentially interfering with microbial symbionts and affecting the sporogony of *Plasmodium* parasites; the effect of insecticides on immunity via oxidative stress could be especially important [112, 182, 188].

1.8. Impact of insecticide resistance on malaria transmission

Following exposure to insecticides, resistant mosquitoes tend to survive longer than susceptible ones and are more likely to live long enough to allow *Plasmodium* parasites to complete sporogony [191, 192]. This can reduce the efficacy of vector control measures and may even reverse the gains already made [160, 193-197]. As well as affecting transmission in these well-established ways, insecticide resistance mechanisms may also have a number of additional effects on resistant insects and on the parasites they transmit.

1.8.1. Resistance-associated fitness costs and mosquito longevity

Vector longevity is crucial for disease transmission, yet mutations causing insecticide resistance are often associated with fitness costs that prevent them from spreading to fixation, and in the absence of insecticide, resistance alleles can be eliminated [143, 198, 199]. But following long-term insecticide exposure genome modifications can evolve that minimize deleterious effects of some resistant alleles, minimizing their fitness costs even in the absence of insecticides [200, 201].

Insecticide resistance caused by insensitive acetylcholinesterase (AChE) was shown to have an impact on longevity and other traits associated with the fitness of *Culex pipiens/Cx. quinquefasciatus*, and was found to affect pupal survival of *An. gambiae* s.s. [202, 203]. The presence of target-site resistance mechanisms such as *kdr-L1014F* and *RDL* can affect the mating competitiveness of male *An. gambiae* in natural populations, especially in homozygous resistant males [204]. Different resistance alleles can also interact to influence the fitness of mosquitoes [9].

Over-expression of detoxification enzymes requires a substantial investment of resources. A resource trade-off due to increased production of proteins can lead to a significant reduction of other resources, such as lipids, with a negative effect on longevity and possibly incurring other fitness costs [58]. In *Cx. pipiens* with overexpressed esterases, energetic reserves were on average 30 % lower than in susceptible mosquitoes; in mosquitoes with modified AChE the depletion of energetic resources is likely not due to resource trade-offs, but instead due to hyperactivation of the nervous system [205].

Cytochrome P450 monooxygenases are a large enzyme family and there is a lot of plasticity regarding the selection of particular enzymes in different resistant populations, which can lead to variation in fitness costs between these populations. Fitness costs associated with CYP-based permethrin detoxification resistance were observed in *Cx. quinquefasciatus*, with the strength of the cost varying depending on the environmental conditions [206].

1.8.2. Impact of resistance on vector competence

Insecticide resistance can compromise vector competence in two ways: by various physiological modifications in insecticide-resistant mosquitoes, or by affecting vector immunity. Vector mosquitoes provide a very specific environment in which parasites complete their development. This environment can become significantly changed if vectors become resistant to insecticides, and can potentially become toxic to the parasites, affecting their development [207-211].

McCarroll *et al* [12, 13] have shown that insecticide resistant *Cx. quinquefaciatus* were less likely to transmit the filarial worms *Wuchereria bancrofti,* due to overproduction of esterases, which prevented the development of the parasite's L3 infective larvae [12, 13].

Insecticide resistance caused by overproduction of esterases or AChE modification in *Cx. pipiens* had no effect on the development of the avian malaria parasite *P. relictum* within the mosquito [212]. However, pyrethroid resistance caused by *CYP6* class of mono-oxygenases in *An. funestus* has been associated with low *P. berghei* infectivity, indicating there may be an association between resistance and parasite infectivity where over-expression of detoxifying enzymes might have affected parasite development [213].

A series of detoxification responses are triggered during *Plasmodium* infection of a mosquito [95, 112] – many of which are also involved in metabolism of insecticides [115] and play a role in insecticide resistance [180]. Reactive oxygen species (ROS) levels can increase not

only due to blood feeding but also due to environmental stress (UV, heat exposure, exposure to insecticides), or infection with bacteria and parasites [110, 112, 119]. In *An. gambiae,* excess ROS production led to increased mortality during *P. berghei* infection [110]. Insecticide resistance mechanisms can additionally alter ROS levels. For example, increased activity of CYPs in insecticide resistant *An. gambiae* s.s. was shown to result in excess production of ROS, longer developmental times of immature stages of mosquitoes and a shorter life span [11].

Indirectly, these enzymes might also affect the midgut microbiota of mosquitoes - bacteria which produce free radicals and trigger the immune response, thereby negatively affecting parasite development. If enhanced expression of resistance-causing enzymes would in some way disturb the midgut bacteria, this might increase mosquito susceptibility to *Plasmodium* parasites [110, 214].

The *L1014F* or *L1014S* SNP variants tag a haplotype of 65 linked genes, two of which are potentially involved in host-defence processes and might affect susceptibility of mosquitoes to parasites. One of the genes with strong prediction of immune function is *ClipC9*, which influences parasite infection intensity. It was also shown that the *para* VGSC gene on its own does not affect parasite development in mosquitoes [215].

1.8.3. Insecticide resistance and *Plasmodium* infection in mosquitoes

Complex interactions have developed between mosquito adaptations to insecticide exposure, the eventual occurrence of resistance, and vector competence. These interactions are further affected by environmental conditions and exposure to insecticides.

Alout *et al* demonstrated that insecticide resistance mutations affect vector competence of *An. gambiae* s.s. mosquitoes for *P. falciparum* parasites in the absence of insecticides [216]. The prevalence of *P. falciparum* infection was significantly higher in insecticide resistant mosquitoes, especially in those carrying the *kdr* allele, compared to those carrying the *ace-1* mutation or susceptible mosquitoes. The parasite burden - especially at oocyst stage - was lower in mosquitoes with *kdr*, while there was no difference between the susceptible strain and the one with *ace-1* mutation. Ndiath *et al* also reported higher infection rates in *kdr*-resistant mosquitoes compared with susceptible ones, although with a different conclusion in terms of the effect on infection intensity [217]. However, a more recent study showed that the cost of *P. falciparum* infection was higher in *kdr* and *ace-1* resistant *An. gambiae* s.s mosquitoes than in insecticide-susceptible mosquitoes, with reduced survival and fecundity

rates, all of which could potentially reduce the vectorial capacity of resistant mosquitoes [218].

Another study explored the impact of *P. falciparum* infection on the level of DDT susceptibility in *kdr* homozygous *An. gambiae* s.s., showing that *Plasmodium* infection increased mortality caused by DDT in *kdr*-resistant mosquitoes [120]. This increased susceptibility was more pronounced if insecticide exposure took place on day 1 after the infectious blood meal which coincides with ookinete development, or on day 7 which is during oocyst maturation. However, there was no difference in DDT-induced mortality on day 15 after the blood meal, when mosquitoes were infected with sporozoites. As insecticide resistance can decrease with age (depending on the mechanism) [219-222], mosquito age might be affecting susceptibility of mosquitoes to insecticides more than infection with sporozoites. Similarly, Saddler *et al* explored the effects of *P. berghei* infection and mosquito age on DDT susceptibility of an *An. gambiae* s.s. strain resistant to DDT due to GST-based metabolic resistance [222]. Mosquitoes were more sensitive to DDT when they fed on *Plasmodium*-infected mice but were not actually infected, whereas susceptibility was not significantly different between infected mosquitoes and those fed on an uninfected mouse. These studies both indicate that infection by *Plasmodium* does not affect the expression of insecticide resistance and suggests that resistance to parasites might incur a fitness cost in mosquitoes due to a trade-off between mounting an effective immune response against the infection and surviving DDT exposure by means of insecticide elimination using detoxification enzymes.

Exposure to insecticides was shown to affect vector-parasite interactions and parasite development in mosquitoes with target site resistance mechanisms [188]. The prevalence of infection was significantly lower in *kdr*- and *ace-1* resistant mosquitoes exposed to insecticides, however oocyst burden was not lower in *kdr* mosquitoes following DDT exposure. Insecticides might affect the parasites developing in vectors directly, exerting direct toxicity through unknown target sites. Alternatively, they might affect the parasites indirectly, by changing the internal mosquito environment to which the parasite is exposed during the sporogonic development, for example by triggering the processes involved in detoxification or by triggering immune pathways [7, 66].

Studies using the natural avian malaria system showed that *Plasmodium* infection is more likely to incur higher costs in insecticide resistant mosquitoes compared with susceptible ones. Furthermore, mosquitoes with a metabolic esterase-overproducing insecticide

resistance suffered a higher cost of infection than mosquitoes with target site resistance and were more affected by unfavourable environmental conditions [223].

Results of field studies are not conclusive, either. While in Bioko Island *An. gambiae* mosquitoes homozygous for *kdr* appeared less likely to transmit malaria as their sporozoite rates were lower than those of heterozygous and homozygous non-*kdr* mosquitoes [224], a study from Tanzania showed that sporozoite rates were significantly higher in *kdr*-homozygotes than in heterozygotes or susceptible mosquitoes [225].

1.9. Entomological and epidemiological impacts of resistance

It is assumed that insecticide resistance will have a significant impact on the efficacy of vector control interventions such as ITNs and IRS, potentially resulting in their failure and in the resurgence of malaria. *Kdr* resistance mechanisms were first identified in *An. gambiae* s.l. more than 15 years ago [172, 173], and are now widespread in Africa [163, 164]. Yet, there is still no clear evidence that resistance is operationally significant, and still very little is known about the epidemiological impact of resistance on currently used control measures and disease transmission.

Three different methods can be used for detection of insecticide resistance, each providing complementary types of information. Susceptibility testing is used to detect phenotypic resistance using WHO paper bioassays or CDC bottle bioassays. In addition, bottle assays can be used to measure the "intensity" of resistance (i.e. comparing the doses needed to kill the resistant wild population, relative to the standard susceptible). Biochemical assays are used to detect the presence of particular metabolic resistance mechanisms; while molecular tests are used to detect mutations in or expression levels of the actual genes [15, 149, 226, 227]. However, none of these alone can signify control failure. Results of the bioassays provide an indication that resistance may have been selected for in a given area, but the diagnostic concentrations used in resistance bioassays do not correspond to the concentrations of insecticides used in vector control. Intensity bioassays provide further information on resistance being selected in field populations, which should be used proactively in the planning of vector control operations [228, 229]. Information on resistance mechanisms obtained from biochemical and molecular assays provides further information on how widespread resistance genes are, the level of resistance, the type of resistance, and the pattern of cross-resistance between insecticides [226, 230].

Entomological indicators obtained when measuring the effects of resistance on mosquitoes can serve as an indicator of resistance having an impact on malaria transmission [231]. However, measuring the effects of resistance in terms of epidemiological outcomes has been more problematic [14].

The best-known example of control failure due to pyrethroid resistance was reported from the border area between Mozambique and South Africa, where the main vector *An. funestus* developed metabolic resistance to pyrethroids (but not to DDT) after the local malaria control programme started using deltamethrin instead of DDT. This change in the use of insecticide, together with an increase in rainfall, led to a sharp increase in the number of malaria cases. After reverting back to the IRS using DDT, a substantial decrease in the number of cases followed (Fig. 1-5).

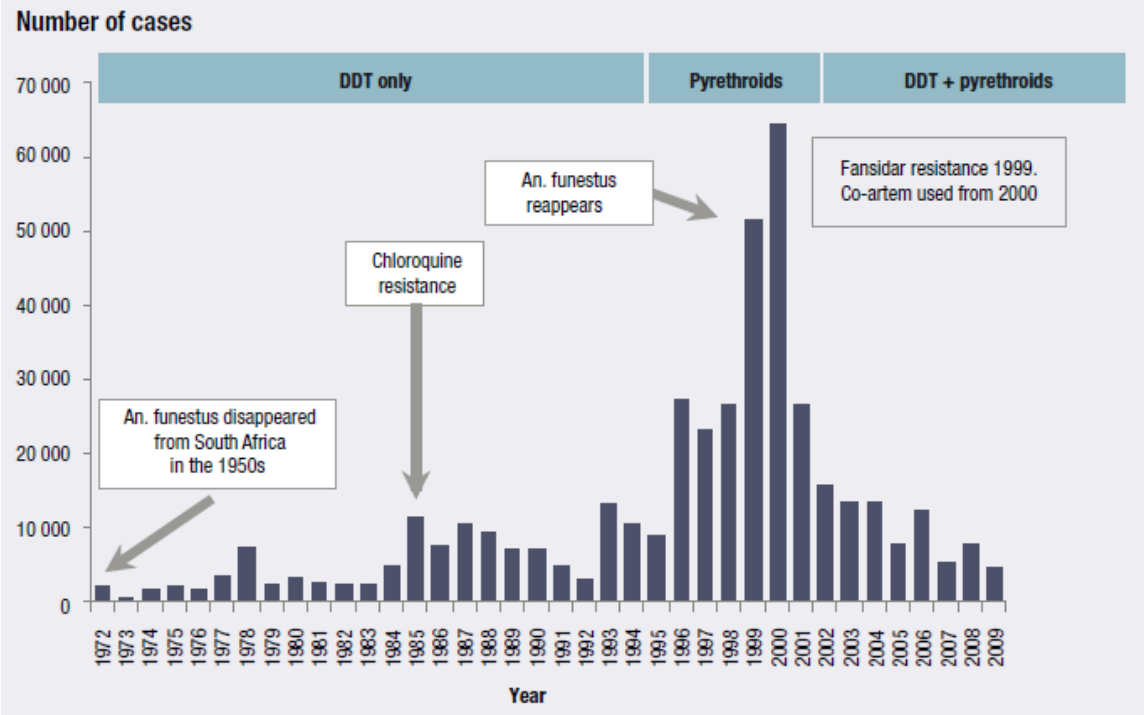

Fig. 1-5. Consequences of control failure – most likely due to insecticide resistance - in South Africa. Reprinted with permission from "Global plan for insecticide resistance management in malaria vectors" by WHO, 2012 [149].

Although the reintroduction of DDT coincided with a change of antimalarial drugs used from sulfadoxine-pyrimethamine (SP) to artemisinin combination therapy Co-artem (ACTs) and both interventions played a part in decreasing malaria incidence, DDT use significantly

decreased the density of *An. funestus* to undetectable levels, leaving *An. arabiensis* as the remaining, but much less efficient, malaria vector [194-196, 232, 233].

The situation elsewhere is not as clear-cut, and resistance has not always had an epidemiologically significant effect on malaria incidence. A reduction in the efficacy of ITNs and IRS due to pyrethroid resistance was recorded in Benin [197]. Reduced susceptibility of field collected mosquitoes to LLINs, as compared to a laboratory-reared susceptible strain, was reported from Burkina Faso [234] and Kenya [235]. Another study from Kenya reported that in the areas where pyrethroid resistant vectors are present, LLINs with holes permit mosquitoes to enter and feed, and LLIN protection is therefore reduced [236]. A study from Senegal claimed that the extensive use of LLINs exerted a strong selective pressure on vector populations, causing an increase in insecticide resistance, and subsequently contributing to an increase in malaria morbidity [237]. In general, the level of insecticide resistance has increased significantly in recent years in many settings [163, 238].

A simple calculation, used by WHO to make a first-approximation estimate of the potential impact of insecticide resistance on malaria burden [149], suggested that at coverage levels of the time (2010) the failure of pyrethroids could result in approximately 120,000 additional child deaths – and if universal coverage was achieved, could lead to approximately 259,000 additional child deaths per year and 55 million additional malaria cases each year in the WHO African Region. This would represent a loss of about 56 % of the benefits that result from vector control. Apart from an increase in diagnostic and treatment costs and loss of life, there would be other secondary costs linked to insecticide failure, such as increases in the prevalence of other vector-borne diseases and potentially increases in the level of drug resistance.

A more advanced transmission dynamics model that was recently published, predicts that the public health impact of pyrethroid resistance will be high, both in terms of the number of clinical cases and the force of infection; however, the meta-analysis part of the study also shows that people using LLINs in areas with intermediate insecticide resistance still benefit from a high level of protection [239].

On the other hand, pyrethroid-based control methods can remain effective even in the presence of *kdr* resistance in local mosquito populations [14, 16, 240-242]. A meta-analysis showed that in the evidence available up to 2014, ITNs were still giving better protection than untreated nets (which can reduce malaria incidence (mild episodes) by about 50%),

despite insecticide resistance [231]. However, it is not clear whether the level of control in the presence of resistance is as high as it would have been if the mosquitoes were susceptible. Moreover, since the concentration of insecticide on an LLIN declines by about ten-fold over its effective life [243], the effect of resistance might be to greatly shorten the effective life of an LLIN, i.e. it still gives some additional protection while new, but after a few months this protection has declined to low levels [149].

A WHO-coordinated multi-country study was undertaken to provide insights on the implications of insecticide resistance on malaria vector control, showing that LLINs still provide personal protection (comparing ITN-users with non-users) even in areas with pyrethroid resistance, and no evidence was found of a village-level association between malaria disease burden and pyrethroid resistance [16]. Results from the Sudanese part of the study did however indicate that pyrethroid resistance may have affected pyrethroid-based IRS, but its impact on LLINs was not assessed [244].

In Bioko island pyrethroid-based IRS failed to reduce mosquito density of pyrethroid-resistant *An. gambiae* with high frequencies of the *L1014F kdr* allele [245, 246]. Nevertheless, deltamethrin IRS was followed by a large reduction in the prevalence of malaria infection in children, and pyrethroid resistance was found not to be operationally significant after further detailed analysis [224].

Resistance has been spreading rapidly in Malawi in both *An. gambiae* s.s. and *An. funestus*, while the use of vector control interventions (ITNs, LLINs and IRS) has been scaled up. Thus far, no major epidemiological impact on malaria transmission has been demonstrated [230], and ITNs remain effective in reducing the incidence of malaria infection in children (comparing ITN-users with non-users) in areas with moderate levels of metabolic pyrethroid resistance in *An. funestus* [247].

In Western Kenya, resistance mechanisms in *An. gambiae* s.s. became more prevalent following the ITN trials but their impact on malaria control in these areas has been minimal [248]. Along the Lake Victoria basin, ITNs continue to be effective despite the dramatic rise of the *kdr-1014S* allele to near fixation point and detection of phenotypic resistance to DDT and pyrethroids [249]. More recently, a study found that LLINs were still effective at reducing transmission in areas of both low- and high insecticide resistance, and that there was no significant association at cluster level between insecticide resistance and malaria parasite infection incidence [250].

Assessing the entomological, epidemiological and operational impacts of resistance has been hindered by differences in data collection methods; lack of DNA-level molecular markers for metabolic resistance mechanisms leading to under-reporting of their presence and spread in the field; inability to associate metabolic resistance genotypes with sporozoite positivity; the use of *kdr* as a surrogate for comprehensive resistance monitoring; and a large number of confounding factors associated with different studies [15, 161, 231]. Moreover, as resistance in the field cannot be randomly assigned to groups or separated from other factors that may influence the effectiveness of various vector control measures, it is impossible to carry out trials directly measuring its impact. There is also a lack of data about the impact of resistance on entomological indicators in the field, including vector survival and longevity [161].

Until alternative insecticides become available, it will be of the utmost importance to continue monitoring the presence of resistance, and its impacts, in the field. To prolong the use of effective vector control measures available today and continue to develop novel interventions, further research is required to better understand the effects of both insecticides and insecticide resistance mechanisms on the parasites, vectors and malaria transmission.

1.10. Placing the study into context

Laboratory studies carried out by Carle *et al* [65], Elissa *et al* [66, 189] and Hill [7] indicated that sub-lethal doses of pyrethroids have a significant effect on *Plasmodium* parasites, while Hill also showed that these effects are confined to a relatively narrow window of time where exposure can affect the parasite.

At the same time, insecticide resistance – and especially pyrethroid resistance - has become widespread in anopheline mosquitoes, potentially posing a major threat to the effectiveness of vector control strategies [15, 149, 161, 239]. However, there is currently no clear evidence that resistance is operationally significant or has had a major epidemiological impact.

This raises the question whether antiparasitic effects of pyrethroids observed in laboratory studies also play a role in field conditions and, if they do, how important such effects are in the context on insecticide resistance. Observing this in the wild would be incredibly difficult, while controlled trials on the effect of insecticide resistance and malaria infection are not possible. However, a study based on a “semi-wild” set up with sympatric wild parasites and

vectors – as described in this thesis – might be a start in hopefully bridging a gap between laboratory findings and the real-world situation.

1.11. Aims and objectives

The aim of this project was to investigate the potential effects of pyrethroids and pyrethroid exposure of *Anopheles gambiae* s.l. vectors on the sporogonic development and transmissibility of *Plasmodium falciparum* parasites.

The objectives of the PhD were as follows:

1. The primary objective was to study the effects of pyrethroids and pyrethroid exposure of female *An. gambiae* s.s. on the sporogonic cycle of *P. falciparum*.

   The effects of sub-lethal doses of deltamethrin on sporogony in wild pyrethroid resistant *An. gambiae* s.l. in Uganda were studied, together with the effect of environmental variables. Preliminary investigations into possible mechanisms underlying the observed effects were made in the laboratory, including determination of insecticide doses which mosquitoes pick up on contact with treated netting, which in turn indicate a range of doses the parasites can be expected to encounter in mosquitoes.

2. The secondary objective was to investigate whether *kdr* insecticide resistance interferes with the development of *P. falciparum* within the vector.

   The effects of *kdr* genotype on *Plasmodium infection* rates in *An. gambiae* s.l. were explored, together with variations in phenotypic and genetic resistance against commonly used insecticides among *An. gambiae* s.l. in mid-western Uganda.

1.12. References

1. World Health Organization. World Malaria Report 2017. Geneva: World Health Organization; 2017.

2. Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U, Battle KE, Moyes CL, Henry A, Eckhoff PA *et al*. The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. Nature. 2015, 526:207-211.

3. Busvine JR. The present status of insecticide resistance. Bull World Health Organ. 1963, 29 Suppl:31-40.

4. Muirhead-Thomson RC. The significance of irritability, behaviouristic avoidance and allied phenomena in malaria eradication. Bull World Health Organ. 1960, 22:721-734.

5. Pampana EJ. Changing strategy in malaria control. Bull World Health Organ. 1954, 11(4-5):513-520.

6. Alonso PL, Brown G, Arevalo-Herrera M, Binka F, Chitnis C, Collins F, Doumbo OK, Greenwood B, Hall BF, Levine MM *et al*. A research agenda to underpin malaria eradication. PLoS Med. 2011, 8(1):e1000406.

7. Hill N. Effects of sublethal doses of pyrethroids on malaria vectors. *PhD Thesis.* London: London School of Hygiene & Tropical Medicine, University of London; 2002.

8. Chaccour CJ, Kobylinski KC, Bassat Q, Bousema T, Drakeley C, Alonso P, Foy BD. Ivermectin to reduce malaria transmission: a research agenda for a promising new tool for elimination. Malar J. 2013, 12:153.

9. Berticat C, Bonnet J, Duchon S, Agnew P, Weill M, Corbel V. Costs and benefits of multiple resistance to insecticides for *Culex quinquefasciatus* mosquitoes. BMC Evol Biol. 2008, 8:104.

10. Brito LP, Linss JG, Lima-Camara TN, Belinato TA, Peixoto AA, Lima JB, Valle D, Martins AJ. Assessing the effects of *Aedes aegypti kdr* mutations on pyrethroid resistance and its fitness cost. PLoS One. 2013, 8(4):e60878.

11. Otali D, Novak RJ, Wan W, Bu S, Moellering DR, De Luca M. Increased production of mitochondrial reactive oxygen species and reduced adult life span in an insecticide-resistant strain of *Anopheles gambiae*. Bull Entomol Res. 2014, 104(3):323-333.

12. McCarroll L, Hemingway J. Can insecticide resistance status affect parasite transmission in mosquitoes? Insect Biochem Mol Biol. 2002, 32(10):1345-1351.

13. McCarroll L, Paton MG, Karunaratne SH, Jayasuryia HT, Kalpage KS, Hemingway J. Insecticides and mosquito-borne disease. Nature. 2000, 407(6807):961-962.

14. Kleinschmidt I, Mnzava AP, Kafy HT, Mbogo C, Bashir AI, Bigoga J, Adechoubou A, Raghavendra K, Knox TB, Malik EM *et al*. Design of a study to determine the impact of insecticide resistance on malaria vector control: a multi-country investigation. Malar J. 2015, 14:282.

15. 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(2):91-98.

16. World Health Organization. Implications of insecticide resistance for malaria vector control. Geneva: World Health Organization; 2016: 4.

17. Cox FE. History of the discovery of the malaria parasites and their vectors. Parasit Vectors. 2010, 3(1):5.

18. CDC GH-DoPDaM. Malaria - About Malaria - Biology - Lifecycle [https://www.cdc.gov/malaria/about/biology/index.html]

19. Ross R. On some peculiar pigmented cells found in two mosquitos fed on malarial blood. Br Med J. 1897, 2(1929):1786-1788.

20. Ross R. The role of the mosquito in the evolution of the malaria parasite. Lancet. 1898, 152(3912):488-490.

21. Angrisano F, Tan YH, Sturm A, McFadden GI, Baum J. Malaria parasite colonisation of the mosquito midgut - Placing the *Plasmodium* ookinete centre stage. Int J Parasitol. 2012, 42(6):519-527.

22. Billker O, Lindo V, Panico M, Etienne AE, Paxton T, Dell A, Rogers M, Sinden RE, Morris HR. Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito. Nature. 1998, 392(6673):289-292.

23. Sinden RE, Butcher GA, Billker O, Fleck SL. Regulation of infectivity of *Plasmodium* to the mosquito vector. Adv Parasitol. 1996, 38:53-117.

24. Meis JF, Wismans PG, Jap PH, Lensen AH, Ponnudurai T. A scanning electron microscopic study of the sporogonic development of *Plasmodium falciparum* in *Anopheles stephensi*. Acta Trop. 1992, 50(3):227-236.

25. Baton LA, Ranford-Cartwright LC. *Plasmodium falciparum* ookinete invasion of the midgut epithelium of *Anopheles stephensi* is consistent with the Time Bomb model. Parasitology. 2004, 129(Pt 6):663-676.

26. Baton LA, Ranford-Cartwright LC. How do malaria ookinetes cross the mosquito midgut wall? Trends Parasitol. 2005, 21(1):22-28.

27. Baton LA, Ranford-Cartwright LC. Morphological evidence for proliferative regeneration of the *Anopheles stephensi* midgut epithelium following *Plasmodium falciparum* ookinete invasion. J Invertebr Pathol. 2007, 96(3):244-254.

28. Rosenberg R. Malaria: some considerations regarding parasite productivity. Trends Parasitol. 2008, 24(11):487-491.

29. Rosenberg R, Rungsiwongse J. The number of sporozoites produced by individual malaria oocysts. Am J Trop Med Hyg. 1991, 45(5):574-577.

30. Vaughan JA. Population dynamics of *Plasmodium* sporogony. Trends Parasitol. 2007, 23(2):63-70.

31. Boyd MF. Malariology: A Comprehensive Survey of All Aspects of this Group of Diseases from a Global Standpoint. Philadelphia and London: W. B. Saunders & Co.; 1949.

32. Billingsley PF, Medley GF, Charlwood D, Sinden RE. Relationship between prevalence and intensity of *Plasmodium falciparum* infection in natural populations of *Anopheles* mosquitoes. Am J Trop Med Hyg. 1994, 51(3):260-270.

33. Drakeley C, Sutherland C, Bousema JT, Sauerwein RW, Targett GA. The epidemiology of *Plasmodium falciparum* gametocytes: weapons of mass dispersion. Trends Parasitol. 2006, 22(9):424-430.

34. Bousema T, Drakeley C. Epidemiology and Infectivity of *Plasmodium falciparum* and *Plasmodium vivax* Gametocytes in Relation to Malaria Control and Elimination. Clin Microbiol Rev. 2011, 24(2):377-410.

35. Carter R, Graves PM. Gametocytes. In: *Malaria: Principles and Practice of Malariology*. Edinburgh: Churchill Livingstone; 1988: 253-305.

36. Churcher TS, Bousema T, Walker M, Drakeley C, Schneider P, Ouedraogo AL, Basanez MG. Predicting mosquito infection from *Plasmodium falciparum* gametocyte density and estimating the reservoir of infection. eLife. 2013, 2:e00626.

37. Schneider P, Bousema JT, Gouagna LC, Otieno S, van de Vegte-Bolmer M, Omar SA, Sauerwein RW. Submicroscopic *Plasmodium falciparum* gametocyte densities frequently result in mosquito infection. Am J Trop Med Hyg. 2007, 76(3):470-474.

38. Paul RE, Bonnet S, Boudin C, Tchuinkam T, Robert V. Aggregation in malaria parasites places limits on mosquito infection rates. Infect Genet Evol. 2007, 7(5):577-586.

39. Medley GF, Sinden RE, Fleck S, Billingsley PF, Tirawanchai N, Rodriguez MH. Heterogeneity in patterns of malarial oocyst infections in the mosquito vector. Parasitology. 1993, 106 (Pt 5):441-449.

40. Pichon G, Awono-Ambene HP, Robert V. High heterogeneity in the number of *Plasmodium falciparum* gametocytes in the bloodmeal of mosquitoes fed on the same host. Parasitology. 2000, 121 (Pt 2):115-120.

41. Da DF, Churcher TS, Yerbanga RS, Yameogo B, Sangare I, Ouedraogo JB, Sinden RE, Blagborough AM, Cohuet A. Experimental study of the relationship between *Plasmodium* gametocyte density and infection success in mosquitoes; implications for the evaluation of malaria transmission-reducing interventions. Exp Parasitol. 2015, 149:74-83.

42. Stone WJ, Eldering M, van Gemert GJ, Lanke KH, Grignard L, van de Vegte-Bolmer MG, Siebelink-Stoter R, Graumans W, Roeffen WF, Drakeley CJ *et al*. The relevance and applicability of oocyst prevalence as a read-out for mosquito feeding assays. Sci Rep. 2013, 3:3418.

43. Churcher TS, Sinden RE, Edwards NJ, Poulton ID, Rampling TW, Brock PM, Griffin JT, Upton LM, Zakutansky SE, Sala KA *et al*. Probability of transmission of malaria from mosquito to human is regulated by mosquito parasite density in naive and vaccinated hosts. PLOS Pathog. 2017, 13(1):e1006108.

44. Rosenberg R, Wirtz RA, Schneider I, Burge R. An estimation of the number of malaria sporozoites ejected by a feeding mosquito. Trans R Soc Trop Med Hyg. 1990, 84(2):209-212.

45. Ponnudurai T, Lensen AH, van Gemert GJ, Bolmer MG, Meuwissen JH. Feeding behaviour and sporozoite ejection by infected *Anopheles stephensi*. Trans R Soc Trop Med Hyg. 1991, 85(2):175-180.

46. Aly AS, Vaughan AM, Kappe SH. Malaria parasite development in the mosquito and infection of the mammalian host. Annu Rev Microbiol. 2009, 63:195-221.

47. Smith RC, Vega-Rodriguez J, Jacobs-Lorena M. The *Plasmodium* bottleneck: malaria parasite losses in the mosquito vector. Mem Inst Oswaldo Cruz. 2014, 109(5):644-661.

48. Clayton AM, Dong Y, Dimopoulos G. The *Anopheles* innate immune system in the defense against malaria infection. J Innate Immun. 2014, 6(2):169-181.

49. Baton LA, Ranford-Cartwright LC. Spreading the seeds of million-murdering death: metamorphoses of malaria in the mosquito. Trends Parasitol. 2005, 21(12):573-580.

50. Churcher TS, Trape JF, Cohuet A. Human-to-mosquito transmission efficiency increases as malaria is controlled. Nat Commun. 2015, 6:6054.

51. Molineaux L. The epidemiology of malaria as an explanation of its distribution, including some implications for its control. In: *Malaria: Principles and Practice of Malariology.* Edited by Wernsdorfer WH, McGregor, I. Edinburgh: Churchill Livingstone; 1988: 913-998.

52. Killeen GF, McKenzie FE, Foy BD, Schieffelin C, Billingsley PF, Beier JC. The potential impact of integrated malaria transmission control on entomologic inoculation rate in highly endemic areas. Am J Trop Med Hyg. 2000, 62(5):545-551.

53. Shaukat AM, Breman JG, McKenzie FE. Using the entomological inoculation rate to assess the impact of vector control on malaria parasite transmission and elimination. Malar J. 2010, 9:122.

54. Tusting LS, Bousema T, Smith DL, Drakeley C. Measuring changes in *Plasmodium falciparum* transmission: precision, accuracy and costs of metrics. Adv Parasitol. 2014, 84:151-208.

55. Garrett-Jones C. The human blood index of malaria vectors in relation to epidemiological assessment. Bull World Health Organ. 1964, 30:241-261.

56. Macdonald G. The Epidemiology and Control of Malaria. London: Oxford University Press; 1957.

57. Dye C. The analysis of parasite transmission by bloodsucking insects. Annu Rev Entomol. 1992, 37:1-19.

58. Rivero A, Vezilier J, Weill M, Read AF, Gandon S. Insecticide control of vector-borne diseases: when is insecticide resistance a problem? PLoS Pathog. 2010, 6(8).

59. Lefevre T, Ohm J, Dabire KR, Cohuet A, Choisy M, Thomas MB, Cator L. Transmission traits of malaria parasites within the mosquito: Genetic variation, phenotypic plasticity, and consequences for control. Evol Appl. 2017;1-14.

60. Sinden RE. Targeting the Parasite to Suppress Malaria Transmission. Adv Parasitol. 2017, 97:147-185.

61. Williams A, Taulane JP, Russell PJ. Comparative inhibition patterns of adenylate kinases from mammals, bird, fish and microorganisms. Comp Biochem Physiol Biochem Mol Biol. 1994, 107(3):489-494.

62. Goncalves D, Hunziker P. Transmission-blocking strategies: the roadmap from laboratory bench to the community. Malar J. 2016, 15(1):95.

63. Sinden RE. Developing transmission-blocking strategies for malaria control. PLOS Pathog. 2017, 13(7):e1006336.

64. Carter R, Mendis K. Transmission immunity in malaria: reflections on the underlying immune mechanisms during natural infections and following artificial immunization. Mem Inst Oswaldo Cruz. 1992, 87 Suppl 3:169-173.

65. Carle PR, Coz J, Elissa N, Gasquet M, Sannier C, Richard A, Timon-David P. [Intravector anti-*Plasmodium* activity of a pyrethrinoid: deltamethrin]. C R Acad Sci III. 1986, 303(13):565-568.

66. Elissa N, Sannier C, Coz J. [Étude de la réceptivité à *Plasmodium yoelii yoelii* d'*Anopheles stephensi* soumis au stade adulte à des doses toxiques de différents pyréthrinoïdes]. Cah ORSTOM, ser Ent méd et Parasitol. 1987, 25(3-4):187-192.

67. Kristan M, Lines J, Nuwa A, Ntege C, Meek SR, Abeku TA. Exposure to deltamethrin affects development of *Plasmodium falciparum* inside wild pyrethroid resistant *Anopheles gambiae* s.s. mosquitoes in Uganda. Parasit Vectors. 2016, 9(1):100.

68. Kobylinski KC, Foy BD, Richardson JH. Ivermectin inhibits the sporogony of *Plasmodium falciparum* in *Anopheles gambiae*. Malar J. 2012, 11:381.

69. Hobbs CV, Tanaka TQ, Muratova O, Van Vliet J, Borkowsky W, Williamson KC, Duffy PE. HIV treatments have malaria gametocyte killing and transmission blocking activity. J Infect Dis. 2013, 208(1):139-148.

70. Blagborough AM, Delves MJ, Ramakrishnan C, Lal K, Butcher G, Sinden RE. Assessing transmission-blockade in *Plasmodium* spp. In: *Malaria: Methods and Protocols.* Edited by Ménard R, vol. 923: Springer Science+Business Media; 2012: 577-600.

71. Churcher TS, Blagborough AM, Delves M, Ramakrishnan C, Kapulu MC, Williams AR, Biswas S, Da DF, Cohuet A, Sinden RE. Measuring the blockade of malaria transmission - An analysis of the Standard Membrane Feeding Assay. Int J Parasitol. 2012, 42(11):1037-1044.

72. Sinden RE. Molecular interactions between *Plasmodium* and its insect vectors. Cell Microbiol. 2002, 4(11):713-724.

73. Huff CG. Studies on the infectivity of *Plasmodia* of birds for mosquitoes, with special reference to the problem of immunity in the mosquito. Am J Epidemiol. 1927, 7(6):706-734.

74. Lefevre T, Vantaux A, Dabire KR, Mouline K, Cohuet A. Non-genetic determinants of mosquito competence for malaria parasites. PLoS Pathog. 2013, 9(6):e1003365.

75. Lazzaro BP, Little TJ. Immunity in a variable world. Philos Trans R Soc Lond B Biol Sci. 2009, 364(1513):15-26.

76. Wolinska J, King KC. Environment can alter selection in host-parasite interactions. Trends Parasitol. 2009, 25(5):236-244.

77. Blandin SA, Wang-Sattler R, Lamacchia M, Gagneur J, Lycett G, Ning Y, Levashina EA, Steinmetz LM. Dissecting the genetic basis of resistance to malaria parasites in *Anopheles gambiae*. Science. 2009, 326(5949):147-150.

78. Riehle MM, Markianos K, Niare O, Xu J, Li J, Toure AM, Podiougou B, Oduol F, Diawara S, Diallo M *et al*. Natural malaria infection in *Anopheles gambiae* is regulated by a single genomic control region. Science. 2006, 312(5773):577-579.

79. Collins FH, Sakai RK, Vernick KD, Paskewitz S, Seeley DC, Miller LH, Collins WE, Campbell CC, Gwadz RW. Genetic selection of a *Plasmodium*-refractory strain of the malaria vector *Anopheles gambiae*. Science. 1986, 234(4776):607-610.

80. Vernick KD, Oduol F, Lazzaro BP, Glazebrook J, Xu J, Riehle M, Li J. Molecular genetics of mosquito resistance to malaria parasites. Curr Top Microbiol Immunol. 2005, 295:383-415.

81. Riehle MM, Markianos K, Lambrechts L, Xia A, Sharakhov I, Koella JC, Vernick KD. A major genetic locus controlling natural *Plasmodium falciparum* infection is shared by East and West African *Anopheles gambiae*. Malar J. 2007, 6:87.

82. Harris C, Lambrechts L, Rousset F, Abate L, Nsango SE, Fontenille D, Morlais I, Cohuet A. Polymorphisms in *Anopheles gambiae* immune genes associated with natural resistance to *Plasmodium falciparum*. PLoS Pathog. 2010, 6(9):e1001112.

83. Horton AA, Lee Y, Coulibaly CA, Rashbrook VK, Cornel AJ, Lanzaro GC, Luckhart S. Identification of three single nucleotide polymorphisms in *Anopheles gambiae* immune signaling genes that are associated with natural *Plasmodium falciparum* infection. Malar J 2010, 9:160.

84. Mitri C, Vernick KD. *Anopheles gambiae* pathogen susceptibility: the intersection of genetics, immunity and ecology. Curr Opin Microbiol. 2012, 15(3):285-291.

85. Jaramillo-Gutierrez G, Rodrigues J, Ndikuyeze G, Povelones M, Molina-Cruz A, Barillas-Mury C. Mosquito immune responses and compatibility between *Plasmodium* parasites and anopheline mosquitoes. BMC Microbiol. 2009, 9:154.

86. Povelones M, Waterhouse RM, Kafatos FC, Christophides GK. Leucine-rich repeat protein complex activates mosquito complement in defense against *Plasmodium* parasites. Science. 2009, 324(5924):258-261.

87. Gupta L, Molina-Cruz A, Kumar S, Rodrigues J, Dixit R, Zamora RE, Barillas-Mury C. The STAT pathway mediates late-phase immunity against *Plasmodium* in the mosquito *Anopheles gambiae*. Cell Host Microbe. 2009, 5(5):498-507.

88. Garver LS, Bahia AC, Das S, Souza-Neto JA, Shiao J, Dong Y, Dimopoulos G. *Anopheles* Imd pathway factors and effectors in infection intensity-dependent anti-*Plasmodium* action. PLoS Pathog. 2012, 8(6):e1002737.

89. Blandin SA, Marois E, Levashina EA. Antimalarial responses in *Anopheles gambiae*: from a complement-like protein to a complement-like pathway. Cell Host Microbe. 2008, 3(6):364-374.

90. Marois E. The multifaceted mosquito anti-*Plasmodium* response. Curr Opin Microbiol. 2011, 14(4):429-435.

91. Cirimotich CM, Dong Y, Garver LS, Sim S, Dimopoulos G. Mosquito immune defenses against *Plasmodium* infection. Dev Comp Immunol. 2010, 34(4):387-395.

92. Gomes PS, Bhardwaj J, Rivera-Correa J, Freire-De-Lima CG, Morrot A. Immune escape strategies of malaria parasites. Front Microbiol. 2016, 7:1617.

93. Dimopoulos G, Seeley D, Wolf A, Kafatos FC. Malaria infection of the mosquito *Anopheles gambiae* activates immune-responsive genes during critical transition stages of the parasite life cycle. EMBO J. 1998, 17(21):6115-6123.

94. Osta MA, Christophides GK, Kafatos FC. Effects of mosquito genes on *Plasmodium* development. Science. 2004, 303(5666):2030-2032.

95. Dimopoulos G. Insect immunity and its implication in mosquito-malaria interactions. Cell Microbiol. 2003, 5(1):3-14.

96. Michel K, Kafatos FC. Mosquito immunity against *Plasmodium*. Insect Biochem Mol Biol. 2005, 35(7):677-689.

97. Christophides GK, Vlachou D, Kafatos FC. Comparative and functional genomics of the innate immune system in the malaria vector *Anopheles gambiae*. Immunol Rev. 2004, 198:127-148.

98. Simoes ML, Mlambo G, Tripathi A, Dong Y, Dimopoulos G. Immune regulation of *Plasmodium* is *Anopheles* species specific and infection intensity dependent. mBio. 2017, 8(5).

99. Bryant WB, Michel K. Blood feeding induces hemocyte proliferation and activation in the African malaria mosquito, *Anopheles gambiae* Giles. J Exp Biol. 2014, 217(Pt 8):1238-1245.

100. Bryant WB, Michel K. *Anopheles gambiae* hemocytes exhibit transient states of activation. Dev Comp Immunol. 2016, 55:119-129.

101. Kumar S, Christophides GK, Cantera R, Charles B, Han YS, Meister S, Dimopoulos G, Kafatos FC, Barillas-Mury C. The role of reactive oxygen species on *Plasmodium* melanotic encapsulation in *Anopheles gambiae*. Proc Natl Acad Sci U S A. 2003, 100(24):14139-14144.

102. Dana AN, Hillenmeyer ME, Lobo NF, Kern MK, Romans PA, Collins FH. Differential gene expression in abdomens of the malaria vector mosquito, *Anopheles gambiae*, after sugar feeding, blood feeding and *Plasmodium berghei* infection. BMC Genomics. 2006, 7:119.

103. Sanders HR, Evans AM, Ross LS, Gill SS. Blood meal induces global changes in midgut gene expression in the disease vector, *Aedes aegypti*. Insect Biochem Mol Biol. 2003, 33(11):1105-1122.

104. Ribeiro JM. A catalogue of *Anopheles gambiae* transcripts significantly more or less expressed following a blood meal. Insect Biochem Mol Biol. 2003, 33(9):865-882.

105. Peterson TM, Luckhart S. A mosquito 2-Cys peroxiredoxin protects against nitrosative and oxidative stresses associated with malaria parasite infection. Free Radic Biol Med. 2006, 40(6):1067-1082.

106. Vlachou D, Schlegelmilch T, Christophides GK, Kafatos FC. Functional genomic analysis of midgut epithelial responses in *Anopheles* during *Plasmodium* invasion. Curr Biol. 2005, 15(13):1185-1195.

107. de Almeida Oliveira G, Lieberman J, Barillas-Mury C. Epithelial nitration by a peroxidase/NOX5 system mediates mosquito antiplasmodial immunity. Science. 2012, 335(6070):856-859.

108. Baton LA, Ranford-Cartwright LC. Do malaria ookinete surface proteins P25 and P28 mediate parasite entry into mosquito midgut epithelial cells? Malar J. 2005, 4:15.

109. Oliveira JH, Goncalves RL, Oliveira GA, Oliveira PL, Oliveira MF, Barillas-Mury C. Energy metabolism affects susceptibility of *Anopheles gambiae* mosquitoes to *Plasmodium* infection. Insect Biochem Mol Biol. 2011, 41(6):349-355.

110. Molina-Cruz A, DeJong RJ, Charles B, Gupta L, Kumar S, Jaramillo-Gutierrez G, Barillas-Mury C. Reactive oxygen species modulate *Anopheles gambiae* immunity against bacteria and *Plasmodium*. J Biol Chem. 2008, 283(6):3217-3223.

111. Kumar S, Gupta L, Han YS, Barillas-Mury C. Inducible peroxidases mediate nitration of anopheles midgut cells undergoing apoptosis in response to *Plasmodium* invasion. J Biol Chem. 2004, 279(51):53475-53482.

112. Felix RC, Muller P, Ribeiro V, Ranson H, Silveira H. *Plasmodium* infection alters *Anopheles gambiae* detoxification gene expression. BMC Genomics. 2010, 11:312.

113. Dong Y, Aguilar R, Xi Z, Warr E, Mongin E, Dimopoulos G. *Anopheles gambiae* immune responses to human and rodent *Plasmodium* parasite species. PLoS Pathog. 2006, 2(6):e52.

114. Djouaka RF, Bakare AA, Coulibaly ON, Akogbeto MC, Ranson H, Hemingway J, Strode C. Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of *Anopheles gambiae* s.s. from Southern Benin and Nigeria. BMC Genomics. 2008, 9:538.

115. Stevenson BJ, Bibby J, Pignatelli P, Muangnoicharoen S, O'Neill PM, Lian LY, Muller P, Nikou D, Steven A, Hemingway J *et al*. Cytochrome P450 6M2 from the malaria vector *Anopheles gambiae* metabolizes pyrethroids: Sequential metabolism of deltamethrin revealed. Insect Biochem Mol Biol. 2011, 41(7):492-502.

116. Muller P, Donnelly MJ, Ranson H. Transcription profiling of a recently colonised pyrethroid resistant *Anopheles gambiae* strain from Ghana. BMC Genomics. 2007, 8:36.

117. Felix R, Silveira H. The Role of *Anopheles gambiae* P450 Cytochrome in Insecticide Resistance and Infection. In: *Insecticides - Pest Engineering.* Edited by Perveen F: InTech; 2012: 503-518.

118. Cuamba N, Morgan JC, Irving H, Steven A, Wondji CS. High level of pyrethroid resistance in an *Anopheles funestus* population of the Chokwe District in Mozambique. PLoS One. 2010, 5(6):e11010.

119. Vontas JG, Small GJ, Hemingway J. Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*. Biochem J. 2001, 357(Pt 1):65-72.

120. Alout H, Yameogo B, Djogbenou LS, Chandre F, Dabire RK, Corbel V, Cohuet A. Interplay between *Plasmodium* infection and resistance to insecticides in vector mosquitoes. J Infect Dis. 2014, 210(9):1464-1470.

121. Farenhorst M, Mouatcho JC, Kikankie CK, Brooke BD, Hunt RH, Thomas MB, Koekemoer LL, Knols BG, Coetzee M. Fungal infection counters insecticide resistance in African malaria mosquitoes. Proc Natl Acad Sci U S A. 2009, 106(41):17443-17447.

122. Koella JC, Saddler A, Karacs TP. Blocking the evolution of insecticide-resistant malaria vectors with a microsporidian. Evol Appl. 2012, 5(3):283-292.

123. Buxton PA. The effect of climatic conditions upon populations of insects. Trans R Soc Trop Med Hyg. 1933, 26(1):325-356.

124. Freeborn SB. The seasonal life history of *Anopheles maculipennis* with reference to humidity requirements and "hibernation". Am J Hyg. 1932, 16(1):215-223.

125. Pal R. On the bionomics of *Anopheles culicifacies* Giles. J Malar Inst India. 1943, 5:77-85.

126. Knowles R, Basu BC. Laboratory studies of the infectivity of *Anopheles stephensi*. J Malar Inst India. 1943, 5:1-29.

127. de Meillon B. Some reactions of *Anopheles gambiae* and *Anopheles funestus* to environmental factors. Publications of the South African Institute for Medical Research. 1937(40):313-327.

128. Murdock CC, Paaijmans KP, Bell AS, King JG, Hillyer JF, Read AF, Thomas MB. Complex effects of temperature on mosquito immune function. Proc Biol Sci. 2012, 279(1741):3357-3366.

129. Murdock CC, Paaijmans KP, Cox-Foster D, Read AF, Thomas MB. Rethinking vector immunology: the role of environmental temperature in shaping resistance. Nat Rev Microbiol. 2012, 10(12):869-876.

130. Murdock CC, Moller-Jacobs LL, Thomas MB. Complex environmental drivers of immunity and resistance in malaria mosquitoes. Proc Biol Sci. 2013, 280(1770):20132030.

131. Detinova TS. Age-grouping methods in Diptera of medical importance with special reference to some vectors of malaria. Monogr Ser World Health Organ. 1962, 47:13-191.

132. Ohm JR, Baldini F, Barreaux P, Lefevre T, Lynch PA, Suh E, Whitehead SA, Thomas MB. Rethinking the extrinsic incubation period of malaria parasites. Parasit Vectors. 2018, 11(1):178.

133. Macdonald G. The analysis of the sporozoite rate. Trop Dis Bull. 1952, 49(6):569-586.

134. Eling W, Hooghof J, van de Vegte-Bolmer M, Sauerwein R, van Gemert G-J. Tropical temperatures can inhibit development of the human malaria parasite *Plasmodium falciparum* in the mosquito. Proc Exper Appl Entomol. 2001, 12:151-156.

135. Noden BH, Kent MD, Beier JC. The impact of variations in temperature on early *Plasmodium falciparum* development in *Anopheles stephensi*. Parasitology. 1995, 111 (Pt 5):539-545.

136. Okech BA, Gouagna LC, Kabiru EW, Walczak E, Beier JC, Yan G, Githure JI. Resistance of early midgut stages of natural *Plasmodium falciparum* parasites to high temperatures in experimentally infected *Anopheles gambiae* (Diptera: Culicidae). J Parasitol. 2004, 90(4):764-768.

137. Paaijmans KP, Blanford S, Bell AS, Blanford JI, Read AF, Thomas MB. Influence of climate on malaria transmission depends on daily temperature variation. Proc Natl Acad Sci U S A. 2010, 107(34):15135-15139.

138. Rastogi M, Pal NL, Sen AB. Effect of variation in temperature on development of *Plasmodium berghei* (NK 65 strain) in *Anopheles stephensi*. Folia Parasitol (Praha). 1987, 34(4):289-297.

139. Vanderberg JP, Yoeli M. Effects of temperature on sporogonic development of *Plasmodium berghei*. J Parasitol. 1966, 52(3):559-564.

140. Glunt KD, Paaijmans KP, Read AF, Thomas MB. Environmental temperatures significantly change the impact of insecticides measured using WHOPES protocols. Malar J. 2014, 13:350.

141. Hodjati MH, Curtis CF. Effects of permethrin at different temperatures on pyrethroid-resistant and susceptible strains of *Anopheles*. Med Vet Entomol. 1999, 13(4):415-422.

142. Cutkomp LK, Subramanyam B. Toxicity of pyrethroids to *Aedes aegypti* larvae in relation to temperature. J Am Mosq Control Assoc. 1986, 2(3):347-349.

143. Harwood AD, You J, Lydy MJ. Temperature as a toxicity identification evaluation tool for pyrethroid insecticides: toxicokinetic confirmation. Environ Toxicol Chem. 2009, 28(5):1051-1058.

144. Cohuet A, Harris C, Robert V, Fontenille D. Evolutionary forces on *Anopheles*: what makes a malaria vector? Trends Parasitol. 2010, 26(3):130-136.

145. Ferguson HM, Mackinnon MJ, Chan BH, Read AF. Mosquito mortality and the evolution of malaria virulence. Evolution. 2003, 57(12):2792-2804.

146. Beerntsen BT, James AA, Christensen BM. Genetics of mosquito vector competence. Microbiol Mol Biol Rev. 2000, 64(1):115-137.

147. Sinden RE, Alavi Y, Raine JD. Mosquito--malaria interactions: a reappraisal of the concepts of susceptibility and refractoriness. Insect Biochem Mol Biol. 2004, 34(7):625-629.

148. Yassine H, Osta MA. *Anopheles gambiae* innate immunity. Cell Microbiol. 2010, 12(1):1-9.

149. World Health Organization. Global Plan for Insecticide Resistance Management in Malaria Vectors. Geneva: World Health Organization; 2012.

150. World Health Organization. WHO recommended insecticides for indoor residual spraying against malaria vectors [http://www.who.int/whopes/Insecticides_IRS_2_March_2015.pdf?ua=1]

151. World Health Organization. WHO recommended insecticide products for treatment of mosquito nets for malaria vector control [http://www.who.int/whopes/Insecticides_ITN_Malaria_Sep_2016.pdf?ua=1]

152. World Health Organization. WHO recommended long-lasting insecticidal nets [http://www.who.int/whopes/Long-lasting_insecticidal_nets_June_2017.pdf?ua=1]

153. Schechter M, Green N, LaForge F. The synthesis of cyclopentenolones of the type of cinerolone. J Am Chem Soc. 1949, 71(4):1517-1517.

154. Elliott M, Farnham AW, Janes NF, Needham PH, Pulman DA, Stevenson JH. A photostable pyrethroid. Nature. 1973, 246(5429):169-170.

155. Davies TG, Field LM, Usherwood PN, Williamson MS. DDT, pyrethrins, pyrethroids and insect sodium channels. IUBMB life. 2007, 59(3):151-162.

156. Soderlund DM, Bloomquist JR. Neurotoxic actions of pyrethroid insecticides. Annu Rev Entomol. 1989, 34:77-96.

157. Palmquist K, Salatas J, Fairbrother A. Pyrethroid Insecticides: Use, Environmental Fate, and Ecotoxicology. In: *Insecticides - Advances in Integrated Pest Management.* Edited by Perveen F: InTech; 2012: 251-278.

158. WHO Expert Committee on Malaria. Assessment of susceptibility to insecticides in anopheline mosquitoes. Geneva, 1957.

159. Busvine JR. The significance of insecticide-resistant strains; with special reference to pests of medical importance. Bull World Health Organ. 1956, 15(3-5):389-401.

160. Busvine JR, Pal R. The impact of insecticide-resistance on control of vectors and vector-borne diseases. Bull World Health Organ. 1969, 40(5):731-744.

161. Hemingway J. The role of vector control in stopping the transmission of malaria: threats and opportunities. Philos Trans R Soc Lond B Biol Sci. 2014, 369(1645):20130431.

162. World Health Organization. World Malaria Report 2014. Geneva: World Health Organization; 2014.

163. 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(3):187-196.

164. Knox TB, Juma EO, Ochomo EO, Pates Jamet H, Ndungo L, Chege P, Bayoh NM, N'Guessan R, Christian RN, Hunt RH *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 Vectors. 2014, 7:76.

165. IVCC. Why do we need 3 new insecticides? [http://www.ivcc.com/creating-solutions/why-do-we-need-3-new-insecticides]

166. World Health Organization. Report of the twentieth WHOPES working group meeting, WHO/HQ, Geneva, 20-24 March 2017: review of Interceptor G2 LN, DawaPlus 3.0 LN, DawaPlus 4.0 LN, SumiLarv 2 MR, Chlorfenapyr 240 SC. Geneva: World Health Organization; 2017: 122.

167. Chandre F, Darriet F, Manguin S, Brengues C, Carnevale P, Guillet P. Pyrethroid cross resistance spectrum among populations of *Anopheles gambiae* s.s. from Cote d'Ivoire. J Am Mosq Control Assoc. 1999, 15(1):53-59.

168. Vulule JM, Beach RF, Atieli FK, Roberts JM, Mount DL, Mwangi RW. Reduced susceptibility of *Anopheles gambiae* to permethrin associated with the use of permethrin-impregnated bednets and curtains in Kenya. Med Vet Entomol. 1994, 8(1):71-75.

169. Elissa N, Mouchet J, Riviere F, Meunier JY, Yao K. Resistance of *Anopheles gambiae* s.s. to pyrethroids in Cote d'Ivoire. Ann Soc Belg Med Trop. 1993, 73(4):291-294.

170. Brown AW. Insecticide resistance in mosquitoes: a pragmatic review. J Am Mosq Control Assoc. 1986, 2(2):123-140.

171. Toe KH, N'Fale S, Dabire RK, Ranson H, Jones CM. The recent escalation in strength of pyrethroid resistance in *Anopheles coluzzii* in West Africa is linked to increased expression of multiple gene families. BMC Genomics. 2015, 16:146.

172. Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Berge JB, Devonshire AL, Guillet P, Pasteur N, Pauron D. Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae* s.s. Insect Mol Biol. 1998, 7(2):179-184.

173. Ranson H, Jensen B, Vulule JM, Wang X, Hemingway J, Collins FH. Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. Insect Mol Biol. 2000, 9(5):491-497.

174. Chandre F, Manguin S, Brengues C, Dossou Yovo J, Darriet F, Diabate A, Carnevale P, Guillet P. Current distribution of a pyrethroid resistance gene (*kdr*) in *Anopheles gambiae* complex from west Africa and further evidence for reproductive isolation of the Mopti form. Parassitologia. 1999, 41(1-3):319-322.

175. Vontas JG, Hejazi MJ, Hawkes NJ, Cosmidis N, Loukas M, Janes RW, Hemingway J. Resistance-associated point mutations of organophosphate insensitive acetylcholinesterase, in the olive fruit fly *Bactrocera oleae*. Insect Mol Biol. 2002, 11(4):329-336.

176. Reimer L, Fondjo E, Patchoke S, Diallo B, Lee Y, Ng A, Ndjemai HM, Atangana J, Traore SF, Lanzaro G *et al*. Relationship between *kdr* mutation and resistance to pyrethroid and DDT insecticides in natural populations of *Anopheles gambiae*. J Med Entomol. 2008, 45(2):260-266.

177. Jones CM, Liyanapathirana M, Agossa FR, Weetman D, Ranson H, Donnelly MJ, Wilding CS. Footprints of positive selection associated with a mutation (*N1575Y*) in the voltage-gated sodium channel of *Anopheles gambiae*. Proc Natl Acad Sci U S A. 2012, 109(17):6614-6619.

178. Diop MM, Moiroux N, Chandre F, Martin-Herrou H, Milesi P, Boussari O, Porciani A, Duchon S, Labbe P, Pennetier C. Behavioral cost and overdominance in *Anopheles gambiae*. PLoS One. 2015, 10(4):e0121755.

179. Silva AP, Santos JM, Martins AJ. Mutations in the voltage-gated sodium channel gene of anophelines and their association with resistance to pyrethroids - a review. Parasit Vectors. 2014, 7:450.

180. Hemingway J, Hawkes NJ, McCarroll L, Ranson H. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem Mol Biol. 2004, 34(7):653-665.

181. Ranson H, Claudianos C, Ortelli F, Abgrall C, Hemingway J, Sharakhova MV, Unger MF, Collins FH, Feyereisen R. Evolution of supergene families associated with insecticide resistance. Science. 2002, 298(5591):179-181.

182. James RR, Xu J. Mechanisms by which pesticides affect insect immunity. J Invertebr Pathol. 2012, 109(2):175-182.

183. Kurt D, Kayis T. Effects of the pyrethroid insecticide deltamethrin on the hemocytes of *Galleria mellonella*. Turk J Zool. 2015, 39:452-457.

184. Nakhleh J, Christophides GK, Osta MA. The serine protease homolog CLIPA14 modulates the intensity of the immune response in the mosquito *Anopheles gambiae*. J Biol Chem. 2017, 292(44):18217-18226.

185. Gassner B, Wuthrich A, Scholtysik G, Solioz M. The pyrethroids permethrin and cyhalothrin are potent inhibitors of the mitochondrial complex I. J Pharmacol Exp Ther. 1997, 281(2):855-860.

186. Prasittisuk C, Curtis CF. Absence of effects of insecticides on susceptibility of anophelines to *Plasmodium yoelii nigeriensis*. Southeast Asian J Trop Med Public Health. 1982, 13(1):127-132.

187. Rifaat MA, Khalil HM, Gad AM, Sadek S. Effect of sublethal concentrations of the insecticides DDT, Abate and Sevin applied to 3rd stage larvae of *Anopheles pharoensis* on malaria cycle in the adult mosquito. J Egypt Public Health Assoc. 1974, 49(6):329-340.

188. Alout H, Djegbe I, Chandre F, Djogbenou LS, Dabire RK, Corbel V, Cohuet A. Insecticide exposure impacts vector-parasite interactions in insecticide-resistant malaria vectors. Proc Biol Sci. 2014, 281(1786).

189. Elissa N, Sannier C, Richard A. [La Receptivite a *Plasmodium yoelii yoelii* d'*Anopheles* Soumis a des Subletales de Deltamethrine]. In: *IVe Congres Sur La Protection De La Sante Humaine Et Des Cultures En Milieu Tropical*. Marseille, France; 1986: 599-604.

190. Chunina LM, Iakubovich V, Ganushkina LA, Zakharova NF, Dadasheva NR. [The absence of an action of the pyrethroids deltamethrin and cypermethrin on mosquito susceptibility to the causative agent of malaria]. Med Parazitol (Mosk). 1990(1):20-23.

191. Molineaux L, Shidrawi GR, Clarke JL, Boulzaguet JR, Ashkar TS. Assessment of insecticidal impact on the malaria mosquito's vectorial capacity, from data on the man-biting rate and age-composition. Bull World Health Organ. 1979, 57(2):265-274.

192. Garrett-Jones C, Grab B. The Assessment of Insecticidal Impact on the Malaria Mosquito's Vectorial Capacity, from Data on the Proportion of Parous Females. Bull World Health Organ. 1964, 31:71-86.

193. Busvine JR. Current problems in the control of mosquitoes. Nature. 1978, 273(5664):604-607.

194. 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(2):181-189.

195. Brooke BD, Kloke G, Hunt RH, Koekemoer LL, Temu EA, Taylor ME, Small G, Hemingway J, Coetzee M. Bioassay and biochemical analyses of insecticide resistance in southern African *Anopheles funestus* (Diptera: Culicidae). Bull Entomol Res. 2001, 91(4):265-272.

196. Maharaj R, Mthembu DJ, Sharp BL. Impact of DDT re-introduction on malaria transmission in KwaZulu-Natal. S Afr Med J. 2005, 95(11):871-874.

197. N'Guessan R, Corbel V, Akogbeto M, Rowland M. Reduced efficacy of insecticide-treated nets and indoor residual spraying for malaria control in pyrethroid resistance area, Benin. Emerg Infect Dis. 2007, 13(2):199-206.

198. Weston DP, You J, Harwood AD, Lydy MJ. Whole sediment toxicity identification evaluation tools for pyrethroid insecticides: III. Temperature manipulation. Environ Toxicol Chem. 2009, 28(1):173-180.

199. Alout H, Djogbenou L, Berticat C, Chandre F, Weill M. Comparison of *Anopheles gambiae* and *Culex pipiens* acetycholinesterase 1 biochemical properties. Comp Biochem Physiol B Biochem Mol Biol. 2008, 150(3):271-277.

200. Simonsen PE, Pedersen EM, Rwegoshora RT, Malecela MN, Derua YA, Magesa SM. Lymphatic filariasis control in Tanzania: effect of repeated mass drug administration with ivermectin and albendazole on infection and transmission. PLoS Negl Trop Dis. 2010, 4(6):e696.

201. McKenzie JA, Whitten MJ, Adena MA. The effect of genetic background on the fitness of diazinon resistance genotypes of the Australian sheep blowfly, *Lucilia cuprina*. Heredity. 1982, 49(1):1-9.

202. Djogbenou L, Noel V, Agnew P. Costs of insensitive acetylcholinesterase insecticide resistance for the malaria vector *Anopheles gambiae* homozygous for the *G119S* mutation. Malar J. 2010, 9:12.

203. Agnew P, Berticat C, Bedhomme S, Sidobre C, Michalakis Y. Parasitism increases and decreases the costs of insecticide resistance in mosquitoes. Evolution. 2004, 58(3):579-586.

204. Platt N, Kwiatkowska RM, Irving H, Diabate A, Dabire R, Wondji CS. Target-site resistance mutations (*kdr* and *RDL*), but not metabolic resistance, negatively impact male mating competiveness in the malaria vector *Anopheles gambiae*. Heredity (Edinb). 2015, 115(3):243-252.

205. Rivero A, Magaud A, Nicot A, Vezilier J. Energetic cost of insecticide resistance in *Culex pipiens* mosquitoes. J Med Entomol. 2011, 48(3):694-700.

206. Hardstone MC, Lazzaro BP, Scott JG. The effect of three environmental conditions on the fitness of cytochrome P450 monooxygenase-mediated permethrin resistance in *Culex pipiens quinquefasciatus*. BMC Evol Biol. 2009, 9:42.

207. Vontas J, Blass C, Koutsos AC, David JP, Kafatos FC, Louis C, Hemingway J, Christophides GK, Ranson H. Gene expression in insecticide resistant and susceptible *Anopheles gambiae* strains constitutively or after insecticide exposure. Insect Mol Biol. 2005, 14(5):509-521.

208. Vontas J, David JP, Nikou D, Hemingway J, Christophides GK, Louis C, Ranson H. Transcriptional analysis of insecticide resistance in *Anopheles stephensi* using cross-species microarray hybridization. Insect Mol Biol. 2007, 16(3):315-324.

209. Reid WR, Zhang L, Gong Y, Li T, Liu N. Gene expression profiles of the Southern house mosquito *Culex quinquefasciatus* during exposure to permethrin. Insect Sci. 2018, 25(3):439-453.

210. Gong Y, Li T, Zhang L, Gao X, Liu N. Permethrin induction of multiple cytochrome P450 genes in insecticide resistant mosquitoes, *Culex quinquefasciatus*. Int J Biol Sci. 2013, 9(9):863-871.

211. Yang T, Liu N. Genome analysis of cytochrome P450s and their expression profiles in insecticide resistant mosquitoes, *Culex quinquefasciatus*. PLoS One. 2011, 6(12):e29418.

212. Vezilier J, Nicot A, Gandon S, Rivero A. Insecticide resistance and malaria transmission: infection rate and oocyst burden in *Culex pipiens* mosquitoes infected with *Plasmodium relictum*. Malar J. 2010, 9:379.

213. Lo TM, Coetzee M. Marked biological differences between insecticide resistant and susceptible strains of *Anopheles funestus* infected with the murine parasite *Plasmodium berghei*. Parasit Vectors. 2013, 6:184.

214. Gendrin M, Rodgers FH, Yerbanga RS, Ouedraogo JB, Basanez MG, Cohuet A, Christophides GK. Antibiotics in ingested human blood affect the mosquito microbiota and capacity to transmit malaria. Nat Commun. 2015, 6:5921.

215. Mitri C, Markianos K, Guelbeogo WM, Bischoff E, Gneme A, Eiglmeier K, Holm I, Sagnon N, Vernick KD, Riehle MM. The *kdr*-bearing haplotype and susceptibility to *Plasmodium falciparum* in *Anopheles gambiae*: genetic correlation and functional testing. Malar J. 2015, 14(1):391.

216. Alout H, Ndam NT, Sandeu MM, Djegbe I, Chandre F, Dabire RK, Djogbenou LS, Corbel V, Cohuet A. Insecticide resistance alleles affect vector competence of *Anopheles gambiae* s.s. for *Plasmodium falciparum* field isolates. PLoS One. 2013, 8(5):e63849.

217. Ndiath MO, Cailleau A, Diedhiou SM, Gaye A, Boudin C, Richard V, Trape JF. Effects of the *kdr* resistance mutation on the susceptibility of wild *Anopheles gambiae* populations to *Plasmodium falciparum*: a hindrance for vector control. Malar J. 2014, 13:340.

218. Alout H, Dabire RK, Djogbenou LS, Abate L, Corbel V, Chandre F, Cohuet A. Interactive cost of *Plasmodium* infection and insecticide resistance in the malaria vector *Anopheles gambiae*. Sci Rep. 2016, 6:29755.

219. Lines JD, Nassor NS. DDT resistance in *Anopheles gambiae* declines with mosquito age. Med Vet Entomol. 1991, 5(3):261-265.

220. Rajatileka S, Burhani J, Ranson H. Mosquito age and susceptibility to insecticides. Trans R Soc Trop Med Hyg. 2011, 105(5):247-253.

221. Glunt KD, Thomas MB, Read AF. The effects of age, exposure history and malaria infection on the susceptibility of *Anopheles* mosquitoes to low concentrations of pyrethroid. PLoS One. 2011, 6(9):e24968.

222. Saddler A, Burda PC, Koella JC. Resisting infection by *Plasmodium berghei* increases the sensitivity of the malaria vector *Anopheles gambiae* to DDT. Malar J. 2015, 14:134.

223. Vezilier J, Nicot A, Gandon S, Rivero A. *Plasmodium* infection decreases fecundity and increases survival of mosquitoes. Proc Biol Sci. 2012, 279(1744):4033-4041.

224. Hemingway J, Vontas J, Poupardin R, Raman J, Lines J, Schwabe C, Matias A, Kleinschmidt I. Country-level operational implementation of the Global Plan for Insecticide Resistance Management. Proc Natl Acad Sci U S A. 2013, 110(23):9397-9402.

225. Kabula B, Tungu P, Rippon EJ, Steen K, Kisinza W, Magesa S, Mosha F, Donnelly MJ. A significant association between deltamethrin resistance, *Plasmodium falciparum* infection and the *Vgsc-1014S* resistance mutation in *Anopheles gambiae* highlights the epidemiological importance of resistance markers. Malar J. 2016, 15(1):1-5.

226. World Health Organization. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. 2nd edn. Geneva, Switzerland: World Health Organization; 2016.

227. Weetman D, Wilding CS, Neafsey DE, Muller P, Ochomo E, Isaacs AT, Steen K, Rippon EJ, Morgan JC, Mawejje HD *et al*. Candidate-gene based GWAS identifies reproducible DNA markers for metabolic pyrethroid resistance from standing genetic variation in East African *Anopheles gambiae*. Scientific reports. 2018, 8(1):2920.

228. Venter N, Oliver SV, Muleba M, Davies C, Hunt RH, Koekemoer LL, Coetzee M, Brooke BD. Benchmarking insecticide resistance intensity bioassays for *Anopheles* malaria vector species against resistance phenotypes of known epidemiological significance. Parasit Vectors. 2017, 10(1):198.

229. Bagi J, Grisales N, Corkill R, Morgan JC, N'Fale S, Brogdon WG, Ranson H. When a discriminating dose assay is not enough: measuring the intensity of insecticide resistance in malaria vectors. Malar J. 2015, 14:210.

230. Wondji CS, Coleman M, Kleinschmidt I, Mzilahowa T, Irving H, Ndula M, Rehman A, Morgan J, Barnes KG, Hemingway J. Impact of pyrethroid resistance on operational malaria control in Malawi. Proc Natl Acad Sci U S A. 2012, 109(47):19063-19070.

231. 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 Med. 2014, 11(3):e1001619.

232. Maharaj R, Raman J, Morris N, Moonasar D, Durrheim DN, Seocharan I, Kruger P, Shandukani B, Kleinschmidt I. Epidemiology of malaria in South Africa: from control to elimination. S Afr Med J. 2013, 103(10 Pt 2):779-783.

233. Hargreaves K, Hunt RH, Brooke BD, Mthembu J, Weeto MM, Awolola TS, Coetzee M. *Anopheles arabiensis* and *An. quadriannulatus* resistance to DDT in South Africa. Med Vet Entomol. 2003, 17(4):417-422.

234. Toé KH, Jones CM, N'Fale S, Ismail HM, Dabire KR, Ranson H. Increased pyrethroid resistance in malaria vectors and decreased bed net effectiveness, Burkina Faso. Emerg Infect Dis. 2014, 20:1691-1696.

235. Wanjala CL, Zhou G, Mbugi J, Simbauni J, Afrane YA, Ototo E, Gesuge M, Atieli H, Githeko AK, Yan G. Insecticidal decay effects of long-lasting insecticide nets and indoor residual spraying on *Anopheles gambiae* and *Anopheles arabiensis* in Western Kenya. Parasit Vectors. 2015, 8:588.

236. Ochomo EO, Bayoh NM, Walker ED, Abongo BO, Ombok MO, Ouma C, Githeko AK, Vulule J, Yan G, Gimnig JE. 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.

237. Trape JF, Tall A, Diagne N, Ndiath O, Ly AB, Faye J, Dieye-Ba F, Roucher C, Bouganali C, Badiane A *et al*. Malaria morbidity and pyrethroid resistance after the introduction of insecticide-treated bednets and artemisinin-based combination therapies: a longitudinal study. Lancet Infect Dis. 2011.

238. Hemingway J, Ranson H, Magill A, Kolaczinski J, Fornadel C, Gimnig J, Coetzee M, Simard F, Roch DK, Hinzoumbe CK *et al*. Averting a malaria disaster: will insecticide resistance derail malaria control? Lancet. 2016, 387(10029):1785-1788.

239. 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.

240. Corbel V, Chandre F, Brengues C, Akogbeto M, Lardeux F, Hougard JM, Guillet P. Dosage-dependent effects of permethrin-treated nets on the behaviour of *Anopheles gambiae* and the selection of pyrethroid resistance. Malar J. 2004, 3:22.

241. Okia M, Ndyomugyenyi R, Kirunda J, Byaruhanga A, Adibaku S, Lwamafa DK, Kironde F. Bioefficacy of long-lasting insecticidal nets against pyrethroid-resistant populations of

Anopheles gambiae s.s. from different malaria transmission zones in Uganda. Parasit Vectors. 2013, 6:130.

242. Tokponnon FT, Ogouyemi AH, Sissinto Y, Sovi A, Gnanguenon V, Cornelie S, Adeothy AA, Osse R, Wakpo A, Gbenou D *et al*. Impact of long-lasting, insecticidal nets on anaemia and prevalence of *Plasmodium falciparum* among children under five years in areas with highly resistant malaria vectors. Malar J. 2014, 13:76.

243. Kilian A, Byamukama W, Pigeon O, Gimnig J, Atieli F, Koekemoer L, Protopopoff N. Evidence for a useful life of more than three years for a polyester-based long-lasting insecticidal mosquito net in Western Uganda. Malar J. 2011, 10:299.

244. Kafy HT, Ismail BA, Mnzava AP, Lines J, Abdin MSE, Eltaher JS, Banaga AO, West P, Bradley J, Cook J *et al*. Impact of insecticide resistance in *Anopheles arabiensis* on malaria incidence and prevalence in Sudan and the costs of mitigation. Proc Natl Acad Sci U S A. 2017, 114(52):E11267-E11275.

245. Kleinschmidt I, Sharp B, Benavente LE, Schwabe C, Torrez M, Kuklinski J, Morris N, Raman J, Carter J. Reduction in infection with *Plasmodium falciparum* one year after the introduction of malaria control interventions on Bioko Island, Equatorial Guinea. Am J Trop Med Hyg. 2006, 74(6):972-978.

246. Sharp BL, Ridl FC, Govender D, Kuklinski J, Kleinschmidt I. Malaria vector control by indoor residual insecticide spraying on the tropical island of Bioko, Equatorial Guinea. Malar J. 2007, 6:52.

247. Lindblade KA, Mwandama D, Mzilahowa T, Steinhardt L, Gimnig J, Shah M, Bauleni A, Wong J, Wiegand R, Howell P *et al*. A cohort study of the effectiveness of insecticide-treated bed nets to prevent malaria in an area of moderate pyrethroid resistance, Malawi. Malar J. 2015, 14:31.

248. Stump AD, Atieli FK, Vulule JM, Besansky NJ. Dynamics of the pyrethroid knockdown resistance allele in western Kenyan populations of *Anopheles gambiae* in response to insecticide-treated bed net trials. Am J Trop Med Hyg. 2004, 70(6):591-596.

249. Mathias DK, Ochomo E, Atieli F, Ombok M, Bayoh MN, Olang G, Muhia D, Kamau L, Vulule JM, Hamel MJ *et al*. Spatial and temporal variation in the *kdr* allele *L1014S* in

Anopheles gambiae s.s. and phenotypic variability in susceptibility to insecticides in Western Kenya. Malar J. 2011, 10:10.

250. Ochomo E, Chahilu M, Cook J, Kinyari T, Bayoh NM, West P, Kamau L, Osangale A, Ombok M, Njagi K *et al*. Insecticide-treated nets and protection against insecticide-resistant malaria vectors in Western Kenya. Emerg Infect Dis. 2017, 23(5):758-764.

Chapter 2. Methods

2.1 Field study in Uganda

Although each chapter contains a Methods section this chapter provides further descriptions and additional details on the methods used.

2.1.1 Study area

For the field study, a suitable field site was selected, taking into account the following characteristics:

- A rural setting with a high malaria transmission potential
- Has a health centre III or IV with a malaria microscopy service
- Has retrospective morbidity and/or entomological data including insecticide resistance
- Has a laboratory space to set up experiments and to use as a temporary insectary for rearing and keeping live mosquitoes

The study was conducted at and in the catchment area of Butemba Health Centre III, Butemba County, Kyankwanzi District, Uganda, between August 2013 and June 2014. The field laboratory and insectary were based at Butemba Health Centre III (coordinates of the Health Centre: 1°8'33.86"N, 31°36'8.79"E; altitude 1,107 m) (Fig. 2-1).

This study was carried out in collaboration with Malaria Consortium, who used the study site for other entomological and epidemiological studies [1, 2].

Fig. 2-1. Map of Uganda showing location of Bukwiri (purple marker), the town nearest to Butemba Health Centre III, Kyankwanzi District (Map data: Google).

2.1.2 Mosquitoes used in transmission experiments

Anopheles gambiae s.l. mosquitoes were collected as larvae from breeding sites in the catchment area of the health centre. The adults reared from collected larvae were used to:

- Study transmissibility of *Plasmodium falciparum* in experiments representing actual field conditions
- Determine resistance to insecticides by using WHO susceptibility tests

A field laboratory was established within the health centre for mosquito rearing and experiments. Both adults and immature stages were kept in the facility.

Collection and rearing of larvae and pupae

Different prolific breeding sites were identified and used for collection (Fig. 2-2).

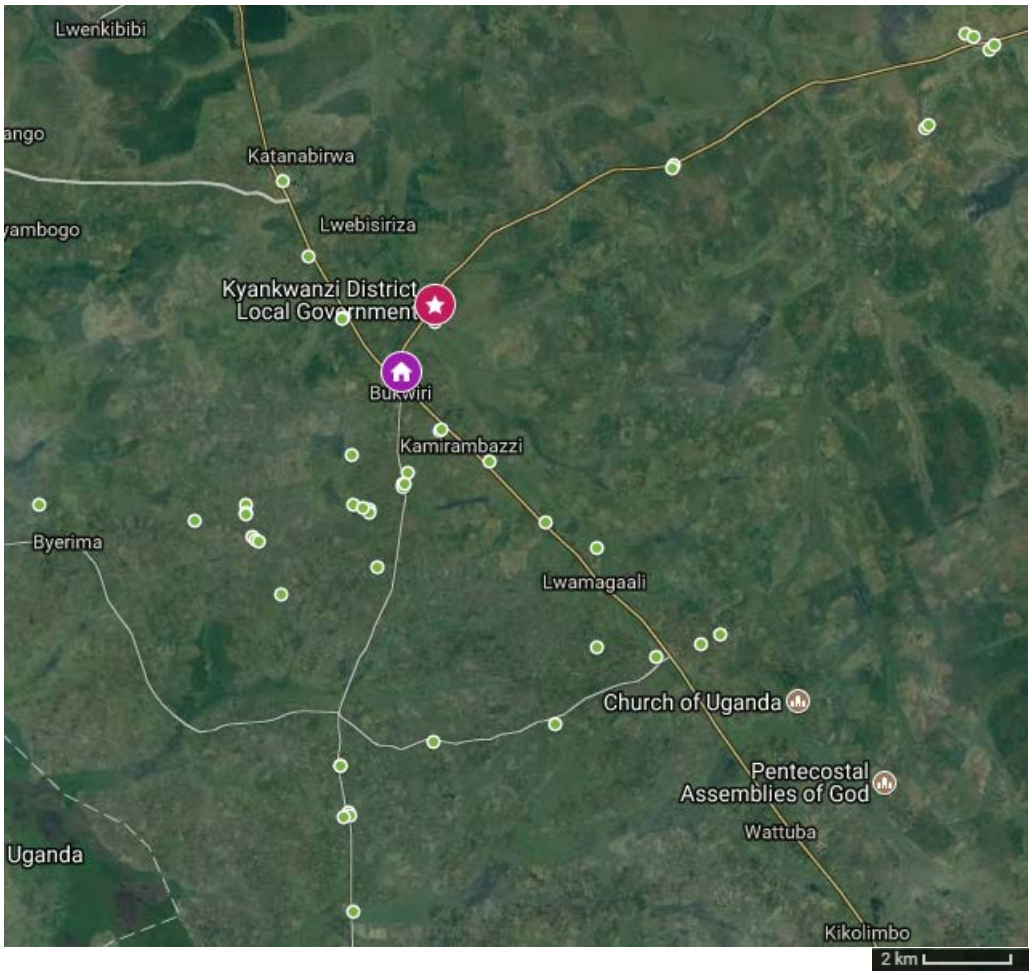

Fig. 2-2. Map of the field site in Kyankwanzi, mid-western Uganda showing location of Bukwiri (purple marker), the town nearest to the field laboratory at Butemba Health Centre III (red marker with star). Larvae were collected in the wider area around Bukwiri, Kyankwanzi (larval sites where larvae were collected for rearing purposes shown with green markers) (Map data: Google, TerraMetrics).

Collected larvae and pupae were kept in plastic containers covered with netting material in the water brought from their breeding sites (Fig. 2-3 and Fig. 2-4). Emerging adults were put into separate cages according to age, to be used in batches for membrane feeding experiments and insecticide susceptibility tests (Fig. 2-3). They were provided with 10 % glucose solution on cotton wool pads which were changed daily. One to two days old adult female mosquitoes were used in WHO susceptibility tests and up to ten days old adult female mosquitoes were used in transmission experiments.

Fig. 2-3. Field laboratory at Butemba Health Centre III where rearing of immature stages took place and emerged adults were kept.

Fig. 2-4. Rearing of immature stages at the field laboratory at Butemba Health Centre III.

2.1.3 Transmission experiments

Field studies were carried out using wild *An. gambiae* s.l. in direct membrane feeding experiments [3, 4], using venous blood samples obtained from local gametocytaemic volunteers.

Selection of gametocytaemic volunteers

In total, forty-seven volunteers taking part in infective membrane feeding experiments were recruited from among the outpatients attending Butemba Health Centre III, who were positive for malaria and had gametocytes in their peripheral blood. Because of exclusion of some feeds from data analysis, the total number of feeds (and hence volunteers) actually used and reported was 42. The first three feeds were excluded because they were not followed by insecticide exposure experiments and were mostly used for setting up the experimental system. Another feed was excluded because no mosquitoes fed. Finally, a feed where a different dose of deltamethrin was used was excluded as the number of mosquitoes in it were not sufficient to be included in analysis.

Patients had to fulfil the following inclusion criteria to be recruited:

- 2 years or older as taking blood samples for transmission experiments was thought to be unacceptable
- *P. falciparum* positive with microscopically detectable gametocytes
- No sign of severe illness
- Not HIV positive as specific HIV treatments were shown to have transmission-blocking and gametocyte killing properties [5]
- Non-pregnant if adult female
- Haemoglobin level of >9.9 g/dl to exclude those with moderate and severe anaemia.

Inclusion criteria were chosen in agreement with the clinical officers at the Health Centre. Written consents of the participating patients or their guardians were sought before they took part in the study. A table showing characteristics of the 42 gametocytaemic volunteers is included in the Appendix 2-1.

Routinely prepared blood smears stained by Giemsa were used to count gametocytes against 200 leucocytes in thick blood smears. Density was calculated assuming a standard leukocyte count of 8,000/µL of blood [6].

Direct membrane feeding experiments

All membrane feeding experiments took place on the day of recruitment. After recruitment, a venous blood sample of approximately 9 ml was collected from volunteers into heparinised tubes. Blood samples were transferred to pre-warmed calibrated membrane feeders (Hemotek Membrane Feeding System, Hemotek Ltd, UK) within 10 minutes and were held at 37.5^{o}C throughout the feed (Fig. 2-5).

Fig. 2-5. Hemotek membrane feeding system was used in direct membrane feeding experiments. Feeders were held at 37.5^{o}C throughout the feed. Parafilm artificial membrane was used.

In three experiments, blood samples were kept for up to 1 ½ hours in a water bath at 37 °C before transfer to the membrane feeders.

Laboratory-reared, previously unfed, up to 10 days old female mosquitoes were allowed to feed through an artificial Parafilm membrane for up to 2 hours. On average 217 mosquitoes were used per infective feed, divided into paper cups with approximately 40 females in each cup (Fig. 2-6).

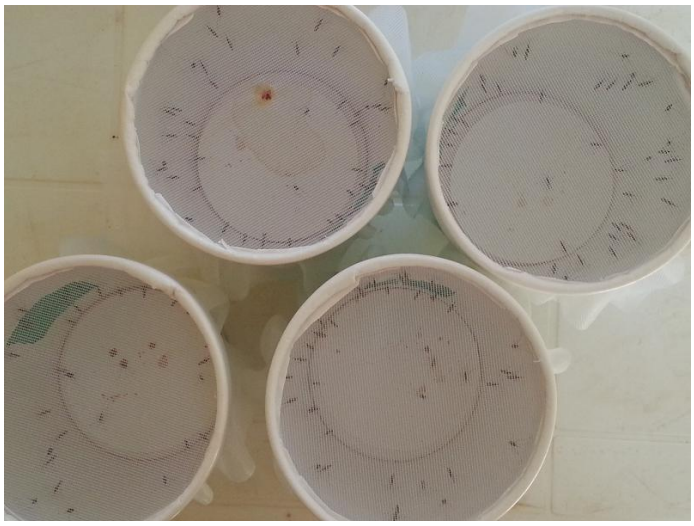

Fig. 2-6. Female mosquitoes were sorted into paper cups prior to the feeds and kept in them throughout the seven day incubation period.

Within 1-3 hours after feeding, mosquitoes were sorted to select only fully fed ones for the experiments. Some of the blood-fed mosquitoes were exposed to a net treated with a sublethal dose of deltamethrin for 5 min using a wire ball frame, while others were exposed to an untreated net as control (Fig. 2-7).

Fig. 2-7. Blood-fed mosquitoes were exposed to untreated control nets or nets treated with a sublethal dose of deltamethrin for 5 min using a wire ball frame. Low dose range was 2.5-5.0mg/m^2 and high dose range was 0.0-16.7mg/m^2 deltamethrin. These doses were chosen to mimic concentrations that can be found on aging used nets.

Following exposure, mosquitoes were kept in paper cups with access to 10 % glucose solution. Mosquito survival was monitored on a daily basis.

Seven days after infection, midguts of surviving females were dissected in 0.25 % mercurochrome in phosphate buffer saline (PBS) solution and examined for oocysts (Fig. 2-8 and Fig. 2-9).

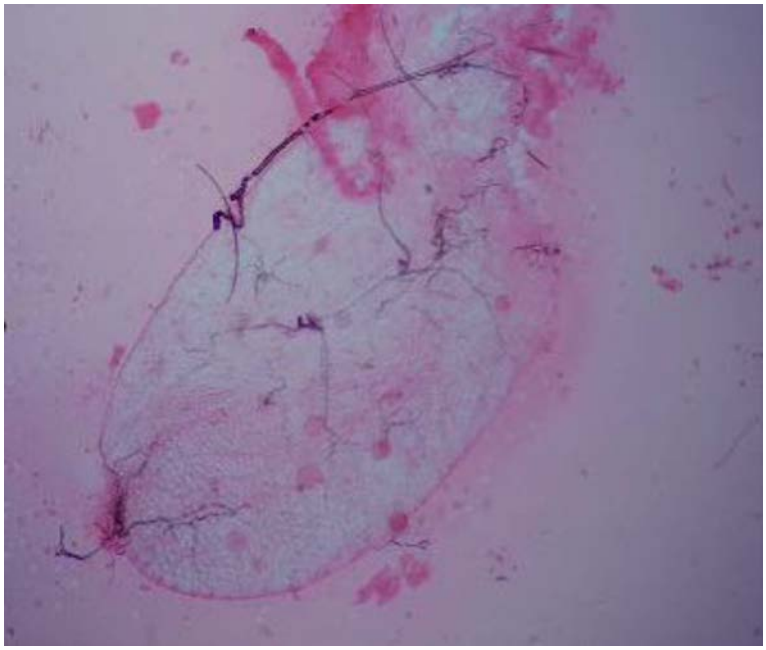

Fig. 2-8. Midguts of surviving females were dissected seven days after the feed in 0.25 % mercurochrome in phosphate buffer saline (PBS) solution and examined for oocysts.

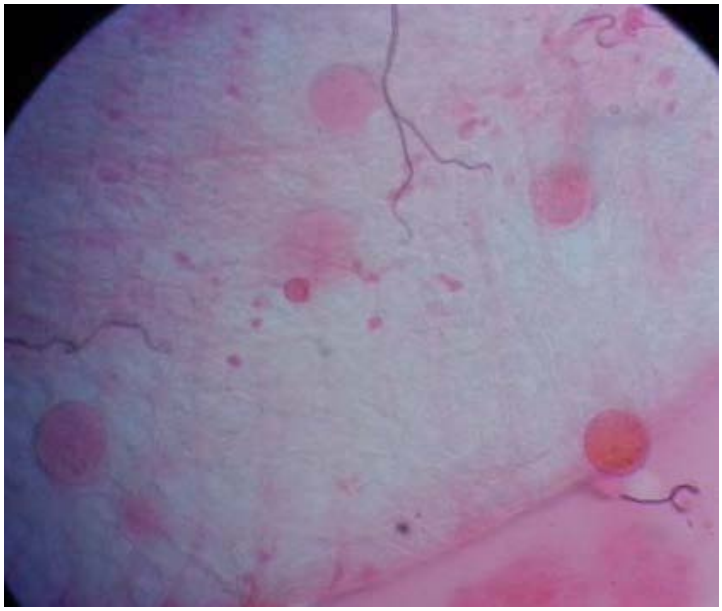

Fig. 2-9. Oocyst presence (i.e. prevalence) and the number of oocysts (i.e. intensity of infection) were recorded after midgut dissections.

Experimental mosquito nets

Sublethal insecticide doses to be used in transmission experiments were determined by treating the nets (untreated polyester nets, Vestergaard Frandsen) with different doses of deltamethrin (K-Othrine/Deltamethrin SC 10B G, Bayer, concentration 9.7g/l). Mosquitoes were exposed to the nets for 5 minutes using a wire ball frame. Knock-down after 5 minutes and 24-hour mortality were recorded.

Temperature and humidity measurement

Temperature and humidity measurements were recorded automatically every 30 minutes during the experiments or incubation periods using EL-USB-2 relative humidity and temperature data loggers (Lascar Electronics).

Summary of number of volunteers and mosquitoes used

Table 2-1 shows the summary of transmission experiments carried out at Butemba for this project. Forty-seven gametocytaemic volunteers were recruited. However, insecticide exposure was carried out from feed four onwards, so mosquitoes used in the first three experiments were not included in any analyses.

Wild mosquitoes were used (i.e. adults emerged from collected larvae) and were therefore not adapted to experimental feeding using artificial membrane, which resulted in low feeding rates. This was also the reason we let mosquitoes feed up to 2 hours instead of the more usual 15-30 minutes as described by most studies using this method. We also used females up to 10 days old to use the maximum number of female mosquitoes available when we recruited a gametocytaemic volunteer.

Table 2-1. Summary of the transmission experiments carried out for this project.

Gametocytaemic volunteers recruited		47
Membrane feeding experiments		47
Post-feed insecticide exposure experiments		43
Membrane feeding experiments included in the analyses		42
Mosquitoes used in transmission experiments		10,207
Mosquitoes fully fed		1,456
Mean blood-feeding rate		14%
Mosquitoes that survived until dissection on day 7		1,023
Mosquitoes successfully dissected:		
	Total	950
	An. gambiae s.s.	845
	An. arabiensis	81
	Others	24
Mosquitoes that died before dissection:		
	Total	434
	An. gambiae s.s.	351
	An. arabiensis	41
	Others	42

2.1.4 WHO insecticide susceptibility tests

Mosquitoes used in susceptibility tests were collected as larvae and reared in the insectary until they emerged. One to two days old unfed adult female mosquitoes were used in susceptibility assays. Mosquitoes were subjected to standard WHO susceptibility tests [7], using the following insecticide-impregnated papers and dosages: deltamethrin (0.05%), DDT (4%), bendiocarb (0.1%), pirimiphos-methyl (0.25%), permethrin (0.75%), and lambda-

cyhalothrin (0.05%). The bioassay kits, and all the insecticide-impregnated and control papers, were supplied by Universiti Sains Malaysia, Penang, Malaysia.

Mosquitoes were exposed to the insecticides for 1 hour. Knock-down was recorded at regular intervals. Mortality was recorded after 24 hour holding period, during which the mosquitoes had access to 10% glucose solution. If the control mortality was between 5-20%, the observed mortality was corrected using Abbott's formula [8].

2.1.5 Entomological surveys

Adult mosquitoes were collected from houses in the vicinity of the health centre during entomological surveys using the following methods: light trap collection (LTC), pyrethrum spray collection (PSC), and human landing catch (HLC). Two rounds of entomological and malariometric surveys were carried out in the vicinity of the health centre, in the village of Kakifulukwa (Fig. 2-10).

Forty households were randomly selected, 18 of which were also used for entomological survey. Data collected during the malariometric survey are not included in this thesis.

- Mosquitoes were collected using the above methods to gather data on entomological indicators including vector density, biting rates, sporozoite rates and parity rates.
- All collected mosquitoes were identified to species level first morphologically and then using molecular methods.
- Resistance against different insecticides in collected mosquitoes was determined using molecular methods.
- Sporozoite rates were determined using circumsporozoite protein enzyme-linked immunosorbent assay (CSP ELISA) procedure [9, 10].

Only data on the species, insecticide resistance genotypes and sporozoite rates are included in this thesis.

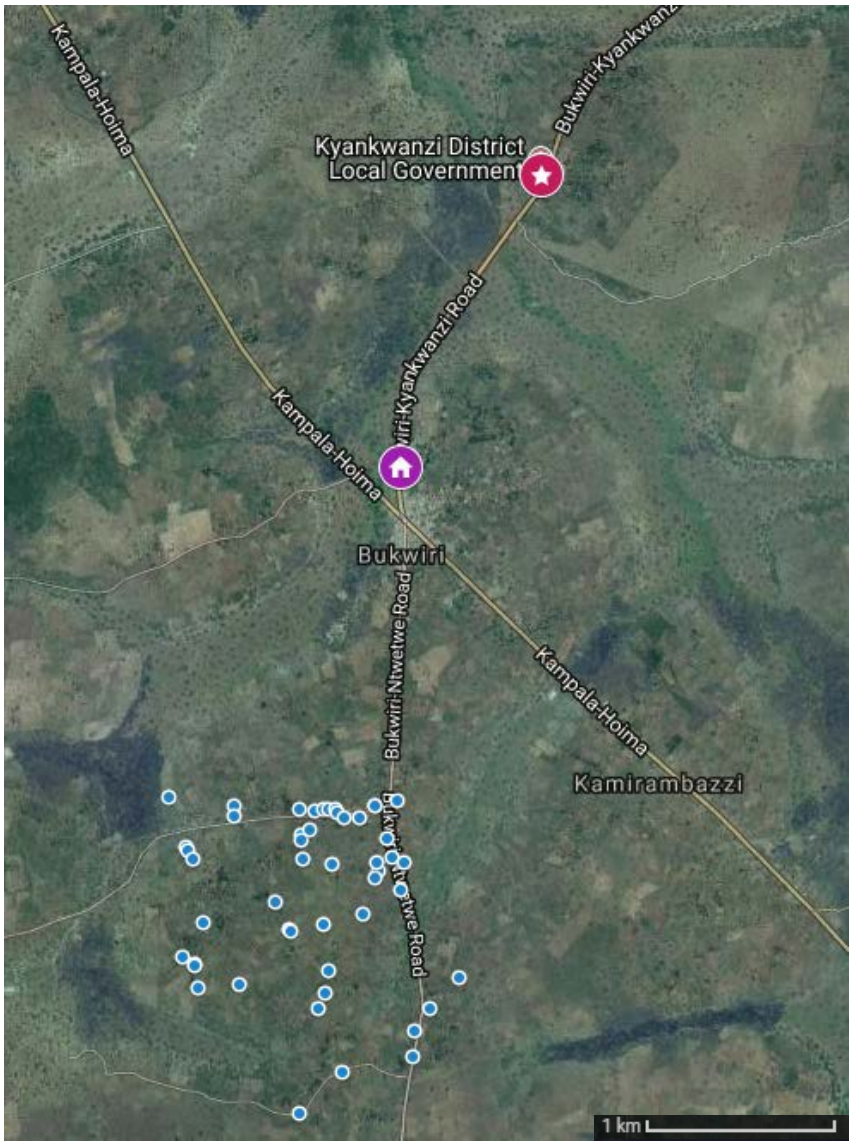

Fig. 2-10. Map of the field site in Kyankwanzi, mid-western Uganda showing location of Bukwiri (purple marker), the town nearest to the field laboratory at Butemba Health Centre III (red marker with star). Malariological and entomological surveys were carried out in Kakifulukwa village (houses used shown with blue markers) (Map data: Google, CNES/Airbus, DigitalGlobe, Landsat/Copernicus).

2.2 Mosquito species identification and resistance genotyping

2.2.1 Storage of samples

Following the field experiments, susceptibility tests and entomological surveys, all mosquitoes were stored dry on silica gel until further laboratory analysis.

2.2.2 DNA extraction

Genomic DNA was extracted from mosquito body parts using Chelex-100 with heat application [11].

2.2.3 Mosquito species identification

Molecular species identification was performed using a multiplex TaqMan real time polymerase chain reaction (rtPCR) assay with three probes for *Anopheles* sibling species identification, to distinguish between *An. gambiae* s.s., *An. arabiensis* and other members of the complex [12].

rtPCR reactions were prepared to give a final reaction volume of 24µl to which 1.0-2.0 µl sample DNA was added (Table 2-2).

Prepared reactions were run on Stratagene MX 3005P (Agilent Technologies) system for 10 min at 95^{o}C, followed by 40 cycles of 95 $^{\circ}$C for 25 seconds and 66 $^{\circ}$C for 60 seconds, measuring the increases in fluorescence of the species-specific FAM and Cy5 fluorophores at the end of each cycle.

gDNA extracted from known *An. gambiae* s.s. and *An. arabiensis* individuals were used on each run as positive controls, together with no template controls (NTCs).

MxPro-Mx3005P v4.00 Build 367 software was used for analysing the results.

Table 2-2. Reagents for the multiplex TaqMan rtPCR with three probes for *Anopheles gambiae* sibling species identification.

Reagent	Volume for 1 reaction [µl]
Sterile H_2O	6.3
SensiMix II Probe Low-ROX kit (Bioline)	12.5
Primer Uni F (800nM) 5'-GTGAAGCTTGGTGCGTGCT-3'	2.0
Primer Uni R (800nM) 5'-GCACGCCGACAAGCTCA-3'	2.0
LNA probe Aa+(200nM) [Cy5]AC+A+T+AG+GATGGA+G+A+AGG[BHQ2]	0.5
TaqMan MGB probe (80 nM) Ag VIC-TGGAGCGGaACAC	0.2
TaqMan MGB probe (200nM) Aq 6FAM-TGGAGCGGgACAC	0.5

2.2.4 Genotyping for *kdr* mutations

Two separate assays were used for the detection of *kdr-w* (L1014F) or *kdr-e* (L1014S) mutations [13].

rtPCR reactions for *kdr-e* (L1014S) were prepared to give a final reaction volume of 19µl to which 1.0 µl sample DNA was added (Table 2-3).

Table 2-3. Reagents for detection of the *kdr-L1014S* mutation.

Reagent	Volume for 1 reaction [µl]
Sterile H_2O	5.0
SensiMix II Probe Low-ROX kit (Bioline)	10.0
Primer kdr-forward (800nM) CATTTTTCTTGGCCACTGTAGTGAT	1.6
Primer kdr-reverse (800nM) CGATCTTGGTCCATGTTAATTTGCA	1.6
TaqMan MGB probe (200 nM) WT VIC-CTTACGACTAAATTTC	0.4
TaqMan MGB probe (200 nM) KdrE 6FAM-ACGACTGAATTTC	0.4

rtPCR reactions for *kdr-w* (L1014F) were prepared to give a final reaction volume of 19µl to which 1.0 µl sample DNA was added (Table 2-4).

Table 2-4. Reagents for detection of the *kdr-L1014F* mutation.

Reagent	**Volume for 1 reaction [µl]**
Sterile H_2O	5.0
SensiMix II Probe Low-ROX kit (Bioline)	10.0
Primer kdr-forward (800nM) CATTTTTCTTGGCCACTGTAGTGAT	1.6
Primer kdr-reverse (800nM) CGATCTTGGTCCATGTTAATTTGCA	1.6
TaqMan MGB probe (200 nM) WT VIC-CTTACGACTAAATTTC	0.4
TaqMan MGB probe (200 nM) KdrW 6FAM-ACGACAAAATTTC	0.4

Prepared reactions were run on Stratagene MX 3005P (Agilent Technologies) system for 10 min at 95°C, followed by 40 cycles of 95 °C for 10 seconds and 60 °C for 45 seconds, measuring fluorescence at the end of each cycle. MxPro-Mx3005P v4.00 Build 367 software was used for analysing the results.

2.2.5 Genotyping for *ace-1* mutations

A further assay to detect the presence of *G119S* mutation in the gene *ace-1* which encodes the acetylcholinesterase enzyme was used [14].

PCR reactions were prepared to give a final reaction volume of 19µl to which 1.0 µl sample DNA was added (Table 2-5).

Prepared reactions were run on Stratagene MX 3005P (Agilent Technologies) system for 10 min at 95°C, followed by 40 cycles of 95 °C for 10 seconds and 60 °C for 35 seconds, measuring fluorescence at the end of each cycle. MxPro-Mx3005P v4.00 Build 367 software was used for analysing the results.

Table 2-5. Reagents for detection of the *G119S* mutation.

Reagent	Volume for 1 reaction [µl]
Sterile H_2O	5.0
SensiMix II Probe Low-ROX kit (Bioline)	10.0
Primer ACE1-F (800nM) GGCCGTCATGCTGTGGAT	1.6
Primer ACE1-R (800nM) GCGGTGCCGGAGTAGA	1.6
TaqMan MGB probe (200 nM) Ace1G119 VIC-TTCGGCGGCGGCT	0.4
TaqMan MGB probe (200 nM) Ace1S119 6FAM-TTCGGCGGCAGCT	0.4

2.2.6 Genotyping for N1575Y mutations

DNA was extracted from individual mosquitoes using the DNeasy Blood and Tissue Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions.

The TaqMan real time rtPCR assay developed by Jones *et al.* [15] was used to test for the presence of N1575Y mutation. PCR reactions were prepared with each reaction containing 10µL of master mix, a final concentration of 1µM of each primer and 0.5µM of each probe, 5µL of PCR grade water and 2µl of sample DNA, to a final reaction volume of 20µL (Table 2-6).

Prepared reactions were run on a Roche LightCycler 96 System for 15 min at 95 °C, followed by 40 cycles of 94 °C for 15 sec and 60 °C for 60 sec. Positive controls from gDNA extracted from known *An. gambiae* s.s. with the N1575 mutation and without the mutation were included on each run, together with no template controls (NTCs). PCR results were analysed using the LightCycler 96 software (Roche Diagnostics).

Table 2-6. Reagents for detection of the *N1575Y* mutation.

Reagent	Volume for 1 reaction [µl]
Sterile H_2O	5.0
Qiagen Quantitect Probes Master mix (QIAGEN)	10.0
Primer 1575-F (800nM) 5'-TGGATCGCTAGAAATGTTCATGACA-3'	1.0
Primer 1575-R (800nM) 5'-CGAGGAATTGCCTTTAGAGGTTTCT-3'	1.0
TaqMan probe N1575 (200 nM) HEX 3'-(NFQ)-ATTTTTTTCATTGCATTATAGTAC-(6-HEX)-5'	0.5
TaqMan probe Y1575 (200 nM) 6FAM 3'-(NFQ)-TTTTTCATTGCATAATAGTAC-(6-FAM)-5'	0.5

2.2.7 Plasmodium detection - CSP ELISA

All the specimens collected during entomological surveys were analysed to detect *P. falciparum* circumsporozoite protein (CSP) by means of enzyme-linked immunosorbent assay (ELISA) [16]. Only head and upper part of the thorax were used [17].

2.3 Ethical considerations

Ethical clearance was obtained from the LSHTM (reference 6454), the Vector Control Division of the Ministry of Health of Uganda (reference VCD-IRC/044), and Uganda National Council of Science and Technology (reference HS 1429).

Volunteers who participated in the transmission experiments were recruited from among the outpatients of the Butemba Health Centre III. All adult volunteers provided written informed consent, and a parent or guardian of any child participant provided written informed consent on their behalf. Written informed consent was obtained from all volunteers after explaining the purpose of the study and all the required procedures in the language understood by each candidate participant or their parent or guardian.

Staff who assisted us with human landing catches also provided written informed consent.

Members of the 40 randomly selected households in Kakifulukwa village who took part in the malariometric surveys - and a subset of 18 household who took part in entomological surveys – also provided informed consent for their participation in the study.

All the necessary laboratory safety procedures were strictly adhered to during the transmission experiments. All the volunteers were treated with appropriate antimalarial drugs. Staff who assisted us with human landing catches were given prophylaxis.

2.4 Mathematical models and statistical analyses

The following section outlines the development and fitting procedures of the mathematical models described in the thesis (Chapters 5, 6, and 7).

Multi-level regression models were used to study the effects of deltamethrin exposure, environmental variables and insecticide resistance status on mosquito infection and mortality.

2.4.1 Variables included in the models

Descriptions of the variables together with descriptive analyses,where appropriate, are presented below. Lists of variables included in the models, categorised as fixed or random in mixed models, and any interaction terms, are then presented with each model.

Deltamethrin dosage

Nets were treated with a range of deltamethrin concentrations chosen to mimic those that can be found on used, aging nets: 2.5, 5, 10, 15, 16.67 mg/m^2 deltamethrin. Because of low feeding rates and mosquito mortality, sample size for some of these doses was inadequate for further analysis at the individual dose level (see Table 2-7 for details).

Table 2-7. Number of *Re/Re* mosquitoes used in each round and at each deltamethrin dose (total n = 692).

Round	Deltamethrin dose on net in mg/m^2					
	0	**2.5**	**5**	**10**	**15**	**16.67**
1	150	7	32	70	0	7
2	119	0	31	97	0	0
3	81	0	38	34	26	0

Data from different doses were therefore pooled into two exposure groups:

- Control (untreated nets)
- Low dose (2.5–5.0 mg/m^2 deltamethrin)
- High dose (10.0–16.7 mg/m^2 deltamethrin)

Gametocyte donor volunteers and associated variables

The total number of volunteers (and feeding experiments) used for data analyses was 42. A table showing characteristics of the 42 gametocytaemic volunteers is included in the Appendix 2-1, whereas summaries of the variables and descriptive analysis are provided in the tables below.

Table 2-8. Gametocytaemic volunteer-related binary and categorical variables.

Variable	Category 1	Value	Category 2	Value	Missing values
Sex	Male	14	Female	28	0
History of fever in the last 48 hours	No fever	1	Fever	40	1
Any antimalarials taken in the last 7 days prior to the clinic visit	Yes	10	No	30	2

Table 2-9. Gametocytaemic volunteer-related continuous variables and descriptive analysis.

Variable	Mean	Standard deviation	Minimum	Maximum	Missing values
Age	17.31	12.19	2	56	0
Weight	41.99	21.81	12	92.7	0
Body temperature [^oC]	36.92	1.07	35.15	39.55	0
HemoCue values [g haemoglobin/dl blood]	12.05	1.45	10.1	15.5	0
Gametocyte density per µl blood	138.69	63.92	34.48	280	2

Table 2-10. Gametocyte densities in different study rounds.

Gametocyte density per µl blood	n	Mean	Standard deviation	95% Confidence interval - lower	95% Confidence interval - upper
Round1	15	167.89	62.86	135.07	200.72
Round 2	14	120.00	62.76	86.07	153.93
Round 3	11	122.66	56.86	87.98	157.34

A one-way ANOVA was used to determine if gametocyte density differed between the three study rounds. Comparison of gametocyte density means between the three rounds shows that they were not significantly different ($F_{2,37} = 2.73$, $p = 0.0782$).

Environmental variables

Temperature and humidity were recorded every 30 minutes throughout the experiments. All the environmental variables included in the models (temperature and relative humidity averages, maximums and minimums, and ranges) for each feed were obtained from the records, providing 42 time-points in total for the feeds included in the analyses.

Some of the summaries are presented below (Tables 2-10, 2-11 and 2-12).

Table 2-11. Mean daily temperature, daily minimum and maximum temperature and daily temperature range shown for rounds 1, 2 and 3.

	Mean daily T						Min daily T						Max daily T						Daily T range					
n	Var	Mean	S.D.	Min	Mdn	Max	Var	Mean	S.D.	Min	Mdn	Max	Var	Mean	S.D.	Min	Mdn	Max	Var	Mean	S.D.	Min	Mdn	Max
266	t0	24.85	0.55	24.16	24.63	26.48	T0min	23.27	0.50	22.00	23.50	24.00	T0max	27.77	1.06	26.00	27.50	30.75	T0dtr	4.50	1.13	2.50	4.00	7.75
266	t1	24.8	0.61	23.86	24.9	25.58	T1min	23.27	0.47	22.50	23.00	24.00	T1max	27.55	1.37	25.50	27.13	30.75	T1dtr	4.29	1.34	2.50	3.88	7.75
266	t2	24.88	0.41	24.16	24.9	25.82	T2min	23.19	0.50	22.00	23.00	24.25	T2max	28.72	1.76	26.00	27.50	30.75	T2dtr	5.53	1.85	2.50	5.50	7.75
266	t3	25.12	0.57	24.24	25.28	25.82	T3min	23.30	0.42	23.00	23.25	24.25	T3max	28.38	1.53	26.50	28.00	30.25	T3dtr	5.08	1.48	2.50	4.50	7.00
266	t4	25.4	0.42	24.58	25.42	25.9	T4min	23.69	0.55	23.00	24.00	24.25	T4max	29.16	1.39	26.50	30.00	30.75	T4dtr	5.47	1.45	2.50	5.75	7.75
266	t5	25.41	0.37	24.53	25.58	25.9	T5min	23.74	0.46	23.00	24.00	24.25	T5max	28.34	1.14	26.50	28.00	30.25	T5dtr	4.60	1.36	2.50	4.00	7.00
266	t6	25.55	0.47	24.24	25.82	25.9	T6min	23.83	0.37	23.00	24.00	24.25	T6max	28.77	1.10	26.50	29.50	30.00	T6dtr	4.94	0.81	3.25	5.50	5.75
237	t7	25.32	0.41	24.24	25.29	25.86	T7min	23.82	0.38	23.00	24.00	24.25	T7max	27.77	0.87	26.50	28.00	30.75	T7dtr	3.95	1.00	3.00	3.75	7.75
Round 2																								
	Mean daily T						Min daily T						Max daily T						Daily T range					
n	Var	Mean	S.D.	Min	Mdn	Max	Var	Mean	S.D.	Min	Mdn	Max	Var	Mean	S.D.	Min	Mdn	Max	Var	Mean	S.D.	Min	Mdn	Max
247	t0	26.89	0.98	25.32	26.44	28.24	T0min	23.37	0.87	22.50	23.00	24.50	T0max	29.62	0.84	27.50	30.00	31.00	T0dtr	6.25	0.95	4.50	6.00	7.50
247	t1	26.09	0.41	25.13	26.13	26.63	T1min	22.75	0.42	22.50	22.50	24.25	T1max	28.89	0.72	27.75	28.50	30.00	T1dtr	6.14	0.84	3.75	5.75	7.50
247	t2	26.29	0.35	25.32	26.24	26.91	T2min	23.12	0.54	22.50	23.00	24.25	T2max	28.93	0.60	27.50	29.00	30.00	T2dtr	5.81	1.02	3.75	6.00	7.50
247	t3	26.42	0.59	25.13	26.44	27.56	T3min	23.27	0.77	22.50	23.00	24.25	T3max	29.18	0.97	27.50	29.50	31.00	T3dtr	5.91	1.43	3.75	6.25	7.50
247	t4	26.51	0.8	25.13	26.44	27.56	T4min	23.18	0.63	22.50	23.00	24.25	T4max	29.50	1.16	27.50	29.75	31.00	T4dtr	6.32	0.99	4.50	7.00	7.25
247	t5	26.36	0.57	25.13	26.6	27.56	T5min	23.29	0.50	22.75	23.00	24.25	T5max	28.90	0.85	26.75	29.00	31.00	T5dtr	5.61	0.71	3.75	6.00	7.00
247	t6	26.42	0.68	24.67	26.52	27.56	T6min	23.52	0.65	22.75	23.50	24.25	T6max	28.77	1.02	26.25	28.75	31.00	T6dtr	5.25	1.03	3.25	5.50	7.00
247	t7	26.3	1.04	23.69	25.98	27.56	T7min	23.73	0.53	22.50	24.00	24.25	T7max	28.98	1.63	25.50	28.50	31.00	T7dtr	5.25	1.44	3.00	5.00	7.00
Round 3																								
	Mean daily T						Min daily T						Max daily T						Daily T range					
n	Var	Mean	S.D.	Min	Mdn	Max	Var	Mean	S.D.	Min	Mdn	Max	Var	Mean	S.D.	Min	Mdn	Max	Var	Mean	S.D.	Min	Mdn	Max
179	t0	25.72	0.48	25.16	25.79	26.71	T0min	23.21	0.60	22.25	23.25	24.00	T0max	28.80	0.65	27.50	28.75	29.75	T0dtr	5.58	0.77	4.25	5.75	6.50
179	t1	25.95	0.22	25.39	25.89	26.39	T1min	23.78	0.35	23.25	23.75	24.25	T1max	28.58	0.62	27.50	28.75	29.25	T1dtr	4.80	0.84	3.25	5.00	5.75
179	t2	25.66	0.4	24.91	25.89	26.16	T2min	23.71	0.30	23.25	23.75	24.25	T2max	28.42	1.07	26.25	29.00	29.75	T2dtr	4.71	1.20	2.50	5.50	6.50
179	t3	25.4	0.61	24.49	25.29	26.71	T3min	23.43	0.61	22.25	23.75	24.00	T3max	27.91	1.22	25.50	28.00	29.75	T3dtr	4.47	1.22	2.50	4.00	6.50
179	t4	25.23	0.59	24.6	25.29	26.31	T4min	23.46	0.46	22.25	23.25	24.25	T4max	27.60	1.27	26.00	28.00	29.75	T4dtr	4.14	1.19	2.75	4.00	6.50
179	t5	25.24	0.62	24.49	25.15	26.39	T5min	23.28	0.36	22.50	23.25	24.00	T5max	27.49	1.16	25.50	27.75	29.75	T5dtr	4.22	1.02	2.50	4.50	6.00
179	t6	25.23	0.51	24.6	25.15	26.03	T6min	23.31	0.52	22.50	23.25	24.25	T6max	27.48	1.01	26.00	27.50	29.25	T6dtr	4.16	0.96	2.75	4.50	5.75
179	t7	24.77	0.71	23.64	24.91	26.16	T7min	23.06	0.48	22.50	23.00	24.00	T7max	27.29	0.96	25.50	27.25	29.75	T7dtr	4.23	0.82	2.50	4.25	6.50

Table 2-12. Average temperature within the first 24 hours (i.e. day 1) post-infectious feed, minimum and maximum temperatures, and temperature range, shown for rounds 1, 2 and 3.

Round 1						
Variable	n	Mean	S.D.	Min	Median	Max
Mean T on day 1	266	24.79	0.59	24.2	24.3	25.53
Min T on day 1	266	23.27	0.47	22.5	23	24
Max T on day 1	266	27.48	0.96	26	27.5	30.25
T range on day 1	266	4.21	0.93	2.5	4	7
Round 2						
Variable	n	Mean	S.D.	Min	Median	Max
Mean T on day 1	247	25.57	0.38	24.73	25.43	26.63
Min T on day 1	247	22.71	0.32	22.5	22.5	23.75
Max T on day 1	247	29.4	0.87	27.5	29.75	31
T range on day 1	247	6.69	0.85	4.5	7.25	7.5
Round 3						
Variable	n	Mean	S.D.	Min	Median	Max
Mean T on day 1	179	25.84	0.34	24.91	25.8	26.56
Min T on day 1	179	23.78	0.35	23.25	23.75	24.25
Max T on day 1	179	28.66	0.57	27.5	28.75	29.75
T range on day 1	179	4.88	0.8	3.25	5	5.75

Table 2-13. Average temperature post day 1 following the infectious feed, minimum and maximum temperatures, and temperature range, shown for rounds 1, 2 and 3.

Round 1						
Variable	n	Mean	S.D.	Min	Median	Max
Mean T post day 1	266	25.21	0.26	24.65	25.16	25.62
Min T post day 1	266	23.60	0.25	23.00	23.54	24.00
Max T post day 1	266	28.65	0.88	26.92	28.88	30.00
T range post day 1	266	5.05	0.73	3.58	5.33	6.25
Round 2						
Variable	n	Mean	S.D.	Min	Median	Max
Mean T post day 1	247	25.88	0.37	24.97	26.06	26.21
Min T post day 1	247	23.37	0.24	23.00	23.29	23.71
Max T post day 1	247	29.12	0.64	27.54	29.54	29.83
T range post day 1	247	5.75	0.61	4.54	5.83	6.50
Round 3						
Variable	n	Mean	S.D.	Min	Median	Max
Mean T post day 1	179	25.20	0.31	24.76	25.18	25.84
Min T post day 1	179	23.38	0.21	23.04	23.42	23.79
Max T post day 1	179	27.80	0.48	27.13	27.63	28.96
T range post day 1	179	4.43	0.35	4.00	4.46	5.25

***Kdr* resistance**

Data analysis in Chapters 5 and 6 includes only *Re/Re* homozygous mosquitoes, whereas mosquitoes of all different *kdr* genotypes (*S/S, Re/S, Rw/S, Re/Re, Rw/Rw, Re/Rw*) that were found in the study area and collected during the study are included in mosquito survival analysis in Chapter 6, and in models in Chapter 7.

Study round

In total 47 feeds were carried out but 42 were included in the analysis, as described.

Table 2-14. Study round description.

Study round	Date	Feeds included
1	September – October 2013	4 – 21 (n = 16)
2	November – December 2013	22 – 35 (n = 14)
3	May – June 2014	36 – 47 (n = 12)

The length of membrane feed

Because of the reluctance of mosquitoes to feed on membrane, they were given access to feeders for as long as the blood was in good condition and some mosquitoes were left feeding.

Table 2-15. The length of membrane feeds of 692 *Re/Re* mosquitoes and descriptive analysis.

Variable	Mean	Standard deviation	Minimum	Maximum	Missing values
Length of feed	1.91 hrs	0.7	0.53 hrs	3.97 hrs	0

2.4.2 Description of models

Full description of the models is provided in the section below, including all model outputs, while the interpretation of the models is provided in subsequent chapters.

Chapter 5

Model: Mixed-effects logistic regression to study the effects of the insecticide on oocyst infection rate (oocyst prevalence).

Melogit procedure in Stata was used with backward elimination.

Table 2-16. Description of variables used in a mixed-effects logistic regression model to study the effects of the insecticide on oocyst infection rate (oocyst prevalence).

Variables and interaction terms	Variable type
Deltamethrin dosage group (*dosecat*)	Fixed-effect categorical variable with three levels: control (untreated nets), low dose (2.5–5.0 mg/m^2 deltamethrin) and high dose (10.0–16.7 mg/m^2 deltamethrin)
Average environmental temperature during incubation (*tempavg*)	Fixed-effect explanatory factor; continuous variable
Gametocyte density (*gamden*)	Fixed-effect explanatory factor; continuous variable
Age of gametocytaemic volunteers (*age*)	Fixed-effect explanatory factor; continuous variable
Blood hemoglobin concentration - hemoCue values (*hemocue*)	Fixed-effect explanatory factor; continuous variable
Weight of gametocytaemic volunteers (*weight*)	Fixed-effect explanatory factor; continuous variable
Whether or not the donor received medication with antimalarials in the previous seven days (*med*)	Fixed-effect explanatory factor; binary categorical variable
Length of membrane feed (*exphrs*)	Fixed-effect explanatory factor; continuous variable
Interaction term: *c.gamden##c.hemocue*	
Interaction term: *c.gamden##c.age*	
Interaction term: *c.gamden##i.fever*	
Interaction term: *c.gamden##i.med*	
Gametocyte donor volunteers (*feed*)	Random (or group) variable to account for the correlation of mosquitoes fed on the same blood sample within each experiment
Outcome variable	
Prevalence of oocyst infection among *An. gambiae* s.s. mosquitoes with *kdr-L1014S* homozygous (*ReRe*) genotype (*pos*)	

The final model output was:

```
. melogit pos i.dosecat tempavg || feed:, or

Fitting fixed-effects model:

Iteration 0:   log likelihood = -383.01049
Iteration 1:   log likelihood = -382.84378
Iteration 2:   log likelihood = -382.84376

Refining starting values:

Grid node 0:   log likelihood =  -340.8983

Fitting full model:

Iteration 0:   log likelihood =  -340.8983
Iteration 1:   log likelihood = -335.70391
Iteration 2:   log likelihood = -335.43105
Iteration 3:   log likelihood = -335.42952
Iteration 4:   log likelihood = -335.42953

Mixed-effects logistic regression               Number of obs     =        692
Group variable:            feed                 Number of groups  =         42

                                                Obs per group:
                                                              min =          1
                                                              avg =       16.5
                                                              max =         60

Integration method: mvaghermite                 Integration pts.  =          7

                                                Wald chi2(3)      =      56.23
Log likelihood = -335.42953                     Prob > chi2       =     0.0000
```

pos	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
dosecat						
1	.4091821	.1348093	-2.71	0.007	.2145257	.7804658
2	.1972657	.0502974	-6.37	0.000	.1196791	.3251507
tempavg	.1794635	.0728436	-4.23	0.000	.0809984	.397627
_cons	5.63e+19	5.88e+20	4.36	0.000	7.40e+10	4.29e+28
feed						
var(_cons)	1.889012	.6718649			.9407793	3.792989

```
Note: Estimates are transformed only in the first equation.
Note: _cons estimates baseline odds (conditional on zero random effects).
LR test vs. logistic model: chibar2(01) = 94.83       Prob >= chibar2 = 0.0000
```

Model: Mixed-effects negative binomial regression to study the effects of the insecticide on oocyst counts (oocyst intensity) in infected mosquitoes only.

Menbreg procedure in Stata was used with backward elimination.

Table 2-17. Description of variables used in mixed-effects negative binomial regression model to study the effects of the insecticide on oocyst counts (oocyst intensity) in infected mosquitoes.

Variables and interaction terms	Variable type
Deltamethrin dosage group (*dosecat*)	Fixed-effect categorical variable with three levels: control (untreated nets), low dose (2.5–5.0 mg/m^2 deltamethrin) and high dose (10.0–16.7 mg/m^2 deltamethrin)
Average environmental temperature during incubation (*tempavg*)	Fixed-effect explanatory factor; continuous variable
Gametocyte density (*gamden*)	Fixed-effect explanatory factor; continuous variable
Age of gametocytaemic volunteers (*age*)	Fixed-effect explanatory factor; continuous variable
Blood hemoglobin concentration - hemoCue values (*hemocue*)	Fixed-effect explanatory factor; continuous variable
Whether the volunteer had fever or not (*fever*)	Fixed-effect explanatory factor; continuous variable
Weight of gametocytaemic volunteers (*weight*)	Fixed-effect explanatory factor; continuous variable
Whether or not the donor received medication with antimalarials in the previous seven days (*med*)	Fixed-effect explanatory factor; binary categorical variable
Length of membrane feed (*exphrs*)	Fixed-effect explanatory factor; continuous variable
Gametocyte donor volunteers (*feed*)	Random (or group) variable to account for the correlation of mosquitoes fed on the same blood sample within each experiment
Outcome variable	
Oocyst infection rate (infection intensity) in *An. gambiae* s.s. infected mosquitoes with *kdr-L1014S* homozygous (*ReRe*) genotype (*oocyst*)	

Oocyst distribution is over-dispersed and follows the negative binomial distribution, where few mosquitoes have many oocysts while most have few or none. Mosquitoes with no detected oocysts were therefore excluded from the analysis of intensity of infection, as this is a commonly used procedure [18-22].

The final model output was:

```
. menbreg oocyst i.dosecat tempavg i.med if pos == 1 || feed:, irr

Fitting fixed-effects model:

Iteration 0:   log likelihood = -1305.7262
Iteration 1:   log likelihood = -1281.3477
Iteration 2:   log likelihood =  -1278.402
Iteration 3:   log likelihood = -1278.3941
Iteration 4:   log likelihood = -1278.3941

Refining starting values:

Grid node 0:   log likelihood =  -1270.103

Fitting full model:

Iteration 0:   log likelihood =  -1270.103  (not concave)
Iteration 1:   log likelihood = -1263.2137
Iteration 2:   log likelihood = -1259.4743
Iteration 3:   log likelihood = -1256.3488
Iteration 4:   log likelihood = -1256.1771
Iteration 5:   log likelihood = -1256.1747
Iteration 6:   log likelihood = -1256.1747

Mixed-effects nbinomial regression              Number of obs     =        421
Overdispersion:            mean
Group variable:            feed                 Number of groups  =         40

                                                Obs per group:
                                                              min =          1
                                                              avg =       10.5
                                                              max =         51

Integration method: mvaghermite                 Integration pts.  =          7

                                                Wald chi2(4)      =      47.72
Log likelihood = -1256.1747                     Prob > chi2       =     0.0000
```

oocyst	IRR	Std. Err.	z	P>|z|	[95% Conf.	Interval]
dosecat						
1	.5862489	.0748619	-4.18	0.000	.4564431	.7529696
2	.6551721	.0679178	-4.08	0.000	.5347076	.8027761
tempavg	.5223533	.0862892	-3.93	0.000	.377878	.7220663
1.med	.5949828	.1560678	-1.98	0.048	.3558187	.9949013
_cons	1.10e+08	4.63e+08	4.40	0.000	28773.08	4.21e+11
/lnalpha	-.6884243	.0876714			-.860257	-.5165916
feed						
var(_cons)	.2433201	.0898784			.1179663	.5018776

```
Note: Estimates are transformed only in the first equation.
Note: _cons estimates baseline incidence rate (conditional on zero random effects).
LR test vs. nbinomial model: chibar2(01) = 44.44      Prob >= chibar2 = 0.0000
```

Chapter 6

Model: Mixed-effects logistic regression model to study the effects of temperature, relative humidity and deltamethrin exposure on oocyst infection rate (oocyst prevalence).

Melogit procedure in Stata was used with backward elimination.

Table 2-18. Description of variables used in a mixed-effects logistic regression model to study the effects of temperature, relative humidity and insecticide exposure on oocyst infection rate (oocyst prevalence).

Variables and interaction terms	**Variable type**
Deltamethrin dosage group (*dosecat*)	Fixed-effect categorical variable with three levels: control (untreated nets), low dose (2.5–5.0 mg/m^2 deltamethrin) and high dose (10.0–16.7 mg/m^2 deltamethrin)
Deltamethrin dose (dose)	Fixed-effect explanatory factor; continuous variable
Gametocyte density (*gamden*)	Fixed-effect explanatory factor; continuous variable
Age of gametocytaemic volunteers (*age*)	Fixed-effect explanatory factor; continuous variable
Whether or not the donor received medication with antimalarials in the previous seven days (*med*)	Fixed-effect explanatory factor; binary categorical variable
Daily temperature range on days 1, 2, 3, 4, 5, 6, and 7 post-infectious feed (*trng000024d1, trng024048d2 trng048072d3, trng072096d4, trng096120d5, trng120144d6, trng144168d7*)	Fixed-effect explanatory factors; continuous variables
Gametocyte donor volunteers (*feed*)	Random (or group) variable to account for the correlation of mosquitoes fed on the same blood sample within each experiment
Daily temperature range on days 1, 2, 3, 4, 5, 6, and 7 post-infectious feed	Fixed-effect explanatory factors; continuous variables
Outcome variables	
Prevalence of oocyst infection among *An. gambiae* s.s. mosquitoes with *kdr-L1014S* homozygous (*ReRe*) genotype (*pos*)	

Model used in Chapter 5, as outlined above, showed the significance of average daily temperature. However, as Paaijmans *et al.* [23] showed that temperature fluctuations immediately following the infectious blood meal can significantly affect parasite

development, a new model was developed based on the original model, using daily temperature ranges on days 1 - 7 post-infectious blood meal.

A model was first developed using deltamethrin dosage group (*dosecat*) as the explanatory variable. However, the same principle was then followed using deltamethrin dose (*dose*) as a continuous explanatory variable instead. The results similarly showed that insecticide dose, temperature range on day 1 and day 4 post-infectious feed had a highly significant effect on risk of infection.

The final model output, as reported in Chapter 6, with deltamethrin dosage group (*dosecat*) was:

```
. melogit pos i.dosecat trng000024d1 trng072096d4 || feed:, or

Fitting fixed-effects model:

Iteration 0:   log likelihood = -390.30736
Iteration 1:   log likelihood = -390.20248
Iteration 2:   log likelihood = -390.20246

Refining starting values:

Grid node 0:   log likelihood = -342.56502

Fitting full model:

Iteration 0:   log likelihood = -342.56502
Iteration 1:   log likelihood = -337.26346
Iteration 2:   log likelihood =  -336.7773
Iteration 3:   log likelihood = -336.76902
Iteration 4:   log likelihood =   -336.769

Mixed-effects logistic regression               Number of obs     =        692
Group variable:            feed                 Number of groups  =         42

                                                Obs per group:
                                                              min =          1
                                                              avg =       16.5
                                                              max =         60

Integration method: mvaghermite                 Integration pts.  =          7

                                                Wald chi2(4)      =      54.33
Log likelihood =   -336.769                     Prob > chi2       =     0.0000
```

pos	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
dosecat						
1	.4227025	.1394643	-2.61	0.009	.2214082	.8070044
2	.1900224	.0484455	-6.51	0.000	.1152907	.3131955
trng000024d1	.6308891	.1330805	-2.18	0.029	.4172531	.9539082
trng072096d4	.6058323	.1372119	-2.21	0.027	.3886584	.9443584
_cons	617.4769	844.1172	4.70	0.000	42.36351	9000.143
feed						
var(_cons)	2.092445	.7292707			1.05679	4.143045

```
Note: Estimates are transformed only in the first equation.
Note: _cons estimates baseline odds (conditional on zero random effects).
LR test vs. logistic model: chibar2(01) = 106.87      Prob >= chibar2 = 0.0000
```

Alternatively, the final model using deltamethrin dose (*dose*) was:

```
. melogit pos dose trng000024d1 trng072096d4 || feed:, or

Fitting fixed-effects model:

Iteration 0:   log likelihood = -390.26388
Iteration 1:   log likelihood = -390.23392
Iteration 2:   log likelihood = -390.23392

Refining starting values:

Grid node 0:   log likelihood = -343.02292

Fitting full model:

Iteration 0:   log likelihood = -343.02292
Iteration 1:   log likelihood = -338.39592
Iteration 2:   log likelihood = -337.98878
Iteration 3:   log likelihood = -337.97412
Iteration 4:   log likelihood = -337.97407
Iteration 5:   log likelihood = -337.97407

Mixed-effects logistic regression               Number of obs     =        692
Group variable:            feed                 Number of groups  =         42

                                                Obs per group:
                                                              min =          1
                                                              avg =       16.5
                                                              max =         60

Integration method: mvaghermite                 Integration pts.  =          7

                                                Wald chi2(3)      =      53.01
Log likelihood = -337.97407                     Prob > chi2       =     0.0000
------------------------------------------------------------------------------
         pos | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
        dose |   .8656418   .0195482    -6.39   0.000     .8281635    .9048162
trng000024d1 |   .6407412    .133193    -2.14   0.032     .4263248    .9629966
trng072096d4 |   .5922594   .1326165    -2.34   0.019     .3818688    .9185647
       _cons |   596.7632   800.7602     4.76   0.000     43.01566    8278.994
-------------+----------------------------------------------------------------
feed         |
   var(_cons) |   2.028389   .7100937                      1.021327     4.02845
------------------------------------------------------------------------------
Note: Estimates are transformed only in the first equation.
Note: _cons estimates baseline odds (conditional on zero random effects).
LR test vs. logistic model: chibar2(01) = 104.52      Prob >= chibar2 = 0.0000
```

Model: Mixed-effects logistic regression model to study the effects the temperature, relative humidity and deltamethrin exposure on mosquito survival through the seven days of incubation.

Melogit procedure in Stata was used with backward elimination.

Table 2-19. Description of variables used in a mixed-effects logistic regression model to study the effects of temperature, relative humidity and insecticide exposure on mosquito survival.

Variables and interaction terms	Variable type
Deltamethrin dosage group (*dosecat*)	Fixed-effect categorical variable with three levels: control (untreated nets), low dose (2.5–5.0 mg/m^2 deltamethrin) and high dose (10.0–16.7 mg/m^2 deltamethrin)
Average environmental temperature during incubation (*tavgall*)	Fixed-effect explanatory factor; continuous variable
Average maximum temperature (*tmaxall*)	Fixed-effect explanatory factor; continuous variable
Average temperature range (*trngall*)	Fixed-effect explanatory factor; continuous variable
Average relative humidity during incubation (*rhavgall*)	Fixed-effect explanatory factor; continuous variable
Average maximum relative humidity (*rhmaxall*)	Fixed-effect explanatory factor; continuous variable
Average relative humidity range (*rhrngall)*	Fixed-effect explanatory factor; continuous variable
Outcome variables	
Mosquito mortality among *An. gambiae* s.s. mosquitoes with *kdr-L1014S* homozygous (*ReRe*) genotype (*dead*)	

The final model output was:

```
. melogit dead i.dosecat tavgall rhavgall || feed:, or

Fitting fixed-effects model:

Iteration 0:   log likelihood = -355.77685
Iteration 1:   log likelihood =  -349.6658
Iteration 2:   log likelihood = -349.59886
Iteration 3:   log likelihood = -349.59883

Refining starting values:

Grid node 0:   log likelihood = -355.67895

Fitting full model:

Iteration 0:   log likelihood = -355.67895  (not concave)
Iteration 1:   log likelihood = -351.67248  (not concave)
Iteration 2:   log likelihood = -349.48931
Iteration 3:   log likelihood = -349.03536
Iteration 4:   log likelihood = -349.01294
Iteration 5:   log likelihood = -349.01286
Iteration 6:   log likelihood = -349.01286

Mixed-effects logistic regression               Number of obs     =        838
Group variable:            feed                 Number of groups  =         42

                                                Obs per group:
                                                              min =          1
                                                              avg =       20.0
                                                              max =         71

Integration method: mvaghermite                 Integration pts.  =          7

                                                Wald chi2(4)      =      57.97
Log likelihood = -349.01286                     Prob > chi2       =     0.0000
```

dead	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
dosecat						
1	5.144258	1.588246	5.31	0.000	2.808804	9.421586
2	5.069617	1.298705	6.34	0.000	3.068452	8.37589
tavgall	8.471604	5.133254	3.53	0.000	2.583399	27.78048
rhavgall	1.248408	.0728436	3.80	0.000	1.113498	1.399663
_cons	2.21e-32	4.28e-31	-3.77	0.000	7.79e-49	6.29e-16
feed						
var(_cons)	.1051055	.122365			.0107312	1.029446

```
Note: Estimates are transformed only in the first equation.
Note: _cons estimates baseline odds (conditional on zero random effects).
LR test vs. logistic model: chibar2(01) = 1.17        Prob >= chibar2 = 0.1395
```

Chapter 7

Model: Mixed-effects logistic regression model to study the effects of insecticide resistance status (*kdr*) on oocyst infection rate (oocyst prevalence).

Melogit procedure in Stata was used with backward elimination.

Table 2-20. Description of variables used in a mixed-effects logistic regression model to study the effects of insecticide resistance status on oocyst infection rate (oocyst prevalence).

Variables and interaction terms	Variable type
Deltamethrin dosage group (*dosecat*)	Fixed-effect categorical variable with three levels: control (untreated nets), low dose (2.5–5.0 mg/m^2 deltamethrin) and high dose (10.0–16.7 mg/m^2 deltamethrin)
Average environmental temperature during incubation (*tempavg*)	Fixed-effect explanatory factor; continuous variable
Gametocyte density (*gamden*)	Fixed-effect explanatory factor; continuous variable
Age of gametocytaemic volunteers (*age*)	Fixed-effect explanatory factor; continuous variable
Blood hemoglobin concentration - hemoCue values (*hemocue*)	Fixed-effect explanatory factor; continuous variable
Weight of gametocytaemic volunteers (*weight*)	Fixed-effect explanatory factor; continuous variable
Whether or not the donor received medication with antimalarials in the previous seven days (*med*)	Fixed-effect explanatory factor; binary categorical variable
Length of membrane feed (*exphrs*)	Fixed-effect explanatory factor; continuous variable
Kdr status (*kdr*)	Fixed-effect explanatory factor; categorical variable with five levels for *S/S, S/Re, Re/Re, Rw/Rw* and *Re/Rw* genotypes
Interaction term: *i.dosecat##i.kdr*	
Gametocyte donor volunteers (*feed*)	Random (or group) variable to account for the correlation of mosquitoes fed on the same blood sample within each experiment
Outcome variable	
Prevalence of oocyst infection among all *An. gambiae* s.s. mosquitoes (*pos*)	

While insecticide exposure and mean ambient temperature had a significant effect on the risk of infection, *kdr* genotype or the interaction term (*i.dosecat##i.kdr*) had no significant effect on oocyst infection rate.

A model was first developed using deltamethrin dosage group (*dosecat*) as the explanatory variable.

An output with deltamethrin dosage group (*dosecat*) as shown in Chapter 7:

```
. melogit pos i.dosecat tempavg i.kdr|| feed:, or
note: 0.kdr != 0 predicts success perfectly
      0.kdr dropped and 2 obs not used

note: 5.kdr omitted because of collinearity

Fitting fixed-effects model:

Iteration 0:   log likelihood = -414.75372
Iteration 1:   log likelihood = -414.56091
Iteration 2:   log likelihood = -414.56089

Refining starting values:

Grid node 0:   log likelihood =  -369.3533

Fitting full model:

Iteration 0:   log likelihood =  -369.3533
Iteration 1:   log likelihood = -361.77852
Iteration 2:   log likelihood = -361.39683
Iteration 3:   log likelihood = -361.39032
Iteration 4:   log likelihood = -361.39032

Mixed-effects logistic regression               Number of obs     =        754
Group variable:            feed                 Number of groups  =         42

                                                Obs per group:
                                                              min =          1
                                                              avg =       18.0
                                                              max =         68

Integration method: mvaghermite                 Integration pts.  =          7

                                                Wald chi2(6)      =      61.20
Log likelihood = -361.39032                     Prob > chi2       =     0.0000
```

pos	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
dosecat						
1	.3838893	.1202681	-3.06	0.002	.2077469	.7093778
2	.1881615	.047366	-6.64	0.000	.1148834	.3081798
tempavg	.1686366	.0686889	-4.37	0.000	.0758999	.3746814
kdr						
0	1	(empty)				
2	.7669149	.9877669	-0.21	0.837	.0614358	9.573553
3	1.400858	.7277826	0.65	0.516	.506026	3.87807
4	1.910466	1.404482	0.88	0.379	.4522485	8.07052
5	1	(omitted)				
_cons	2.04e+20	2.13e+21	4.46	0.000	2.48e+11	1.68e+29
feed						
var(_cons)	1.979858	.6933344			.9966631	3.932962

```
Note: Estimates are transformed only in the first equation.
Note: _cons estimates baseline odds (conditional on zero random effects).
LR test vs. logistic model: chibar2(01) = 106.34      Prob >= chibar2 = 0.0000
```

The final model output, with deltamethrin dosage group (*dosecat*) was:

```
. melogit pos i.dosecat tempavg || feed:, or

Fitting fixed-effects model:

Iteration 0:   log likelihood = -417.24659
Iteration 1:   log likelihood = -417.06229
Iteration 2:   log likelihood = -417.06226

Refining starting values:

Grid node 0:   log likelihood = -369.65974

Fitting full model:

Iteration 0:   log likelihood = -369.65974
Iteration 1:   log likelihood = -363.81923
Iteration 2:   log likelihood = -363.53646
Iteration 3:   log likelihood = -363.53483
Iteration 4:   log likelihood = -363.53483

Mixed-effects logistic regression               Number of obs     =        756
Group variable:            feed                 Number of groups  =         42

                                                Obs per group:
                                                              min =          1
                                                              avg =       18.0
                                                              max =         68

Integration method: mvaghermite                 Integration pts.  =          7

                                                Wald chi2(3)      =      61.76
Log likelihood = -363.53483                     Prob > chi2       =     0.0000
```

pos	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
dosecat						
1	.3762375	.1171996	-3.14	0.002	.2043192	.6928114
2	.1870486	.0468383	-6.69	0.000	.1145005	.3055634
tempavg	.169149	.0675442	-4.45	0.000	.0773339	.3699723
_cons	2.66e+20	2.73e+21	4.58	0.000	4.89e+11	1.45e+29
feed						
var(_cons)	1.881874	.6596098			.9467572	3.740611

```
Note: Estimates are transformed only in the first equation.
Note: _cons estimates baseline odds (conditional on zero random effects).
LR test vs. logistic model: chibar2(01) = 107.05      Prob >= chibar2 = 0.0000
```

However, the same principle was then followed using deltamethrin dose (*dose*) as a continuous explanatory variable instead – with similar results. The model using deltamethrin dose (*dose*) was:

```
. melogit pos dose tempavg i.kdr || feed:, or
note: 0.kdr != 0 predicts success perfectly
      0.kdr dropped and 2 obs not used

note: 5.kdr omitted because of collinearity

Fitting fixed-effects model:

Iteration 0:   log likelihood = -414.36122
Iteration 1:   log likelihood = -414.25724
Iteration 2:   log likelihood = -414.25723

Refining starting values:

Grid node 0:   log likelihood = -369.62736

Fitting full model:

Iteration 0:   log likelihood = -369.62736
Iteration 1:   log likelihood = -363.11416
Iteration 2:   log likelihood = -362.78163
Iteration 3:   log likelihood = -362.77535
Iteration 4:   log likelihood = -362.77535

Mixed-effects logistic regression               Number of obs     =        754
Group variable:            feed                 Number of groups  =         42

                                                Obs per group:
                                                              min =          1
                                                              avg =       18.0
                                                              max =         68

Integration method: mvaghermite                 Integration pts.  =          7

                                                Wald chi2(5)      =      60.01
Log likelihood = -362.77535                     Prob > chi2       =     0.0000
```

pos	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
dose	.8645381	.0192178	-6.55	0.000	.8276806	.9030368
tempavg	.1712251	.0684228	-4.42	0.000	.0782386	.3747259
kdr						
0	1	(empty)				
2	.7649735	.9754245	-0.21	0.834	.0628452	9.311529
3	1.432653	.7413311	0.69	0.487	.519618	3.950009
4	1.988104	1.461869	0.93	0.350	.4704823	8.401071
5	1	(omitted)				
_cons	1.24e+20	1.27e+21	4.50	0.000	2.24e+11	6.81e+28
feed						
var(_cons)	1.9132	.674982			.9581954	3.820027

```
Note: Estimates are transformed only in the first equation.
Note: _cons estimates baseline odds (conditional on zero random effects).
LR test vs. logistic model: chibar2(01) = 102.96      Prob >= chibar2 = 0.0000
```

Mixed-effects negative binomial regression to study the effects of insecticide resistance status (*kdr*) on oocyst counts (oocyst intensity) in infected mosquitoes.

Menbreg procedure in Stata was used with backward elimination.

Table 2-21. Description of variables used in mixed-effects negative binomial regression model to study the effects of insecticide resistance status on oocyst counts (oocyst intensity) in infected mosquitoes.

Variables and interaction terms	**Variable type**
Deltamethrin dosage group (*dosecat*)	Fixed-effect categorical variable with three levels: control (untreated nets), low dose (2.5–5.0 mg/m^2 deltamethrin) and high dose (10.0–16.7 mg/m^2 deltamethrin)
Average environmental temperature during incubation (*tempavg*)	Fixed-effect explanatory factor; continuous variable
Gametocyte density (*gamden*)	Fixed-effect explanatory factor; continuous variable
Age of gametocytaemic volunteers (*age*)	Fixed-effect explanatory factor; continuous variable
Blood hemoglobin concentration - hemoCue values (*hemocue*)	Fixed-effect explanatory factor; continuous variable
Weight of gametocytaemic volunteers (*weight*)	Fixed-effect explanatory factor; continuous variable
Whether or not the donor received medication with antimalarials in the previous seven days (*med*)	Fixed-effect explanatory factor; binary categorical variable
Length of membrane feed (*exphrs*)	Fixed-effect explanatory factor; continuous variable
Kdr status (*kdr*)	Fixed-effect explanatory factor; categorical variable with five levels for *S/S, S/Re, Re/Re, Rw/Rw* and *Re/Rw* genotypes
Interaction term: *i.dosecat##i.kdr*	
Gametocyte donor volunteers (*feed*)	Random (or group) variable to account for the correlation of mosquitoes fed on the same blood sample within each experiment
Outcome variable	
Oocyst infection rate (infection intensity) in all *An. gambiae* s.s. infected mosquitoes (*oocyst*)	

While insecticide exposure and mean ambient temperature had a significant effect on oocyst burden, *kdr* genotype or the interaction term (*i.dosecat##i.kdr*) had no significant effect on the number of oocysts per infected mosquito:

```
. menbreg oocyst i.dosecat tempavg i.med i.kdr || feed:, irr

Fitting fixed-effects model:

Iteration 0:   log likelihood = -1418.2233
Iteration 1:   log likelihood = -1391.9144
Iteration 2:   log likelihood =  -1388.7707
Iteration 3:   log likelihood = -1388.7616
Iteration 4:   log likelihood = -1388.7616

Refining starting values:

Grid node 0:   log likelihood = -1379.5434

Fitting full model:

Iteration 0:   log likelihood = -1379.5434  (not concave)
Iteration 1:   log likelihood = -1372.0563
Iteration 2:   log likelihood = -1367.5165
Iteration 3:   log likelihood = -1364.0384
Iteration 4:   log likelihood = -1364.0024
Iteration 5:   log likelihood = -1364.0023

Mixed-effects nbinomial regression              Number of obs     =        456
Overdispersion:            mean
Group variable:            feed                 Number of groups  =         40

                                                Obs per group:
                                                              min =          1
                                                              avg =       11.4
                                                              max =         58

Integration method: mvaghermite                 Integration pts.  =          7

                                                Wald chi2(8)      =      60.24
Log likelihood = -1364.0023                     Prob > chi2       =     0.0000
```

oocyst	IRR	Std. Err.	z	P>|z|	[95% Conf.	Interval]
dosecat						
1	.5916514	.07262	-4.28	0.000	.465145	.7525639
2	.6296028	.063247	-4.61	0.000	.5170814	.7666099
tempavg	.503054	.0832461	-4.15	0.000	.3637111	.695781
1.med	.5922334	.1564858	-1.98	0.047	.3528417	.9940447
kdr						
2	3.361107	3.122602	1.30	0.192	.5441042	20.76263
3	2.29555	1.869196	1.02	0.307	.4653498	11.32385
4	2.044489	1.748587	0.84	0.403	.382454	10.92926
5	3.73626	3.140337	1.57	0.117	.7194486	19.40325
_cons	1.25e+08	5.41e+08	4.33	0.000	26892.54	5.85e+11
/lnalpha	-.6917543	.0839976			-.8563865	-.5271221
feed						
var(_cons)	.2602009	.0929436			.1291993	.5240315

```
Note: Estimates are transformed only in the first equation.
Note: _cons estimates baseline incidence rate (conditional on zero random effects).
LR test vs. nbinomial model: chibar2(01) = 49.52      Prob >= chibar2 = 0.0000
```

The final model output was:

```
. menbreg oocyst i.dosecat tempavg i.med || feed:, irr

Fitting fixed-effects model:

Iteration 0:   log likelihood =  -1426.381
Iteration 1:   log likelihood = -1401.2152
Iteration 2:   log likelihood = -1398.3544
Iteration 3:   log likelihood = -1398.3471
Iteration 4:   log likelihood = -1398.3471

Refining starting values:

Grid node 0:   log likelihood = -1382.9158

Fitting full model:

Iteration 0:   log likelihood = -1382.9158  (not concave)
Iteration 1:   log likelihood = -1376.3009
Iteration 2:   log likelihood =   -1369.02
Iteration 3:   log likelihood = -1368.0554
Iteration 4:   log likelihood = -1368.0118
Iteration 5:   log likelihood = -1368.0117

Mixed-effects nbinomial regression              Number of obs     =        456
Overdispersion:            mean
Group variable:            feed                 Number of groups  =         40

                                                Obs per group:
                                                              min =          1
                                                              avg =       11.4
                                                              max =         58

Integration method: mvaghermite                 Integration pts.  =          7

                                                Wald chi2(4)      =      50.37
Log likelihood = -1368.0117                     Prob > chi2       =     0.0000
```

oocyst	IRR	Std. Err.	z	P>|z|	[95% Conf.	Interval]
dosecat						
1	.596593	.073018	-4.22	0.000	.4693514	.7583297
2	.6382158	.0644639	-4.45	0.000	.5235893	.7779369
tempavg	.4975471	.0856312	-4.06	0.000	.3550886	.6971588
1.med	.5805824	.1595607	-1.98	0.048	.338789	.9949436
_cons	3.80e+08	1.67e+09	4.51	0.000	70378.86	2.05e+12
/lnalpha	-.6764266	.0834117			-.8399104	-.5129427
feed						
var(_cons)	.2922024	.09957			.1498411	.5698186

```
Note: Estimates are transformed only in the first equation.
Note: _cons estimates baseline incidence rate (conditional on zero random effects).
LR test vs. nbinomial model: chibar2(01) = 60.67      Prob >= chibar2 = 0.0000
```

Mixed-effects logistic regression to study the effects of insecticide resistance status (*kdr*) on sporozoite rates.

Melogit procedure in Stata was used.

Table 2-22. Description of variables used in a mixed-effects logistic regression model to study the effects of insecticide resistance status on sporozoite rates.

Variables and interaction terms	Variable type
Kdr status (*kdr*)	Fixed-effect explanatory factor; categorical variable with five levels for *S/S, S/Re, Re/Re, Rw/Rw* and *Re/Rw* genotypes
Study round	Random (or group) variable to account for the correlation of mosquitoes collected during the same entomological survey (the same study round)
Outcome variables	
Prevalence of sporozoite infection among *An. gambiae* s.s. mosquitoes caught during entomological surveys (*csp*)	

CS ELISA was used to test for sporozoite presence in 244 *An. gambiae* s.s. mosquitoes collected during two entomological surveys (in round 1 and round 3). Only mosquitoes with *Re/Re* (n = 219) and *Re/Rw* (n = 23) *kdr* genotypes were found to be positive and were therefore included in the model.

The final model output was:

```
. melogit csp i.kdr || round:, or

Fitting fixed-effects model:

Iteration 0:   log likelihood = -86.758712
Iteration 1:   log likelihood = -86.326236
Iteration 2:   log likelihood = -86.325352
Iteration 3:   log likelihood = -86.325352

Refining starting values:

Grid node 0:   log likelihood = -87.103152

Fitting full model:

Iteration 0:   log likelihood = -87.103152  (not concave)
Iteration 1:   log likelihood = -86.540725  (not concave)
Iteration 2:   log likelihood = -86.311712
Iteration 3:   log likelihood = -86.282559
Iteration 4:   log likelihood = -86.230774
Iteration 5:   log likelihood = -86.229278
Iteration 6:   log likelihood = -86.229273

Mixed-effects logistic regression               Number of obs     =        242
Group variable:           round                 Number of groups  =          2

                                                Obs per group:
                                                              min =         70
                                                              avg =      121.0
                                                              max =        172

Integration method: mvaghermite                 Integration pts.  =          7

                                                Wald chi2(1)      =       0.80
Log likelihood = -86.229273                     Prob > chi2       =     0.3714
------------------------------------------------------------------------------
         csp | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       5.kdr |   1.699665   1.008515     0.89   0.371     .5312432    5.437926
       _cons |   .1122585   .0397307    -6.18   0.000     .0560997    .2246351
-------------+----------------------------------------------------------------
round        |
   var(_cons) |   .0673047   .2119926                      .0001403    32.29328
------------------------------------------------------------------------------
Note: Estimates are transformed only in the first equation.
Note: _cons estimates baseline odds (conditional on zero random effects).
LR test vs. logistic model: chibar2(01) = 0.19        Prob >= chibar2 = 0.3306
```

2.5 References

1. Abeku TA, Helinski ME, Kirby MJ, Kefyalew T, Awano T, Batisso E, Tesfaye G, Ssekitooleko J, Nicholas S, Erdmanis L *et al*. Monitoring changes in malaria epidemiology and effectiveness of interventions in Ethiopia and Uganda: Beyond Garki Project baseline survey. Malar J. 2015, 14(1):337.

2. Helinski M, Nuwa A, Protopopoff N, Feldman M, Ojuka P, Oguttu DW, Abeku TA, Meek S. Entomological surveillance following a long-lasting insecticidal net universal coverage campaign in Midwestern Uganda. Parasit Vectors. 2015, 8:458.

3. Bousema T, Churcher TS, Morlais I, Dinglasan RR. Can field-based mosquito feeding assays be used for evaluating transmission-blocking interventions? Trends Parasitol. 2013, 29(2):53-59..

4. Bousema T, Dinglasan RR, Morlais I, Gouagna LC, van Warmerdam T, Awono-Ambene PH, Bonnet S, Diallo M, Coulibaly M, Tchuinkam T *et al*. Mosquito feeding assays to determine the infectiousness of naturally infected *Plasmodium falciparum* gametocyte carriers. PLoS One. 2012, 7(8):e42821.

5. Hobbs CV, Tanaka TQ, Muratova O, Van Vliet J, Borkowsky W, Williamson KC, Duffy PE. HIV treatments have malaria gametocyte killing and transmission blocking activity. J Infect Dis. 2013, 208(1):139-148.

6. World Health Organization. Malaria parasite counting. Malaria microscopy standard operating procedure - MM-SOP-09. Geneva: World Health Organization; 2016: 5.

7. World Health Organization. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. Geneva, Switzerland: World Health Organization; 2013.

8. World Health Organization. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. 2nd edn. Geneva, Switzerland: World Health Organization; 2016.

9. Beier JC, Perkins PV, Wirtz RA, Whitmire RE, Mugambi M, Hockmeyer WT. Field evaluation of an enzyme-linked immunosorbent assay (ELISA) for *Plasmodium falciparum* sporozoite detection in anopheline mosquitoes from Kenya. Am J Trop Med Hyg. 1987, 36(3):459-468.

10. Wirtz RA, Duncan JF, Njelesani EK, Schneider I, Brown AE, Oster CN, Were JB, Webster HK. ELISA method for detecting *Plasmodium falciparum* circumsporozoite antibody. Bull World Health Organ. 1989, 67(5):535-542.

11. Lardeux F, Tejerina R, Aliaga C, Ursic-Bedoya R, Lowenberger C, Chavez T. Optimization of a semi-nested multiplex PCR to identify *Plasmodium* parasites in wild-caught *Anopheles* in Bolivia, and its application to field epidemiological studies. Trans R Soc Trop Med Hyg. 2008, 102(5):485-492.

12. Bass C, Williamson MS, Field LM. Development of a multiplex real-time PCR assay for identification of members of the *Anopheles gambiae* species complex. Acta Trop. 2008, 107(1):50-53.

13. Bass C, Nikou D, Donnelly MJ, Williamson MS, Ranson H, Ball A, Vontas J, Field LM. Detection of knockdown resistance (*kdr*) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. Malar J. 2007, 6:111.

14. Bass C, Nikou D, Vontas J, Williamson MS, Field LM. Development of high-throughput real-time PCR assays for the identification of insensitive acetylcholinesterase (*ace-1*^{R}) in *Anopheles gambiae*. Pestic Biochem Phys. 2010, 96(2):80-85.

15. Jones CM, Liyanapathirana M, Agossa FR, Weetman D, Ranson H, Donnelly MJ, Wilding CS. Footprints of positive selection associated with a mutation (*N1575Y*) in the voltage-gated sodium channel of *Anopheles gambiae*. Proc Natl Acad Sci U S A. 2012, 109(17):6614-6619.

16. Wirtz RA, Zavala F, Charoenvit Y, Campbell GH, Burkot TR, Schneider I, Esser KM, Beaudoin RL, Andre RG. Comparative testing of monoclonal antibodies against *Plasmodium falciparum* sporozoites for ELISA development. Bull World Health Organ. 1987, 65(1):39-45.

17. Foley DH, Harrison G, Murphy JR, Dowler M, Rueda LM, Wilkerson RC. Mosquito bisection as a variable in estimates of PCR-derived malaria sporozoite rates. Malar J. 2012, 11(1):145.

18. Billingsley PF, Medley GF, Charlwood D, Sinden RE. Relationship between prevalence and intensity of *Plasmodium falciparum* infection in natural populations of *Anopheles* mosquitoes. Am J Trop Med Hyg. 1994, 51(3):260-270.

19. Alout H, Djegbe I, Chandre F, Djogbenou LS, Dabire RK, Corbel V, Cohuet A. Insecticide exposure impacts vector-parasite interactions in insecticide-resistant malaria vectors. Proc Biol Sci. 2014, 281(1786).

20. Alout H, Ndam NT, Sandeu MM, Djegbe I, Chandre F, Dabire RK, Djogbenou LS, Corbel V, Cohuet A. Insecticide resistance alleles affect vector competence of *Anopheles gambiae* s.s. for *Plasmodium falciparum* field isolates. PLoS One. 2013, 8(5):e63849.

21. Ndiath MO, Cailleau A, Diedhiou SM, Gaye A, Boudin C, Richard V, Trape JF. Effects of the *kdr* resistance mutation on the susceptibility of wild *Anopheles gambiae* populations to *Plasmodium falciparum*: a hindrance for vector control. Malar J. 2014, 13:340.

22. Vezilier J, Nicot A, Gandon S, Rivero A. Insecticide resistance and malaria transmission: infection rate and oocyst burden in *Culex pipiens* mosquitoes infected with *Plasmodium relictum*. Malar J. 2010, 9:379.

23. Paaijmans KP, Blanford S, Bell AS, Blanford JI, Read AF, Thomas MB. Influence of climate on malaria transmission depends on daily temperature variation. Proc Natl Acad Sci U S A. 2010;107:15135-15139.

Appendix 2-1

Table A2-1. Characteristics of the 42 gametocytaemic volunteers.

Round	Volunteer/ Experiment number	Sex	Age	Weight [kg]	T [°C]	Medication taken	Haemoglobin [g/dl]	Gametocyte density/µl blood
1	4	F	25	56	37.05	None	12.2	480
1	5	F	24	74	35.35	None	10.9	120
1	6	M	56	52	36.25	None	11.7	280
1	7	F	5	18	37.25	None	11.2	N/a
1	8	F	19	39	36.65	None	12.1	120
1	9	M	38	64	35.25	None	14.6	200
1	10	M	18	66	35.65	Coartem	15.1	160
1	11	F	23	68	37.1	None	14.1	120
1	12	M	12	31	36.65	None	11.5	280
1	13	F	18	53	35.8	Quinine	11.5	200
1	14	F	14	52	38.5	None	11.6	160
1	16	F	20	58	35.75	None	10.5	120
1	17	F	34	49	37	None	13.3	78
1	18	F	26	50	35.8	None	12.5	240
1	19	F	20	76	36.65	None	12.6	120
1	20	M	5	14	37.3	None	11.0	200
1	21	M	5	20	36.6	N/a	11.8	120
2	22	F	30	49	36.55	None	12.2	40
2	23	F	21	59	38.95	N/a	13.8	80
2	24	F	9	33	37.1	None	10.3	80
2	25	F	25	69	35.8	None	12.8	80
2	26	M	26	46	36.65	None	13.6	40
2	27	M	30	57	38.8	Coartem	13.9	80
2	28	F	21	60	35.15	None	11.9	200
2	29	F	13	40	35.5	None	12	160
2	30	F	18	48	38.15	Coartem	10.3	80
2	31	F	6	16	39.55	None	11.6	240
2	32	F	27	57	37.1	Quinine	11.2	120
2	33	M	6	20	37.35	None	11.3	120

Round	Volunteer/ Experiment number	Sex	Age	Weight [kg]	T [^{o}C]	Medication taken	Haemoglobin [g/dl]	Gametocyte density/µl blood
2	34	F	5	16	38.1	None	11.3	160
2	35	M	4	21	37.05	Nil	11.3	200
3	36	F	7	19	37.8	None	14.3	117
3	37	M	37	92.7	35.65	None	14.0	172
3	38	F	12	32.1	36.25	None	12.1	102
3	39	F	2	12	37.65	Coartem	10.3	152
3	40	F	2	13.1	37	None	10.3	69
3	41	F	34	70	37.35	None	15.5	81
3	42	F	5	16	36.6	Coartem	10.1	240
3	43	M	8	24	37.1	None	10.6	155
3	44	F	6	16.6	36.65	None	10.2	34
3	45	M	5	16.4	38.05	None	12.9	0
3	46	M	7	22	38.75	None	10.7	138
3	47	F	4	16.8	36.7	Coartem	10.7	89

Notes:

Round: Study round 1 = September-October 2013; study round 2 = November-December 2013; study round 3 = May-June 2014

Sex: F = female, M = male

Medication taken: whether the patient has taken any antimalarial drugs in the last 7 days prior to the day they attended the Health Centre

Haemoglobin levels were measured using the HemoCue.

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SECTION A – Student Details

Student	Mojca Kristan
Principal Supervisor	Jo Lines
Thesis Title	Effects of pyrethroid exposure and insecticide resistance on the sporogonic development of Plasmodium falciparum in Anopheles gambiae s.l.

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

SECTION B – Paper already published

Where was the work published?			
When was the work published?			
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
Have you retained the copyright for the work?*	Choose an item.	Was the work subject to academic peer review?	Choose an item.

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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Malaria Journal
Please list the paper's authors in the intended authorship order:	M. Kristan, J. Lines, H. Kaur
Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	With the input from co-authors, I designed the study. I reared the mosquitoes, exposed them to deltamethrin and prepared samples for HPLC and rapid colorimetric tests. HK carried out HPLC, and we together performed rapid colorimetric tests. I analysed the data with assistance of HK. I wrote the manuscript

	with inputs from co-authors.

Student Signature: _____ **Date:** 28/3/2018

Supervisor Signature: _____ **Date:** 28.3.18

Chapter 3. Determination of the amount of insecticide picked up by mosquitoes from treated surfaces

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Abstract

Little is known about the amount of insecticide picked up by malaria vectors after they come into contact with a treated surface. Determining the amount is not only relevant to understanding doses that are lethal to the mosquito but also to studies involving exposure of parasites to insecticides such as pyrethroids used in insecticide-treated nets (ITNs). In order to understand potential transmission-blocking effects of pyrethroids, it is important to identify the likely sites of action where parasites could come into contact with doses of insecticides high enough to harm them. These doses would inevitably depend on how much insecticide mosquitoes come into contact with as a result of vector control interventions.

Three to five days old non-blood fed female *Anopheles coluzzii* mosquitoes were exposed to a long-lasting insecticidal net (PermaNet 2.0 containing 55 mg/m^2 deltamethrin), using a wire ball frame, for 0.5-5.0 minutes. High performance liquid chromatography-photodiode array assay (HPLC-PDA) was used to determine the amount of insecticide mosquitoes pick up from the net, whereas colorimetric tests were used to explore whether deltamethrin could be visually detected from mosquitoes following exposure to the net.

Using HPLC-PDA analysis we showed that mosquitoes pick up to approximately 10 ng of deltamethrin following exposure to PermaNet 2.0 and that the final dose depends on the length of exposure. Colorimetric tests, which are used for detection of deltamethrin on ITNs and sprayed walls, were successfully used for the first time to detect deltamethrin on mosquitoes following exposure to the net.

The study demonstrated the potential of colorimetric tests and HPLC to determine the quantity of insecticide that mosquitoes pick up on contact with treated surfaces, which also determines the amount of insecticide parasites would be exposed to, and implications for detection of specific active ingredients that cause the greatest mosquito mortality in circumstances where mixtures of insecticides might be used to maximise effectiveness of interventions.

3.1 Introduction

Recent declines in malaria incidence across sub-Saharan Africa (SSA) have largely been attributed to a scale-up of insecticide-based vector control interventions, such as insecticide-treated nets (ITNs) and indoor residual spraying (IRS) [1]. Only a small number of insecticides can be used for these interventions and pyrethroids are currently the only insecticides used on all ITNs, either alone or in combination with synergists or a non-pyrethroid insecticide, chlorfenapyr [2]. Pyrethroids were first approved for use in mosquito control by the World Health Organization (WHO) in the 1970s [3]. They are neurotoxins affecting the *para* voltage-gated sodium channels (VGSC) on the mosquito's neurons [4, 5]. They work well on nets and sprayed surfaces because of their rapid knock-down effect, killing properties, and a long residual action [6].

Anopheles mosquitoes in SSA generally feed every 2-3 days, once per gonotrophic cycle [7]. They come into contact with pyrethroids with the tips of their legs when they rest on a sprayed wall after taking a blood meal or come into contact with an ITN while trying to blood feed [8]. There are several possible ways pyrethroids can enter the mosquito's body [9]. It is not known precisely how these insecticides enter and reach their target site VGSCs [10], nor whether they accumulate in tissues or are immediately metabolised, and whether their metabolites exhibit any insecticidal activity [11] or if they might be potentially sporontocidal.

Uptake of insecticides from treated nets or surfaces is variable due to formulation, active ingredient availability, contact time, knockdown, temperature and irritant effects. Insecticides for IRS are available as different formulations, which should provide long lasting residual effect and bioavailability on a number of different surfaces [12, 13], whereas insecticide is incorporated within the netting or bound around the net fibers in long-lasting insecticidal nets (LLINs) [14]. There is a lack of data about the amount of insecticide picked up by the mosquitoes after they come into contact with a treated surface, either a net or a

wall. Only two studies attempted to measure the amounts using gas chromatography in the laboratory (dieldrin) or field (DDT) conditions [15, 16].

Knockdown resistance (*kdr*) mechanism is associated with reduced irritant effects of pyrethroids, meaning that resistant mosquitoes tend to search longer to feed, remain in contact with treated surfaces longer before taking off and acquire more insecticide through contact. This might result in a total dose high enough to kill even homozygous *kdr* resistant mosquitoes [17, 18, 19]. Developing new vector control tools, including the next-generation LLINs, will require a more thorough understanding of how they function in terms of their physiological mode of action and mosquito behavior around them [8]. The minimum duration of LLIN contact necessary to deliver an effective insecticide dose is not known, but mosquito-LLIN interactions have been described and average contact times measured [8, 20].

The degree to which the insecticide is lost from LLINs to make them ineffective and the length of their useful life can vary considerably [21]. Measuring the rate of insecticide loss or, in case of spraying, monitoring of insecticide residues reaching the surface, can provide valuable information to vector control programs. Different types of tests have been developed for this purpose. High performance liquid chromatography (HPLC) and gas chromatography (GC) are techniques often used for characterizing pyrethroids but are reliant on sophisticated laboratorybased equipment and need both expertise and experience to use [22, 23, 24]. More recently, colorimetric assays for detection of pyrethroids [25, 26, 27, 28] and carbamates [29], biosensors using glutathione-S-transferase for pyrethroids [30, 31, 32] and DDT [33], and DDT dipstick assays [34] became available.

However, these methods have not been widely used to detect insecticides on exposed mosquitoes. Being able to determine the quantity of insecticide mosquitoes picked up on contact with treated surfaces, and which active ingredient caused the greatest mosquito mortality in circumstances where mixtures of insecticides might be used, would be important in order to assess and maximize the effectiveness of interventions. Our aim was therefore to test whether rapid colorimetric tests can be used to detect the presence of deltamethrin on mosquitoes, to precisely measure how much insecticide mosquitoes pick up during contact with a LLIN using HPLC, and to compare the results of both methods.

3.2 Methods

3.2.1 Mosquito insecticide exposure

Three to five days old non-blood fed female *Anopheles coluzzii* mosquitoes (susceptible N'gousso strain [35]) were exposed to PermaNet 2.0 (55 mg/m^2 deltamethrin), using a wire ball frame.

To confirm whether deltamethrin could be detected on either whole mosquitoes or different body parts using rapid colorimetric tests, mosquitoes were exposed to the net for 5 minutes.

For HPLC-PDA and associated rapid colorimetric tests, exposure times varied between 0.5-3 minutes, in 0.5-minute increments.

The mosquitoes, together with unexposed controls, were then killed by freezing within 5 minutes of exposure to prevent the enzymes from degrading the insecticides and were stored at -20^{o}C until further processing.

3.2.2 High Performance Liquid Chromatography-photodiode array (HPLC-PDA) analysis

Amount of insecticide on the bed net

PermaNet 2.0 with the manufacturer's claimed level of deltamethrin at 55 mg/m^2 was used in the experiment by firstly determining the amount of deltamethrin on the net using the HPLC method as previously described [22] (also see Appendix, Fig. A3-1 showing a deltamethrin peak as obtained by HPLC-PDA, before a calibration curve was produced using standard deltamethrin dilutions as shown in Fig. A3-2). Briefly, the deltamethrin concentration was determined for the LLIN in the bio-analytical laboratory at the London School of Hygiene and Tropical Medicine (LSHTM), London, UK by using HPLC-PDA. Four squares (2.5 x 2.5 cm^2) were cut from the LLIN and each extracted using acetonitrile (1 ml) under sonication for 5 min. The supernatant was then injected into the HPLC column and the quantity of deltamethrin present was determined. Quantitative analyses were carried out using Dionex Ultimate 3000 HPLC-PDA system (Thermofisher, Hemel Hempstead, UK) and separation achieved using a Acclaim^{R} C_{18} 120 Å (250 X 4.6 mm, Dionex, UK) column eluting with water/acetonitrile (90:10%; v/v) at a flow rate of 2 ml/min and passed through the photodiode array detector (PDA-100, Dionex) set at 275 nm. The authenticity of the detected peaks was determined by comparison of retention time, spectral extraction at 275 nm and

spiking the sample with commercially available standard of the insecticide. A calibration curve of insecticide was generated by Chromeleon (Dionex software) using known amounts of the standard deltamethrin (0.05, 0.04, 0.03, 0.02, 0.01, 0.005 mg/ml) in acetonitrile injected onto the column. Acetonitrile on its own was used as control. From this curve the amount of insecticide in the matrix was calculated. Approximate doses of insecticide per m^2 were calculated from the quantities detected in each of 6.25 cm^2 pieces.

Mosquito sample preparation

Mosquitoes which were exposed to the net for 5 minutes were used only in rapid colorimetric tests as pooled samples of different sizes (Table 3-1).

Table 3-1. Details of the content and size of sample pools following 5 minute exposure to a treated net. These samples were used in rapid colorimetric tests.

Sample pool content	Sample pool size
Whole mosquitoes Sets of mosquito legs Mosquito heads Mosquito thoraces Mosquito abdomens Whole control mosquitoes (not exposed)	10, 20, 30, 40 or 50

For HPLC-PDA analysis and related rapid colorimetric tests, the exposed and control mosquitoes were placed in Eppendorf tubes in pools of 10, depending on their exposure time (Table 3-2).

Table 3-2. Details of exposure times and the number of mosquitoes pooled in each sample used for HPLC-PDA and rapid colorimetric test experiments.

		No. of mosquitoes per pool				
		10	20	30	40	50
Exposure time (mins)	0.5	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**
	1.0	**Sample 6**	**Sample 7**	**Sample 8**	**Sample 9**	**Sample 10**
	1.5	**Sample 11**	**Sample 12**	**Sample 13**	**Sample 14**	**Sample 15**
	2.0	**Sample 16**	**Sample 17**	**Sample 18**	**Sample 19**	**Sample 20**
	2.5	**Sample 21**	**Sample 22**	**Sample 23**	**Sample 24**	**Sample 25**
	3.0	**Sample 26**	**Sample 27**	**Sample 28**	**Sample 29**	**Sample 30**

400 µl of acetonitrile (CH_3CN) were added to each sample and mosquitoes were first roughly ground using plastic pestles, then sonicated for at least 20 minutes making sure all

mosquitoes in each sample were completely crushed. Samples were then vortexed for 10 seconds and centrifuged for 4 minutes. The supernatant was removed into new tubes and used for HPLC-PDA and rapid colorimetric tests.

Measuring the amount of deltamethrin on mosquitoes

Each sample contained a different number of mosquitoes, with different length of exposure to the net (see Table 3-2 for details). The total amount of deltamethrin extracted from each sample was measured using HPLC-PDA in mg deltamethrin/ml, and then recalculated for each sample as follows:

For example, for sample number 30 (50 mosquitoes, exposed for 3 minutes), the HPLC measurement was 0.0002 mg deltamethrin/ml = 0.20 µg/ml. All samples were extracted in 400 µl of acetonitrile, which for sample 30 means 0.08 µg deltamethrin / 400 µl. This quantity was extracted from 50 exposed mosquitoes, therefore giving the final result of 0.0016 µg deltamethrin/ 1 mosquito.

3.2.3 Using the Colorimetric test

Following HPLC-PDA analysis, tubes with supernatant were left open for the acetonitrile to evaporate, leaving any deltamethrin residues behind. A method described by Kaur and Eggelte was used for colorimetric detection of the insecticide [25].

200 µl of solution A (para nitrobenzaldehyde) and 200 µl of solution B (acetonitrile) were added to each supernatant sample tube to produce a colour change in the presence of deltamethrin. After 5 minutes, 100 µl of solution C (aqueous NaOH solution) was added to stop the reaction, resulting in pink colour in the presence of deltamethrin.

Solutions containing deltamethrin at known concentrations (0.05, 0.04, 0.03, 0.02, 0.01, 0.005, 0.0008, 0.0006, 0.0004, 0.0002, 0.0001 mg/ml) were used for comparison purposes, while acetonitrile on its own was used as control blank (no colour). Results were read by eye.

3.3 Results

3.3.1 Amount of insecticide on the bed net

PermaNet 2.0 was used in the experiment first to determine the amount of deltamethrin on it, using the HPLC-PDA method. We detected 56.3 mg/m^2 of deltamethrin whereas the manufacturer's specifications state there is 55 mg/m^2 of deltamethrin.

3.3.2 Rapid colorimetric test for detection of deltamethrin on mosquitoes

The rapid colorimetric test showed that deltamethrin can be detected from whole mosquitoes and mosquito body parts (Fig. 3-1 and 3-2). Although whole mosquitoes produced the most intense colour, changes in the depth of colour could also be detected from different body parts, most often legs, heads and thoraces, but this was not consistent between different batches (Fig. 3-1).

Differences between body parts are not completely unexpected as they differ in surface area, and some (eg. legs) are more likely to come into contact with the net than others. During exposure to the net mosquitoes were at times seen trying to "crawl" through the net or were attempting to probe, which could explain stronger colouration of thorax or head samples.

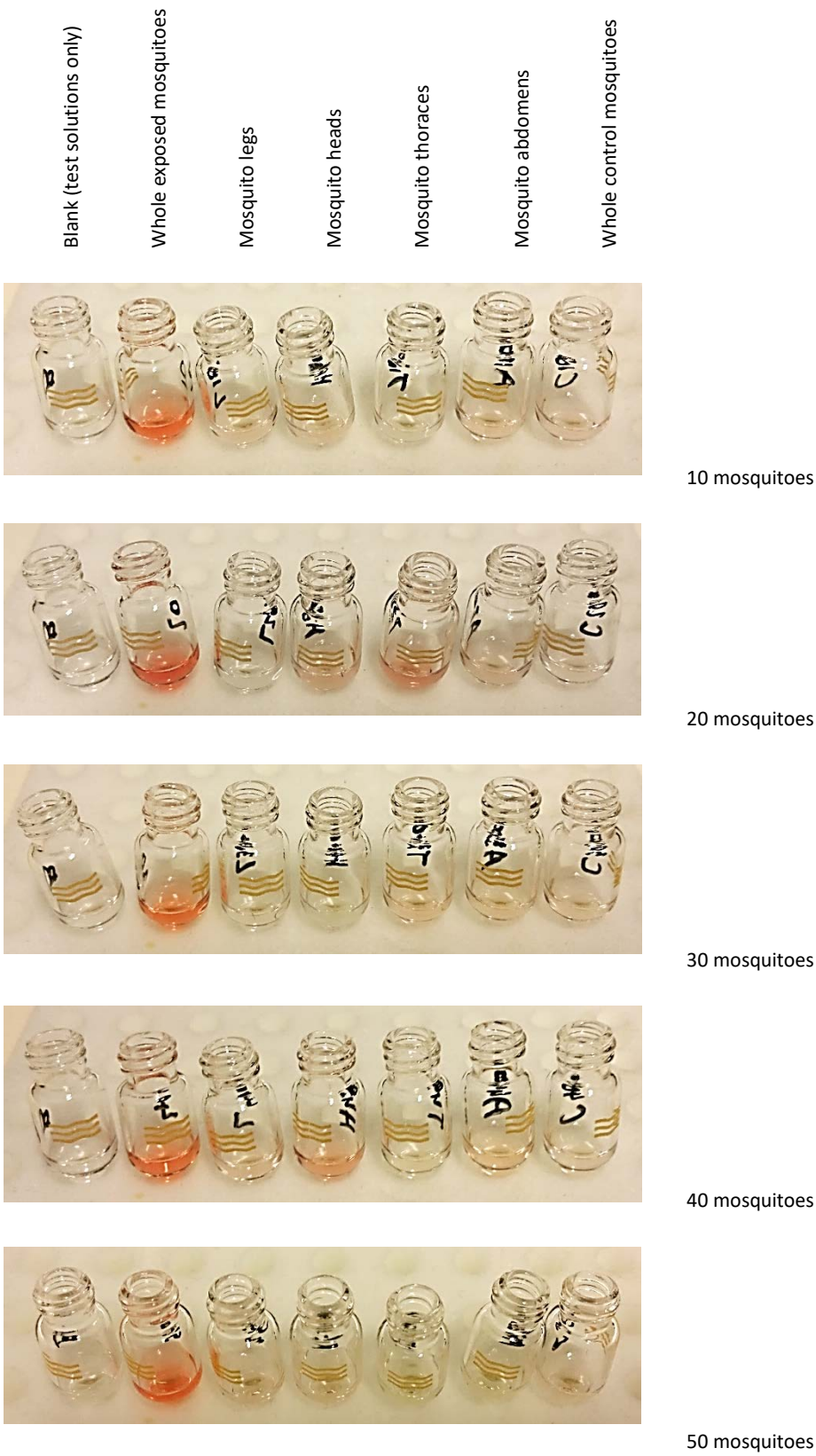

Fig. 3-1. Results of the rapid colorimetric test after 5 min exposure to PermaNet 2.0 (55 mg deltamethrin/m^2). Whole body extracts (second vial from the left in each row) produced the strongest reaction.

3.3.3 HPLC-PDA and rapid colorimetric test experiments to measure the amount of deltamethrin on mosquitoes

Deltamethrin was detected from pools of 10 or more mosquitoes, which were exposed to a LLIN for as little as 0.5 minute (Fig. 3-2; samples 1 – 5 in the second row). Intensity of colour noticeably increased with the number of mosquitoes per tube and with the length of exposure time (see Table 3-2 for details). For example, the intensity of colour increased from sample 6 (10 mosquitoes exposed for 1 min) to sample 10 (50 mosquitoes exposed for 1 min).

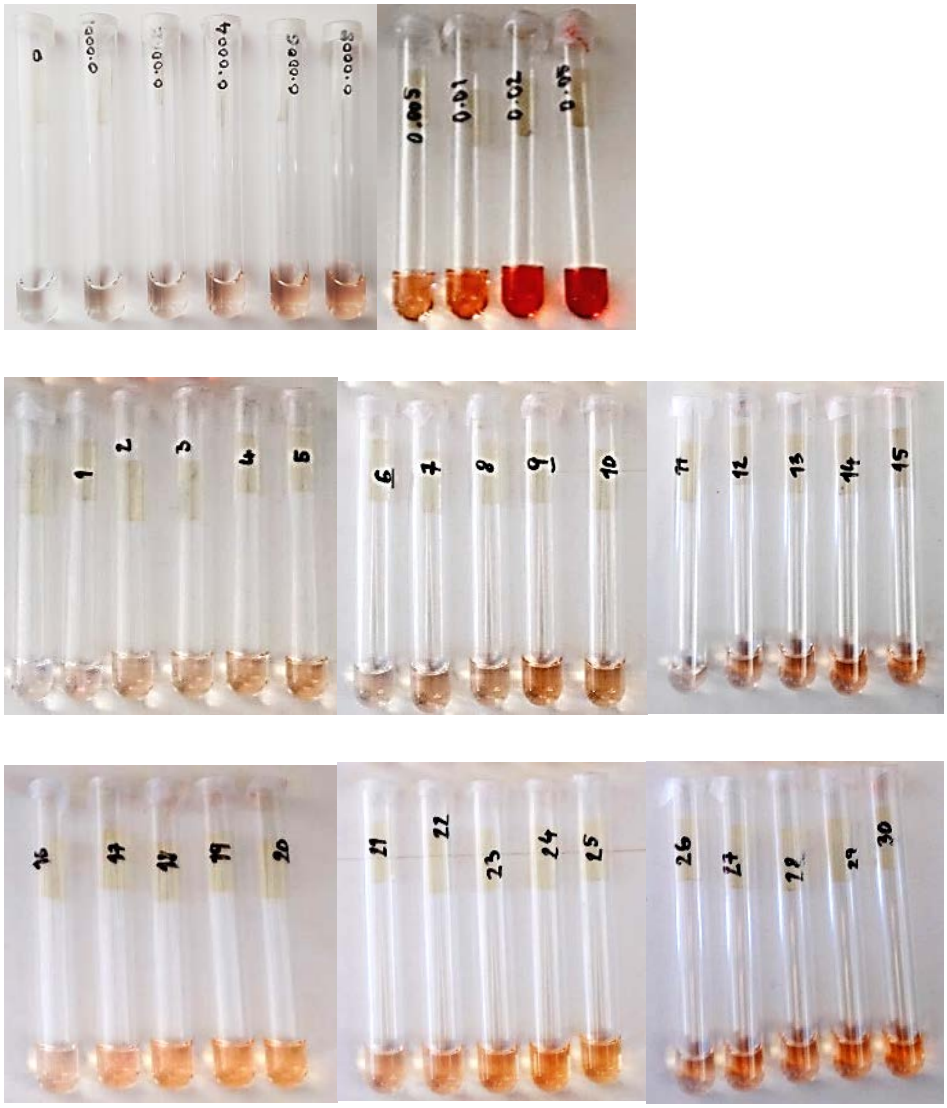

Fig. 3-2. Results of the rapid colorimetric test. Top row left to right: deltamethrin standards at concentrations 0 (control), 0.0001, 0.0002, 0.0004, 0.0006, 0.0008, 0.005, 0.01, 0.02, 0.05 mg/ml. Second row left to right: control; samples 1-5 (0.5 min exposure), 6-10 (1 min exposure), 10-15 (1.5 min exposure). Bottom row left to right: samples 16-20 (2 min exposure), 21-25 (2.5 min exposure), 26-30 (3 min exposure).

Similarly, we observed an increase in the intensity of colour where pool size remained constant but the length of exposure changed: between sample 1 (10 mosquitoes, 0.5 min) to sample 6 (10 mosquitoes, 1 min), sample 11 (10 mosquitoes, 1.5 min), sample 16 (10 mosquitoes, 2 min), sample 21 (10 mosquitoes, 2.5 min), to sample 26 (10 mosquitoes, 3 min).

Samples used in rapid colorimetric test (as shown in Fig. 3-2) were first processed by HPLC-PDA. The amount of deltamethrin was measured for pools of mosquitoes (10, 20, 30, 40 or 50 mosquitoes per pool), then recalculated per mosquito (Fig. 3-3).

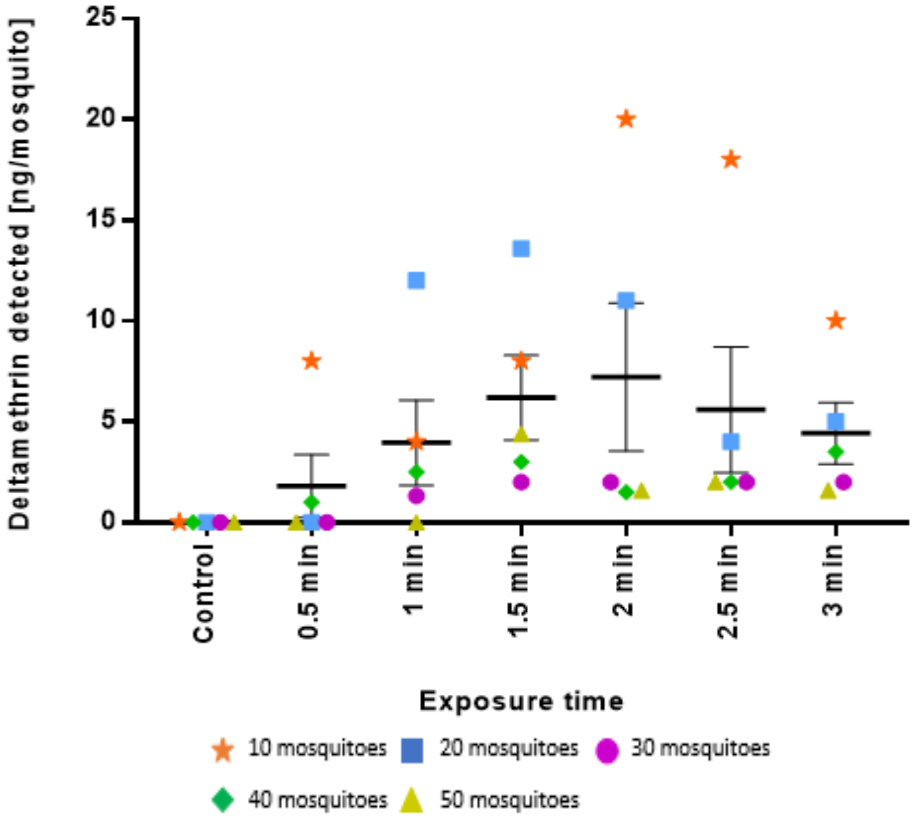

Fig. 3-3. The amount of deltamethrin detected on mosquitoes exposed to PermaNet 2.0 netting for different lengths of time. Thick horizontal lines represent the mean amount of deltamethrin [ng/mosquito] for each exposure time group. Error bars represent standard error of mean.

There was good correspondence between the amount of deltamethrin measured by HPLC-PDA and intensity of colour obtained in rapid colorimetric tests for majority of samples. There was a lot of variation for each exposure time, but the amounts measured were not significantly different (One-way ANOVA, $F_{6,28} = 1.22$, $p = 0.3256$). The highest values at each exposure time point were always measured in the smallest groups, i.e. pools of 10 or 20 mosquitoes. However, there was an increasing trend with more deltamethrin present on

mosquitoes that were exposed to the net for longer, between 0.5 minute exposure (1.8 ng/mosquito) and 2.0 minute exposure (7.2 ng/mosquito), whereas less deltamethrin was detected on mosquitoes that were exposed to the net for 2.5 minutes (5.6 ng/mosquito) and 3 minutes (4.4 ng/mosquito).

3.4 Discussion

While pyrethroids are currently the only insecticide class used on all ITNs, next-generation LLINs treated with a combination of pyrethroids (eg. alpha-cypermethrin) and chlorfenapyr, an N-substituted halogenated pyrrole, are currently under review [36]. Alternatively, pyrethroid LLINs can be used in combination with non-pyrethroid IRS. Mixtures of active ingredients, and combinations of interventions have been proposed as available strategies for insecticide resistance management [37]. Various methods can be used to determine the quantity of insecticides present on the ITNs or sprayed walls for the purposes of quality control, operational monitoring of spraying operations or to monitor degradation of insecticides on ITNs over time [22, 23, 28, 29, 34, 38, 39]. As the new combined tools are introduced, detection of active ingredients that actually come into contact with mosquitoes and cause the greatest mortality might be additionally used in assessing the effectiveness of interventions. Furthermore, measuring how much insecticide mosquitoes pick up when they come in contact with treated surfaces might inform future decisions on the doses of active ingredients used in vector control tools and might be a part of insecticide resistance management.

Pyrethroids need to penetrate through the mosquito cuticle to reach their target sites in the nervous system. Their point of entry is either through the mosquito tarsi when the insects land on the treated surfaces, or through the mosquito body if they collide with the net [8, 40]. Mosquito – LLIN interactions have been characterized using infrared video tracking, showing that susceptible mosquitoes made between 11.0 and 57.1 seconds of contact with a LLIN during the initial 10-minute period of most intense mosquito activity around the net [8]. Mean time spent on deltamethrin-treated net by a susceptible mosquito, causing knockdown and death, was measured to be 70.1 seconds, with the minimum required to cause knockdown just 0.4 seconds [20]. The exposure time in our experiments was chosen accordingly, starting at 30 seconds, whereas the longest exposure time (3 minutes) was the same as that used in WHO standard method for LLIN evaluation [41].

Not much is known about the actual amount of insecticide the mosquitoes pick up after contact with treated surfaces. Previous studies measured the amount of DDT on mosquitoes which entered sprayed huts (sprayed with 200 µg/cm^2 active ingredient) using gas chromatography [16]. The amount of DDT on dead *An. gambiae* and *An. funestus* was in the range of 7–20 ng/mosquito, whereas much lower levels of DDT (around 1.5 ng/mosquito) were found on surviving mosquitoes. Another study measured the amount of dieldrin picked up by *Culex quinquefasciatus* during the exposure in standard WHO bioassay tubes, using different concentrations on papers and different exposure times [15]. The authors concluded that pick-up of insecticide is a linear function of both the concentration and exposure time. It appears that at least some insecticide becomes internalised rapidly after exposure. When deltamethrin was topically applied to mosquito legs, about 5% of the initial applied amount could be detected in the mosquito body after 15-minute exposure (i.e. 0.048 ng/ susceptible mosquito body) [42]. When mosquitoes fed through a radio-labelled permethrin net the insecticide was shown to reach the midgut and was detected in the blood meal within an hour after feeding using a scintillation counter [43].

We have shown in this study that the amount of deltamethrin detected per mosquito using HPLC-PDA increased between 0.5 - 2 minute exposure but then levelled off during longer exposure times (Fig. 3-3), which is similar to the observations by Pennell *et al* of the amount of insecticide dieldrin present on the exterior of mosquitoes as opposed to "internal" amount which increased with increasing exposure time and concentration [15]. The type of insecticide used, its penetration through the cuticle, possible accumulation in the hemolymph and internal organs, and its metabolism within the insect will all determine what happens to insecticides after the initial contact [10]. Pyrethroids are known to associate with hemolymph carrier proteins and with lipids [9]. Although the mosquito samples used in our experiments for HPLC-PDA analysis were broken up by grinding and prolonged sonication, it is possible that deltamethrin on the exterior of mosquitoes was extracted into acetonitrile, while a proportion of it remained bound in lipid and protein-rich debris and therefore remained undetected.

In this study we have demonstrated that colorimetric tests can be used to detect the presence of deltamethrin not only on ITNs [27] and sprayed walls [28] but also on mosquitoes which came into contact with treated PermaNet 2.0 netting. A change in the depth of colour was detected in all samples, including the sample with the fewest pooled mosquitoes (10) and the shortest exposure time (30 seconds). Moreover, pyrethroids were

also detected in pools of different mosquito body parts, but with less consistency as the amounts of insecticide were that much smaller. Other studies have shown that mosquitoes obtain particles across their entire body in a standard 3-minute WHO cone bioassay and that particles can be transferred to their legs even following short contact periods [40]. Using only parts of collected mosquitoes could be advantageous during field work when the rest of the mosquito is required for other tests (eg.blood meal analysis, or to test for the presence of *P. falciparum* infection) but would need to be tested in large pools. The lowest amount of deltamethrin detected on whole mosquitoes was 1.33 ng/mosquito, measured in a pool of 30 mosquitoes following 1-minute exposure to a treated net. As this approached our limit of detection with HPLC-PDA, we did not endeavor to measure deltamethrin on separate mosquito body parts where the amounts would be even lower. However, more precise measurements of insecticide quantities on mosquito abdomens should be carried out in the future as our work (as described in Chapter 4) showed that deltamethrin affects ookinetes in a concentration-dependent manner. Work carried out previously using a scintillation counter showed that radio-labelled permethrin can be detected in the blood meal, but the amounts of permethrin present were not measured [43].

There was some disparity between the two analysis methods used. Whilst HPLC-PDA did not detect deltamethrin in some of the samples (2, 3, 5 and 10), these still produced colour change during the colorimetric test, indicating the presence of deltamethrin. Furthermore, with HPLC the highest values at each insecticide exposure time point were measured in the smallest groups, i.e. pools of 10 or 20 mosquitoes. Binding of the insecticide to cellular debris or the presence of large quantities of mosquito material might not affect rapid colorimetric tests in the same way it possibly affects HPLC. Some of the disparity observed between the two methods and the variation in the amount of deltamethrin detected for each exposure time might be reduced or eliminated if a larger number of replicates were used. The lack of replicates, which occurred due to time and financial constraints, therefore represents a limitation of this study.

The intensity of colour in the colorimetric test correlates with the amount of deltamethrin present in the sample. Although this method can be deployed in the field as is and results can be assessed visually, a mechanical absorbance plate reader could be used to more precisely quantify the results in the laboratory. Alternatively, a chart indicating what colour should be produced by a given amount of insecticide present on a batch of for example 10 mosquitoes, could be produced for use in field where no plate readers are available. Similar

charts are already available for use with colorimetric assays for detection of pyrethroids on treated bed nets [25] (see Appendix, Fig. A3-3 for example). More testing will be required using HPLC-PDA to fine-tune the procedure but in future either smaller pool sizes and/or shorter exposure times should be used, while the method of extracting deltamethrin from the debris is improved.

We have also shown for the first time that the amount of deltamethrin mosquitoes acquire after coming into contact with a LLIN is in the range of up to 10 ng/mosquito. This information could be further used to improve dosing of insecticides on treated nets or sprayed walls and will be used in future insecticide transmission-blocking studies.

3.5 References

1. 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;526:207-211.

2. World Health Organization: WHO recommended long-lasting insecticidal nets. http://www.who.int/whopes/Long-lasting_insecticidal_nets_June_2017.pdf?ua=1 (2017). Accessed 12 July 2017.

3. Nauen R. Insecticide resistance in disease vectors of public health importance. Pest Manag Sci. 2007;63:628-633.

4. Davies TG, Field LM, Usherwood PN, Williamson MS. DDT, pyrethrins, pyrethroids and insect sodium channels. IUBMB life. 2007;59:151-162.

5. Soderlund DM, Bloomquist JR. Neurotoxic actions of pyrethroid insecticides. Annu Rev Entomol. 1989;34:77-96.

6. Palmquist K, Salatas J, Fairbrother A. Pyrethroid Insecticides: Use, Environmental Fate, and Ecotoxicology. In: Perveen F, editor. Insecticides - Advances in Integrated Pest Management: InTech; 2012. p. 251-278.

7. Gillies MT, Wilkes TJ. A study of the age-composition of populations of *Anopheles gambiae* Giles and *A. funestus* Giles in North-Eastern Tanzania. Bull Entomol Res. 1965;56:237-262.

8. Parker JE, Angarita-Jaimes N, Abe M, Towers CE, Towers D, McCall PJ. Infrared video tracking of *Anopheles gambiae* at insecticide-treated bed nets reveals rapid decisive impact after brief localised net contact. Sci Rep. 2015;5:13392.

9. Schleier III JJ, Peterson RKD. Pyrethrins and Pyrethroid Insecticides. In: Lopez O, Fernandez-Bolanos JG, editors. Green Trends in Insect Control: Royal Society of Chemistry; 2011. p. 94-131.

10. Gerolt P. Insecticides: their route of entry, mechanism of transport and mode of action. Biol Rev Camb Philos Soc. 1983;58:233-274.

11. 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 Biochem Mol Biol. 2011;41:492-502.

12. World Health Organization: WHO recommended insecticides for indoor residual spraying against malaria vectors. http://www.who.int/whopes/Insecticides_IRS_2_Mar_2015.pdf (2015). Accessed 1 March 2018.

13. World Health Organization: Malaria vector control. Inseciticdes for indoor residual spraying. World Health Organisation; 2001.

14. World Health Organization: Insecticide-treated mosquito nets: a WHO Position Statement. http://www.ivcc.com/sites/ivcc.mrmdev.co.uk/files/content/itnspospaperfinal.pdf (2007). Accessed 1 March 2018.

15. Pennell JT, Miskus R, Craig R. The use of gas chromatography for the quantitative determination of micro-amounts of insecticide picked up by mosquitos. Bull World Health Organ. 1964;30:91-95.

16. Smith A, Webley DJ. A verandah-trap hut for studying the house-frequenting habits of mosquitoes and for assessing insecticides. 3. The effect of DDT on behavior and mortality. Bull Entomol Res. 1968;59:33-46.

17. Chandre F, Darriet F, Duchon S, Finot L, Manguin S, Carnevale P*, et al*. Modifications of pyrethroid effects associated with *kdr* mutation in *Anopheles gambiae*. Med Vet Entomol. 2000;14:81-88.

18. Hodjati MH, Curtis CF. Dosage differential effects of permethrin impregnated into bednets on pyrethroid resistant and susceptible genotypes of the mosquito *Anopheles stephensi*. Med Vet Entomol. 1997;11:368-372.

19. Porciani A, Diop M, Moiroux N, Kadoke-Lambi T, Cohuet A, Chandre F*, et al*. Influence of pyrethroid-treated bed net on host seeking behavior of *Anopheles gambiae* s.s. carrying the *kdr* allele. PLoS One. 2017;12:e0164518.

20. Spitzen J, Ponzio C, Koenraadt CJ, Pates Jamet HV, Takken W. Absence of close-range excitorepellent effects in malaria mosquitoes exposed to deltamethrin-treated bed nets. Am J Trop Med Hyg. 2014;90:1124-1132.

21. Kilian A, Byamukama W, Pigeon O, Gimnig J, Atieli F, Koekemoer L*, et al*. Evidence for a useful life of more than three years for a polyester-based long-lasting insecticidal mosquito net in Western Uganda. Malar J. 2011;10:299.

22. Graham K, Kayedi MH, Maxwell C, Kaur H, Rehman H, Malima R*, et al*. Multi-country field trials comparing wash-resistance of PermaNet and conventional insecticide-treated nets against anopheline and culicine mosquitoes. Med Vet Entomol. 2005;19:72-83.

23. Jenkins DW, Hensens A, Lloyd J, Payne M, Cizmarik P, Hamel S. Development and validation of a 'universal' HPLC method for pyrethroid quantification in long-lasting insecticidal mosquito nets for malaria control and prevention. Trop Med Int Health. 2013;18:2-11.

24. Ouattara JP, Pigeon O, Spanoghe P. Validation of a multi-residue method to determine deltamethrin and alpha-cypermethrin in mosquito nets by gas chromatography with electron capture detection (GC-muECD). Parasit Vectors. 2013;6:77.

25. Kaur H, Eggelte T: Colorimetric assay for pyrethroid insecticides on solid items. Google Patents; 2013.

26. Green MD: Colorimetric assay for measuring type I pyrethroids on treated objects. Google Patents; 2016.

27. Green MD, Atieli F, Akogbeto M. Rapid colorimetric field test to determine levels of deltamethrin on PermaNet surfaces: association with mosquito bioactivity. Trop Med Int Health. 2009;14:381-388.

28. Russell TL, Morgan JC, Ismail H, Kaur H, Eggelte T, Oladepo F*, et al*. Evaluating the feasibility of using insecticide quantification kits (IQK) for estimating cyanopyrethroid levels for indoor residual spraying in Vanuatu. Malar J. 2014;13:178.

29. Thawer NG, Ngondi JM, Mugalura FE, Emmanuel I, Mwalimu CD, Morou E*, et al*. Use of insecticide quantification kits to investigate the quality of spraying and decay rate of bendiocarb on different wall surfaces in Kagera region, Tanzania. Parasit Vectors. 2015;8:242.

30. Dowd AJ, Morou E, Steven A, Ismail HM, Labrou N, Hemingway J*, et al*. Development of a colourimetric pH assay for the quantification of pyrethroids based on glutathione-S-transferase. Int J Environ An Ch. 2010;90:922-933.

31. Enayati AA, Lengeler C, Erlanger T, Hemingway J. Field evaluation of a recombinant glutathione S-transferase-based pyrethroid quantification assay. Trans R Soc Trop Med Hyg. 2005;99:369-378.

32. Enayati AA, Vontas JG, Small GJ, McCarroll L, Hemingway J. Quantification of pyrethroid insecticides from treated bednets using a mosquito recombinant glutathione S-transferase. Med Vet Entomol. 2001;15:58-63.

33. Morou E, Ismail HM, Dowd AJ, Hemingway J, Labrou N, Paine M*, et al*. A dehydrochlorinase-based pH change assay for determination of DDT in sprayed surfaces. Anal Biochem. 2008;378:60-64.

34. Ismail HM, Kumar V, Singh RP, Williams C, Shivam P, Ghosh A*, et al*. Development of a Simple Dipstick Assay for Operational Monitoring of DDT. PLoS Negl Trop Dis. 2016;10:e0004324.

35. Habtewold T, Duchateau L, Christophides GK. Flow cytometry analysis of the microbiota associated with the midguts of vector mosquitoes. Parasit Vectors. 2016;9:167.

36. World Health Organization: Report of the twentieth WHOPES working group meeting, WHO/HQ, Geneva, 20-24 March 2017: review of Interceptor G2 LN, DawaPlus 3.0 LN, DawaPlus 4.0 LN, SumiLarv 2 MR, Chlorfenapyr 240 SC. Geneva: World Health Organization; 2017: 122.

37. World Health Organization. Global Plan for Insecticide Resistance Management in Malaria Vectors. Geneva: World Health Organization; 2012.

38. Anshebo GY, Graves PM, Smith SC, Wills AB, Damte M, Endeshaw T*, et al*. Estimation of insecticide persistence, biological activity and mosquito resistance to PermaNet(R) 2 long-lasting insecticidal nets over three to 32 months of use in Ethiopia. Malar J. 2014;13:80.

39. Green MD, Mayxay M, Beach R, Pongvongsa T, Phompida S, Hongvanthong B*, et al*. Evaluation of a rapid colorimetric field test to assess the effective life of long-lasting insecticide-treated mosquito nets in the Lao PDR. Malar J. 2013;12:57.

40. Andriessen R, Snetselaar J, Suer RA, Osinga AJ, Deschietere J, Lyimo IN*, et al*. Electrostatic coating enhances bioavailability of insecticides and breaks pyrethroid resistance in mosquitoes. Proc Natl Acad Sci U S A. 2015;112:12081-12086.

41. World Health Organization: Guidelines for laboratory and field testing of long-lasting insecticidal nets. Geneva: World Health Organization; 2013: vii, 89.

42. Yahouedo GA, Chandre F, Rossignol M, Ginibre C, Balabanidou V, Mendez NGA*, et al*. Contributions of cuticle permeability and enzyme detoxification to pyrethroid resistance in the major malaria vector *Anopheles gambiae*. Sci Rep. 2017;7:11091.

43. Hill N. Effects of sublethal doses of pyrethroids on malaria vectors. *PhD Thesis.* London: London School of Hygiene & Tropical Medicine, University of London; 2002.

Appendix 3-1

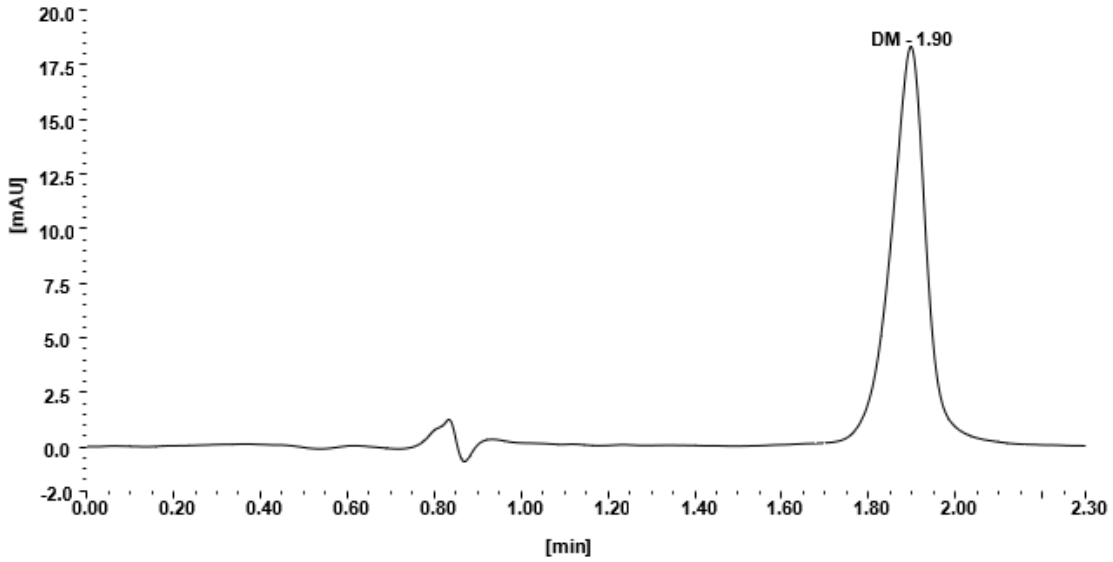

Fig. A3-1. High performance liquid chromatography separation of deltamethrin.

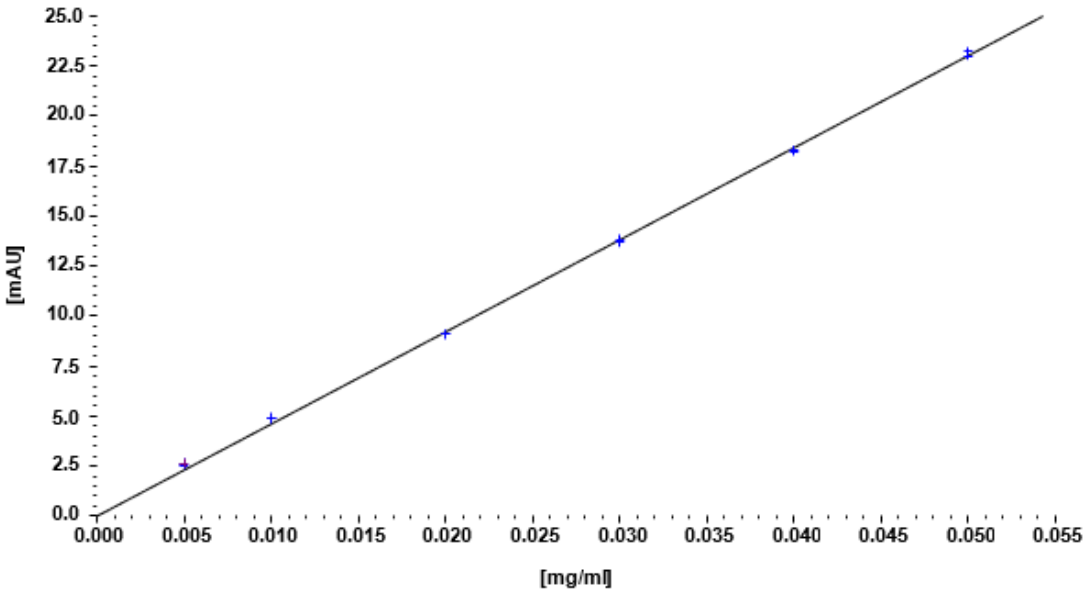

Fig. A3-2. Calibration plot of deltamethrin. Solutions containing deltamethrin at known concentrations (0.005, 0.010, 0.020, 0.030, 0.040, and 0.050 mg/ml) were used.

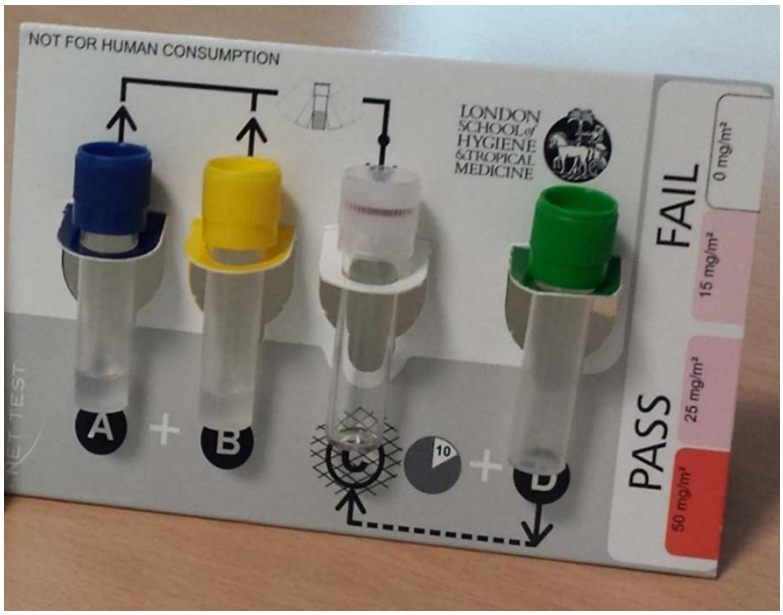

Fig. A3-3. A colorimetric test for detection of pyrethroids on treated bed nets [25].

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SECTION A – Student Details

Student	Mojca Kristan
Principal Supervisor	Jo Lines
Thesis Title	Effects of pyrethroid exposure and insecticide resistance on the sporogonic development of Plasmodium falciparum in Anopheles gambiae s.l.

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

SECTION B – Paper already published

Where was the work published?			
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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Parasites & Vectors
Please list the paper's authors in the intended authorship order:	M. Kristan, J. T. Dessens, R. W. Moon, J. Lines
Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	With the input from co-authors, I designed the study. J. T. D. set up the ookinete cultures. I carried out the microscopy and scoring of specimens, with assistance from J. T. D. and R. W. M.. I analysed the data with suggestions from R. W. M.. I wrote the manuscript with inputs from co-authors.

Student Signature: [redacted] Date: 28/3/2018

Supervisor Signature: [redacted] Date: 28.3.18

Chapter 4. Exploration of possible direct mechanisms underlying the effects of sub-lethal doses of pyrethroids on sporogony

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Abstract

Pyrethroids are neurotoxins affecting the voltage-gated sodium channels on the insect's neurons and a number of other sites or receptors. Some similar receptors might also be putative targets for pyrethroids against parasites. The parasites may come into contact with pyrethroids if the mosquitoes blood feed through a treated net or rest on a sprayed surface after feeding, as at least some of the insecticide becomes internalised rapidly after exposure. *Plasmodium berghei* ookinete cultures were used to investigate whether pyrethroids directly affect the parasites or if the interaction with a mosquito is necessary to produce the effects on sporogony previously reported in laboratory and field conditions.

Plasmodium berghei ookinetes were exposed to deltamethrin at a series of dilutions (100 µg/ml to 1pg/ml). Ookinete conversion assay was used to compare parasite transformation from gamete to ookinete stage between control and exposed groups. Ookinete motility assays were performed using the basement membrane matrix Matrigel® to compare ookinete movement between control and exposed groups.

The results showed that deltamethrin exposure significantly impairs both ookinete conversion and motility at doses that are close to what parasites could be exposed to through mosquitoes in the field. While this study demonstrated that direct effects are possible, it is more likely that mosquito immune responses to both, the infection and

insecticide exposure, as well as other mosquito-related factors are responsible for indirect transmission-blocking effects observed in laboratory and field conditions.

4.1 Introduction

Plasmodium malaria parasites undergo sporogonic development in their definitive hosts – *Anopheles* mosquitoes - before they can be transmitted on to another person. Sporogony generally lasts 8 to >20 days in the tropics [1, 2, 3], and is dependent on *Plasmodium* species, mosquito genetics, external factors such as temperature, humidity and the presence of chemicals, and a range of biotic factors [4].

Pyrethroids have been used for mosquito control since the 1970s [5]. They have become especially important with the scale-up of malaria control interventions as they are the only insecticide class currently used on all insecticide treated nets (ITNs) due to their low mammalian toxicity and rapid insecticidal activity [6]. However, insecticide resistance has become widespread and although the operational and epidemiological consequences of resistance are not yet clear, it is considered to be a major threat to malaria control interventions [7, 8, 9].

Pyrethroids are neurotoxins affecting the *para* voltage-gated sodium channels (VGSC) on the insect's neurons [10, 11], and a number of other channels, enzymes and receptors, contributing to their overall toxicity. Some of these are not insect-specific and could potentially be target sites affecting several vector-borne parasites [12, 13, 14]. It is not known whether pyrethroids accumulate in tissues or are immediately metabolised, and whether their metabolites exhibit any insecticidal or potentially sporontocidal activity [15]. There are several possible ways pyrethroids can enter the mosquito body [14]. The parasites may come into contact with pyrethroids if the mosquitoes blood feed through an ITN or rest on a sprayed surface after the feed. At least some insecticide becomes internalised rapidly after exposure and is transported from the point of contact on mosquito legs to the mosquito body [16]. When mosquitoes fed through a treated net, permethrin was shown to reach the midgut where it could be detected in the blood meal within an hour after feeding [17].

Sub-lethal doses of pyrethroids have been shown to impair sporogonic development of *Plasmodium* parasites in laboratory conditions by Elissa *et al* and by Hill [17, 18, 19, 20], while under field conditions oocyst prevalence and intensity were reduced following

exposure to sub-lethal doses of deltamethrin [21]. These effects could be due to one or more of the following mechanisms: direct effect of insecticides on the parasite inside the mosquito; indirect effect of insecticides on the parasite caused by the physiological changes of the environment inside the mosquito as a consequence of insecticide exposure; differential insecticidal killing of infected mosquitoes if infection restored phenotypic susceptibility in genotypically resistant mosquitoes.

By varying the time of exposure of mosquitoes to pyrethroids and infective bloodmeal, Hill also showed that pyrethroids most likely disrupt ookinete formation and migration of mature ookinetes through the midgut wall [17].

In order to test whether pyrethroids can affect *Plasmodium* sporogony directly, without any involvement of the mosquito immune system or parasite-vector interactions, a series of experiments was planned using parasite cultures. Pyrethroids can set off generation of oxygen reactive species (ROS), causing oxidative stress [22], which may in turn lead to ookinete apoptosis [23]. Deltamethrin was shown to be a potent calcium (Ca^{2+}) channel agonist [12] and calcineurin inhibitor [24], in which case it might have an impact on ookinetes. The aim of this study was to explore the possible mechanisms underlying the effects of sub-lethal doses of pyrethroids on sporogony, focusing on direct effects of insecticides on ookinete development and motility.

4.2 Methods

4.2.1 Deltamethrin stock preparation and serial dilution

Deltamethrin (Sigma Aldrich, product D 9315) was dissolved in 1:1 dimethyl sulfoxide (DMSO):100% ethanol (EtOH) mixture to obtain a 5 mg/ml stock solution.
10 µl of stock deltamethrin solution were added to 0.5 ml culture medium containing parasites to obtain the highest test concentration of 100 µg/ml. Serial ten-fold dilutions of deltamethrin were then carried out to obtain the lowest test concentration of 1 pg/ml deltamethrin.

4.2.2 *Plasmodium berghei* ookinete exposure to deltamethrin

Plasmodium berghei parasites (ANKA isolate, clone 507 – a transgenic, Green Fluorescent Protein (GFP)-expressing parasite line [25]) were maintained by mechanical blood passage

and regular mosquito transmission using *An. stephensi* mosquitoes (SDA500 strain [26]) and female Tuck CD1 mice (Charles River), or as cryopreserved stabilates.

Ookinete cultures were set up overnight from gametocytaemic blood in ookinete medium, as previously described [27], using a 24-well plate, containing 0.5 ml culture medium per well (Fig. 4-1). A 10 µl stock solution of deltamethrin was added to the first wells (A1 and B1), giving the highest concentration of 100 µg/ml. Serial ten-fold dilutions of deltamethrin were then created by transferring 50 µl from one well to the next (A1 → A6 →C1 →C3 and equally B1 → B6 →D1 →D3), ending with the lowest concentration of 1 pg/ml.

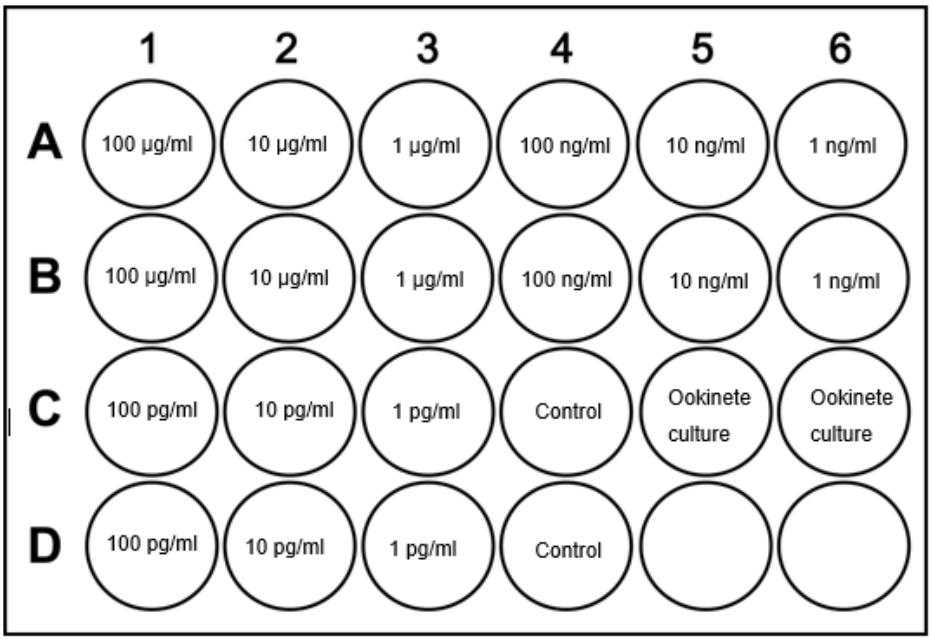


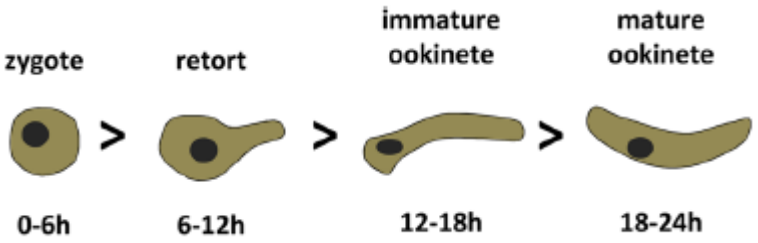
Fig. 4-1. Twenty-four well plate template showing experimental set up with different deltamethrin concentrations.

10 µl of DMSO:EtOH (1:1) was added as a negative control. The culture was incubated at 20^{o}C. The experiment was performed in duplicate (wells A1 → A6 →C1 →C3 and B1 → B6 →D1 →D3) and repeated twice.

4.2.3 *Plasmodium berghei* ookinete conversion assay

A 10 µl sample from each well was placed on a slide. Zeiss LSM510 inverted laser scanning confocal microscope and Zeiss LSM image browser software were used to count different developmental forms (Fig. 4-2) in the absence and presence of deltamethrin.

(a)



(b)

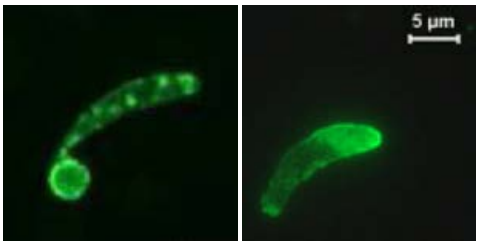


Fig. 4-2. (a) Schematic presentation of zygote-to-ookinete transformation and times-post-infection during which different steps occur. Reproduced from Guerreiro *et al*, 2014 [28] (Creative Commons Attribution License: http://creativecommons.org/licenses/by/4.0). **(b)** Representative images of a retort (left – reproduced from Guerreiro *et al*, 2014 [28] (Creative Commons Attribution License: http://creativecommons.org/licenses/by/4.0)) and an ookinete (right – reproduced from Nacer *et al.*, 2008 [29] (Creative Commons Attribution License: http://creativecommons.org/licenses/by/2.0)).

Ookinete conversion rates were calculated as [30]:

$$\frac{\text{number of gametes,zygotes,retorts or ookinetes}}{\text{total number of macrogametes,zygotes,retorts and ookinetes}} \times 100\%$$

4.2.4 *Plasmodium berghei in vivo* ookinete conversion assay

Ookinete cultures were set up as described above, on a 24-well plate, using only 10 µg/ml deltamethrin concentration and control. After 24 hours cultures were centrifuged, and old medium was removed in order to remove deltamethrin. Cultures were resuspended in the same volume of ookinete medium as was removed and fed to *An. stephensi* mosquitoes (SDA500 strain). Mosquitoes were provided with 10% glucose-0.05% p-aminobenzoic acid and were going to be maintained at 20°C and 80% relative humidity until required for oocyst or sporozoite dissection.

Plasmodium berghei ookinete motility assay

Ookinete motility assays were performed as previously described, using the basement membrane matrix Corning® Matrigel® (VWR) [31]. Briefly, an aliquot of frozen Matrigel® was defrosted prior to the assay. Ookinete culture was checked for ookinetes. To test the effect of deltamethrin on ookinete motility, the highest concentration that had no visible effect on ookinete conversion (i.e. 10 μg/ml) was used in the assay in comparison with control. Equal volumes of ookinete cultures and Matrigel® were gently mixed while kept on ice. A drop of mixture was applied onto a microscope slide, covered with a Vaseline-rimmed cover slip and sealed with nail varnish. The ookinete/ Matrigel® mixture was allowed to set at room temperature for 30 minutes, then slides were examined at 40x magnification to check that the Matrigel® had set.

Time-lapse videos of ookinetes were taken using the Nikon Eclipse Ti-E inverted microscope with attached Hamamatsu Digital Camera at 40x and 60x magnification using differential interference contrast (DIC) and confocal fluorescence settings, and analysed using NIS-Elements Imaging Software. Videos were composed by taking a picture every 5 seconds for 5 minutes, after first identifying a field of view with ookinetes.

The mid-front point on an ookinete (apical leading end) was first marked during each frame and the software calculated the distance it travelled during 5 minutes as a sum of distances between locations in subsequent images. The total distance is then divided by the total travel time [32]. Only ookinetes which remained in the field of view for the entire 5 minutes with the mid-front point clearly visible were scored. Several videos were taken of each sample to provide enough scored ookinetes for statistical analysis.

4.2.5 Statistical analysis

Statistical analysis was performed using the GraphPad Prism 7 software (GraphPad Software, Inc.). Statistical significance was determined with a two tailed, unpaired Student's t-test.

4.2.6 Ethics statement

All animal work was carried out by Dr. Johannes Dessens to produce material for ookinete cultures as part of his work. Animal work was conducted under UK Home Office license and approval in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 implementing European Directive 2010/63 for the protection of animals used for experimental purposes. All methods were carried out in accordance with relevant guidelines

and regulations and approval was obtained from the LSHTM Animal Welfare Ethics Review Board, with animal welfare assessed daily.

4.3 Results

The potential transmission blocking properties of pyrethroids were evaluated using *in vitro P. berghei* ookinete cultures incubated with different concentrations of deltamethrin.

4.3.1 *Plasmodium berghei* ookinete conversion assay

In vitro P. berghei ookinete conversion assays were used to test if deltamethrin affects parasite development. When ookinete cultures were incubated with deltamethrin, ookinete conversion was inhibited at the highest deltamethrin concentration but not at lower concentrations (Table 4-1 and Fig. 4-3).

Table 4-1. Counts of different forms in each experimental condition used to calculate conversion rates (as shown in Fig. 4–3 below).

	Control (95% CI)	10 µg/ml (95% CI)	100 µg/ml (95% CI)
Gametocyte/gamete	62 (59.1, 64.9)	53 (50.2, 54.8)	94 (89.5, 97.5)
Zygote	8 (5.9, 10.1)	9 (6.1, 11.9)	1 (0.5, 0.5)
Retort	4 (1.5, 6.5)	7 (4.9, 9.1)	11 (7.2, 14.8)
Ookinete	38 (32.9, 42.6)	38 (33.7, 41.3)	0
Total forms observed	223	212	210

Note: Counts were made in two wells per experiment (eg. A1 and C1 to get a number of different forms when culture was exposed to 100 µg/ml deltamethrin in duplicate 1). The experiment was repeated twice (n = 4).

Ookinete conversion rates were significantly different between control and 100 µg/ml deltamethrin groups ($p < 0.0001$), and 10 µg/ml and 100 µg/ml deltamethrin groups ($p < 0.0001$), but not between control and 10 µg/ml ($p > 0.05$) or lower concentration (not shown) deltamethrin groups. The largest differences were observed at the gametocyte/gamete and ookinete levels. None of the concentrations below 100 µg/ml deltamethrin affected ookinete conversion rates.

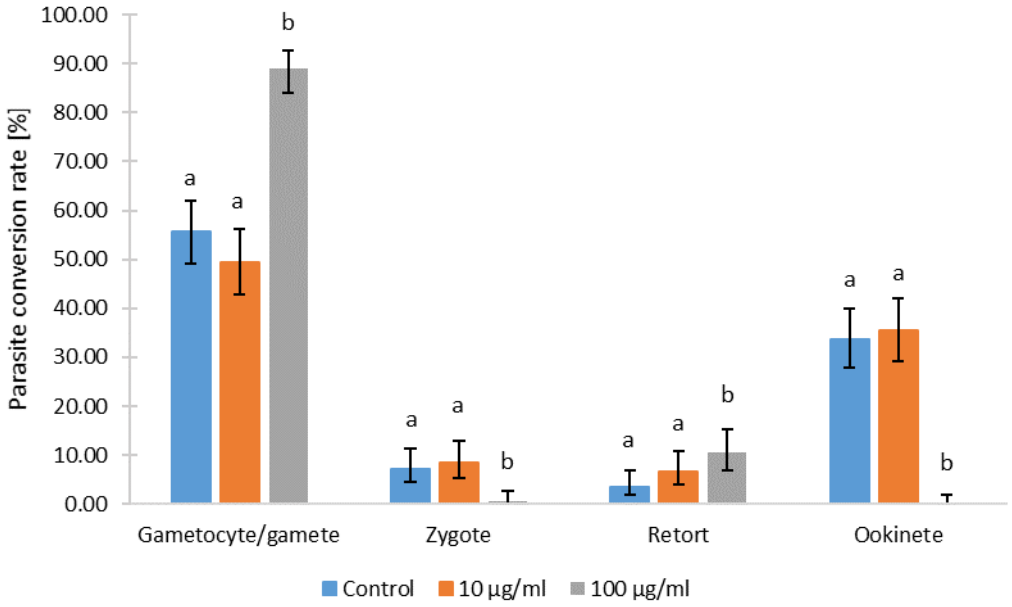


Fig. 4-3. The effect of deltamethrin on *P. berghei* gamete/zygote/retort/ookinete conversion rates *in vitro*, as observed in a 24-hour ookinete culture. Error bars indicate 95% confidence intervals for comparison within each form. The same letter above bars indicates groups are not significantly different; Student's t test $p<0.05$.

No normal ookinetes were visible in 100 µg/ml deltamethrin group while the majority of observed forms were gametocytes/gametes. A number of retorts were present, and some of these had unusually elongated long parts or more pronounced "swollen" round parts in the 100 µg/ml deltamethrin group, unlike those in the other two groups.

4.3.2 *Plasmodium berghei in vivo* ookinete conversion assay

All mosquitoes fed with ookinetes exposed to 10 µg/ml deltamethrin concentration died within 24 hours of the feed. The experiment was then discontinued.

4.3.3 *Plasmodium berghei* ookinete motility assay

To assess whether deltamethrin affects ookinete motility, *in vitro P. berghei* ookinete motility assays were used. Gliding of deltamethrin-exposed ookinetes was characteristically helical and was not visually affected (Fig. 4-4).

However, their average gliding speed was significantly reduced ($p = 0.0128$) (Fig. 4-5).

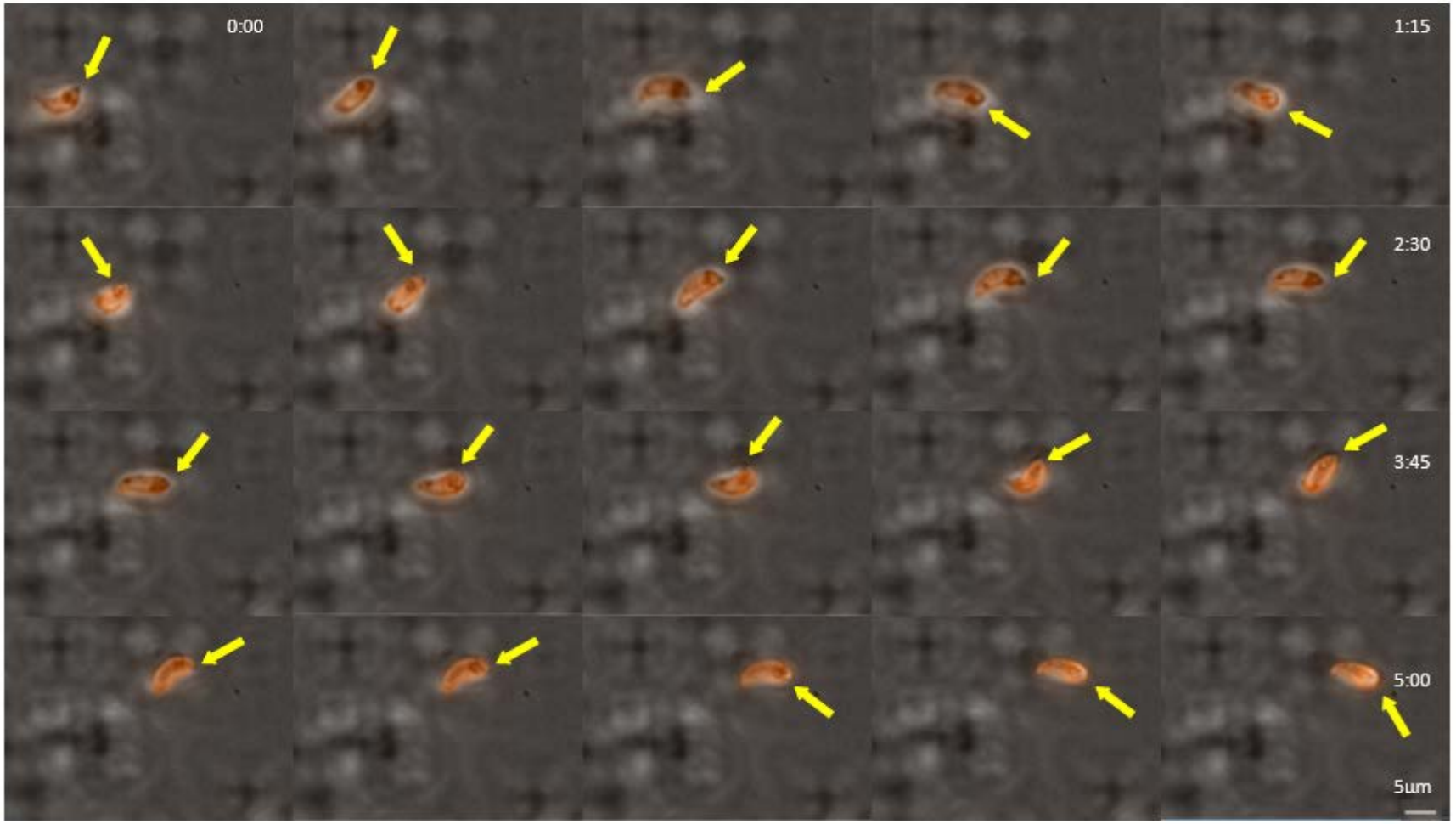


Fig. 4-4. Selected still pictures from a 5-minute-long time-lapse video of an ookinete moving through the Matrigel® membrane matrix showing characteristic movement. This ookinete travelled 55.24 µm in 5 minutes of filming. Movement shown in rows from left to right, with time indicated in top right corner. A yellow arrow marks the apical (i.e. leading) end of the ookinete.

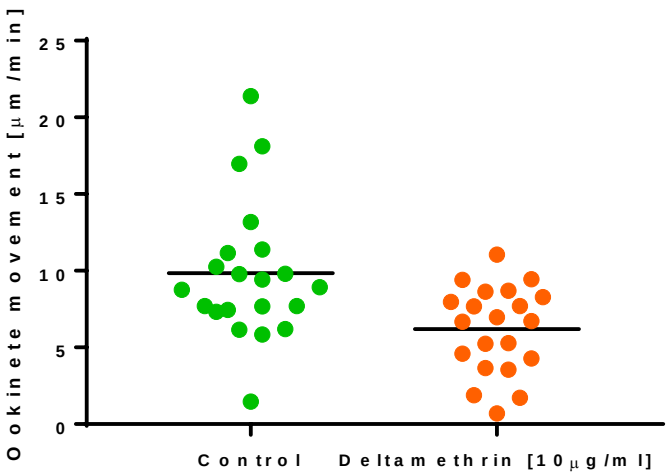


Fig. 4-5. Speed of individual ookinetes from 24-hour ookinete cultures in the presence or absence of deltamethrin (10 µg/ml), measured over 5 min. The thick black line denotes mean. Mean and SEM of control group: 10.05 ± 1.178; mean and SEM of deltamethrin group: 6.507 ± 0.677. Groups significantly different, two-tailed Student's t test, p = 0.0128.

4.4 Discussion

In this study, it was demonstrated that exposure to pyrethroids directly affects *Plasmodium* parasites during sporogony. Both ookinete conversion and motility were affected. The effects were significant in experimental conditions and were observed at concentrations of pyrethroids (10 – 100 µg/ml) that might be biologically relevant. As previously shown (in Chapter 3), mosquitoes pick up nanogram quantities of deltamethrin when exposed to ITNs, which if recalculated to the same scale would be about 2.5 µg/ml, a concentration not significantly lower than those tested. It is therefore possible that the parasites might encounter such concentrations inside mosquitoes in the field. As a follow up, precise measurements of insecticide quantities on whole mosquitoes and especially on mosquito abdomens – where ookinetes are - should be carried out in the future, especially on mosquitoes exposed to LLINs in the field.

After a mosquito ingests gametocytes with an infective blood meal, fertilization occurs and is followed by a complex differentiation process during which parasites transform from spherical zygotes via characteristically-shaped retorts into banana-shaped motile ookinetes within approximately 18-24 hours [33, 34]. Development of motile ookinetes is crucial for

malaria transmission as they must egress from the blood meal inside the midgut, and cross the midgut epithelium wall to form oocysts, where motile sporozoites will eventually form, while untransformed retorts are unable to form oocysts. It is thought that Ca^{2+}/calmodulin signaling plays an important role in this process [35], while the motion is thought to be dependent on actin and myosin [36, 37]. Ookinetes show three distinct modes of motility: stationary rotation, directional spiralling and straight-segment motility [38]. This can be observed using an *in vitro* Matrigel® assay [31, 32]. The ookinete speed of our control group (approximately 10 μm/min) is in line with that measured by Kan *et al* [32], while following the exposure to 10 μg/ml deltamethrin, ookinete average gliding speed was significantly reduced by about 35%. Measuring the speed of ookinetes depends on following the ookinete movement over a given length of time and obtaining a sum of distances between track locations (i.e. marking the mid-front point on an ookinete) in subsequent images. Ookinete motion is random in 3D space, which means that some of the ookinetes cannot be tracked for the entire period of time and therefore that observation had to be discarded, which could generate a bias. However, gliding of deltamethrin-exposed ookinetes was characteristically helical and visually not different from ookinetes in the control group, so scoring bias would affect both groups equally. While Matrigel® provides a suitable environment for study of ookinetes as it supports ookinete motility [31, 32], its density can be inconsistent and variable, presenting a limitation to this assay.

In the presence of 100 μg/ml deltamethrin the majority of observed forms were gametocytes/gametes, followed by retorts, while no zygotes and normal ookinetes were observed. Gametocytes/gametes were also present in control and 10 μg/ml deltamethrin groups, but at significantly lower levels. Retorts form when a rounded zygote starts elongating before it turns into an ookinete. While they normally occur around 10 hours following infected blood meal, with some still visible around 24 hours, the majority should have completed their transformation into elongated ookinetes by this point.

Another question is whether these retorts and ookinetes, which form in the presence of lower concentrations of deltamethrin, are viable and capable of forming oocysts. We intended to test this using an *in vivo* assay. Ookinete cultures were exposed to 10 μg/ml deltamethrin as described above, then washed to remove any residual insecticide. This concentration was used as it had no visible effects on ookinete formation in the *in vitro* assay. The deltamethrin-exposed and control ookinetes were fed to *An. stephensi* (SDA500) mosquitoes, to check for oocyst and sporozoite formation. However, mosquito strain used is

pyrethroid-susceptible and none of the mosquitoes that were fed ookinetes treated with deltamethrin survived more than 24 hours following the feed. It is possible that deltamethrin metabolites which form in mosquitoes following their exposure are toxic to both parasites and susceptible mosquitoes, potentially even more than the insecticide itself. Other indirect effects could play an even bigger role, such as mosquito immune system which is triggered by both, parasite presence and insecticide exposure [39, 40]. Using a negative control (i.e. feeding mosquitoes with deltamethrin-treated uninfected blood) would have allowed us to distinguish between the effects of parasites and the effects of deltamethrin on their own. A lack of negative control therefore represents a limitation of this experiment.

Apart from the best-known effects of pyrethroids on VGSCs, their inhibitory effects have also been described for voltage-gated calcium channels (VGCC), potassium channels, calmodulin and protein kinases, peripheral benzodiazepine receptors, ATPases and Na/Ca exchangers, nicotinic acetylholine receptors, GABA receptors, GABA-activated channels, phosphoinositides and phospholipase C, the βγ subunit of heterotrimeric G-proteins, and the voltage-gated chloride channels (VGClC), contributing to their overall neurotoxicity [12]. Inhibitory effects of type I and type II pyrethroids on mitochondrial Complex I have also been observed [13]. Some of these targets are also present in vector-borne parasites such as *Plasmodium*, so the insecticides could potentially inhibit growth of developing parasites.

A distant relative of parasitic Apicomplexa (eg. *Plasmodium* spp), *Paramecium tetraurelia* is a free living aquatic ciliate that lacks VGSCs yet was found to be highly sensitive to deltamethrin and other pyrethroids, which act as potent calcium (Ca^{2+}) channel agonists on the ciliary VGCC of *P. tetraurelia* [12]. There are a number of differences between Apicomplexa and *Paramecium* in their Ca^{2+} channels but some homologues have been found [41].

Type II pyrethroids such as deltamethrin, cypermethrin and fenvalerate act as calcineurin inhibitors [24]. Calcineurin is a *Plasmodium* phosphatase which modulates several Ca^{2+}-dependent processes and is crucial at key transition points of *Plasmodium* life cycle, regulating male gametogenesis, gamete fertilisation, colonisation of mosquito midgut cells by ookinetes and of hepatocytes by sporozoites [42]. Calcium-regulated signalling cascades have an important role in the regulation of Apicomplexan parasite development, response to environmental cues and invasive motility [43]. Ca^{2+} signalling is vital for gliding of *Plasmodium* sporozoites and ookinetes, the two zoite forms which colonise their vertebrate host and their mosquito vector, respectively [31, 44].

Mitochondria are organelles found in eukaryotic cells, including in *Plasmodium* parasites, where they are vital for metabolism in both asexual and sexual stages [45]. They differ from typical mitochondria and undergo restructuring in gametocytes, in preparation for transmission to mosquitoes and sporogony [46, 47]. Metabolic activity of mitochondria also contributes to the development of zygotes into elongated ookinetes and is vital for successful sporogony [47, 48, 49, 50].

As outlined above there are several putative targets for pyrethroids against parasites. However, parasites are only exposed to insecticides at low concentrations when in mosquitoes. While we demonstrated that direct effects are possible, it is more likely that the mosquito immune response to both the infection and insecticide exposure, and other mosquito-related factors, are responsible for the indirect transmission-blocking effects, as those already observed in the laboratory and field studies. These effects could be one of the reasons why pyrethroid-based interventions such as ITNs have not yet completely failed despite the widespread insecticide resistance [51]. Further *in vitro* and *in vivo* experiments will be required to unravel the underlying mechanisms.

4.5 References

1. Smith RC, Vega-Rodriguez J, Jacobs-Lorena M. The *Plasmodium* bottleneck: malaria parasite losses in the mosquito vector. Mem Inst Oswaldo Cruz. 2014;109:644-661.

2. Angrisano F, Tan YH, Sturm A, McFadden GI, Baum J. Malaria parasite colonisation of the mosquito midgut - Placing the *Plasmodium* ookinete centre stage. Int J Parasitol. 2012;42:519-527.

3. World Health Organization: Manual on Practical Entomology in Malaria. Part II - Methods and Techniques. Geneva: World Health Organisation; 1975: 197.

4. Lefevre T, Vantaux A, Dabire KR, Mouline K, Cohuet A. Non-genetic determinants of mosquito competence for malaria parasites. PLoS Pathog. 2013;9:e1003365.

5. Nauen R. Insecticide resistance in disease vectors of public health importance. Pest Manag Sci. 2007;63:628-633.

6. Zaim M, Aitio A, Nakashima N. Safety of pyrethroid-treated mosquito nets. Med Vet Entomol. 2000;14:1-5.

7. 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.

8. Malaria Policy Advisory Committee: WHO Malaria Policy Advisory Committee (MPAC) meeting report. World Health Organization; 2016: 13.

9. Ranson H, Lissenden N. Insecticide resistance in African *Anopheles* mosquitoes: a worsening situation that needs urgent action to maintain malaria control. Trends Parasitol. 2016;32:187-196.

10. Davies TG, Field LM, Usherwood PN, Williamson MS. DDT, pyrethrins, pyrethroids and insect sodium channels. IUBMB life. 2007;59:151-162.

11. Soderlund DM, Bloomquist JR. Neurotoxic actions of pyrethroid insecticides. Annu Rev Entomol. 1989;34:77-96.

12. Symington SB, Zhang A, Karstens W, Van Houten J, Clark JM. Characterization of pyrethroid action on ciliary calcium channels in *Paramecium tetraurelia*. Pestic Biochem Physiol. 1999;65:181-193.

13. Gassner B, Wuthrich A, Scholtysik G, Solioz M. The pyrethroids permethrin and cyhalothrin are potent inhibitors of the mitochondrial complex I. J Pharmacol Exp Ther. 1997;281:855-860.

14. Schleier III JJ, Peterson RKD. Pyrethrins and Pyrethroid Insecticides. In: Lopez O, Fernandez-Bolanos JG, editors. Green Trends in Insect Control: Royal Society of Chemistry; 2011. p. 94-131.

15. 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 Biochem Mol Biol. 2011;41:492-502.

16. Yahouedo GA, Chandre F, Rossignol M, Ginibre C, Balabanidou V, Mendez NGA*, et al*. Contributions of cuticle permeability and enzyme detoxification to pyrethroid resistance in the major malaria vector *Anopheles gambiae*. Sci Rep. 2017;7:11091.

17. Hill N. Effects of sublethal doses of pyrethroids on malaria vectors. *PhD Thesis.* London: London School of Hygiene & Tropical Medicine, University of London; 2002.

18. Carle PR, Coz J, Elissa N, Gasquet M, Sannier C, Richard A*, et al*. [Intravector anti-*Plasmodium* activity of a pyrethrinoid: deltamethrin]. C R Acad Sci III. 1986;303:565-568.

19. Elissa N, Sannier C, Coz J. [Étude de la réceptivité à *Plasmodium yoelii yoelii* d'*Anopheles stephensi* soumis au stade adulte à des doses toxiques de différents pyréthrinoïdes]. Cah ORSTOM, ser Ent méd et Parasitol. 1987;25:187-192.

20. Elissa N, Sannier C, Richard A: [La Receptivite a *Plasmodium yoelii yoelii* d'*Anopheles* Soumis a des Subletales de Deltamethrine]. In: *IVe Congres Sur La Protection De La Sante Humaine Et Des Cultures En Milieu Tropical*. Marseille1986: 599-604.

21. Kristan M, Lines J, Nuwa A, Ntege C, Meek SR, Abeku TA. Exposure to deltamethrin affects development of *Plasmodium falciparum* inside wild pyrethroid resistant *Anopheles gambiae* s.s. mosquitoes in Uganda. Parasit Vectors. 2016;9:100.

22. Tu HT, Silvestre F, Meulder BD, Thome JP, Phuong NT, Kestemont P. Combined effects of deltamethrin, temperature and salinity on oxidative stress biomarkers and acetylcholinesterase activity in the black tiger shrimp (*Penaeus monodon*). Chemosphere. 2012;86:83-91.

23. Matthews H, Ali M, Carter V, Underhill A, Hunt J, Szor H*, et al*. Variation in apoptosis mechanisms employed by malaria parasites: the roles of inducers, dose dependence and parasite stages. Malar J. 2012;11:297.

24. Enan E, Matsumura F. Specific inhibition of calcineurin by type II synthetic pyrethroid insecticides. Biochem Pharmacol. 1992;43:1777-1784.

25. Janse CJ, Franke-Fayard B, Mair GR, Ramesar J, Thiel C, Engelmann S*, et al*. High efficiency transfection of *Plasmodium berghei* facilitates novel selection procedures. Mol Biochem Parasitol. 2006;145:60-70.

26. Neafsey DE, Waterhouse RM, Abai MR, Aganezov SS, Alekseyev MA, Allen JE*, et al*. Mosquito genomics. Highly evolvable malaria vectors: the genomes of 16 *Anopheles* mosquitoes. Science. 2015;347:1258522.

27. Arai M, Billker O, Morris HR, Panico M, Delcroix M, Dixon D*, et al*. Both mosquito-derived xanthurenic acid and a host blood-derived factor regulate gametogenesis of *Plasmodium* in the midgut of the mosquito. Mol Biochem Parasitol. 2001;116:17-24.

28. Guerreiro A, Deligianni E, Santos JM, Silva PA, Louis C, Pain A*, et al*. Genome-wide RIP-Chip analysis of translational repressor-bound mRNAs in the *Plasmodium* gametocyte. Genome Biol. 2014;15:493.

29. Nacer A, Underhill A, Hurd H. The microneme proteins CTRP and SOAP are not essential for *Plasmodium berghei* ookinete to oocyst transformation *in vitro* in a cell free system. Malar J. 2008;7:82.

30. Zheng W, Kou X, Du Y, Liu F, Yu C, Tsuboi T*, et al*. Identification of three ookinete-specific genes and evaluation of their transmission-blocking potentials in *Plasmodium berghei*. Vaccine. 2016;34:2570-2578.

31. Moon RW, Taylor CJ, Bex C, Schepers R, Goulding D, Janse CJ*, et al*. A cyclic GMP signalling module that regulates gliding motility in a malaria parasite. PLoS Pathog. 2009;5:e1000599.

32. Kan A, Tan YH, Angrisano F, Hanssen E, Rogers KL, Whitehead L*, et al*. Quantitative analysis of *Plasmodium* ookinete motion in three dimensions suggests a critical role for cell shape in the biomechanics of malaria parasite gliding motility. Cell Microbiol. 2014;16:734-750.

33. Carter V, Nacer AM, Underhill A, Sinden RE, Hurd H. Minimum requirements for ookinete to oocyst transformation in *Plasmodium*. Int J Parasitol. 2007;37:1221-1232.

34. Janse CJ, Rouwenhorst RJ, Van der Klooster PF, Van der Kaay HJ, Overdulve JP. Development of *Plasmodium berghei* ookinetes in the midgut of *Anopheles atroparvus* mosquitoes and *in vitro*. Parasitology. 1985;91 (Pt 2):219-225.

35. Silva-Neto MA, Atella GC, Shahabuddin M. Inhibition of Ca^{2+}/calmodulin-dependent protein kinase blocks morphological differentiation of *Plasmodium gallinaceum* zygotes to ookinetes. J Biol Chem. 2002;277:14085-14091.

36. Siden-Kiamos I, Pinder JC, Louis C. Involvement of actin and myosins in *Plasmodium berghei* ookinete motility. Mol Biochem Parasitol. 2006;150:308-317.

37. Angrisano F, Delves MJ, Sturm A, Mollard V, McFadden GI, Sinden RE*, et al*. A GFP-actin reporter line to explore microfilament dynamics across the malaria parasite lifecycle. Mol Biochem Parasitol. 2012;182:93-96.

38. Vlachou D, Zimmermann T, Cantera R, Janse CJ, Waters AP, Kafatos FC. Real-time, *in vivo* analysis of malaria ookinete locomotion and mosquito midgut invasion. Cell Microbiol. 2004;6:671-685.

39. James RR, Xu J. Mechanisms by which pesticides affect insect immunity. J Invertebr Pathol. 2012;109:175-182.

40. Smith RC, Jacobs-Lorena M. *Plasmodium*-mosquito interactions: a tale of roadblocks and detours. Adv In Insect Phys. 2010;39:119-149.

41. Prole DL, Taylor CW. Identification of intracellular and plasma membrane calcium channel homologues in pathogenic parasites. PLoS One. 2011;6:e26218.

42. Philip N, Waters AP. Conditional degradation of *Plasmodium* calcineurin reveals functions in parasite colonization of both host and vector. Cell Host Microbe. 2015;18:122-131.

43. Billker O, Lourido S, Sibley LD. Calcium-dependent signaling and kinases in apicomplexan parasites. Cell Host Microbe. 2009;5:612-622.

44. Ishino T, Orito Y, Chinzei Y, Yuda M. A calcium-dependent protein kinase regulates *Plasmodium* ookinete access to the midgut epithelial cell. Mol Microbiol. 2006;59:1175-1184.

45. MacRae JI, Dixon MW, Dearnley MK, Chua HH, Chambers JM, Kenny S*, et al*. Mitochondrial metabolism of sexual and asexual blood stages of the malaria parasite *Plasmodium falciparum*. BMC Biol. 2013;11:67.

46. Fisher N, Bray PG, Ward SA, Biagini GA. The malaria parasite type II NADH:quinone oxidoreductase: an alternative enzyme for an alternative lifestyle. Trends Parasitol. 2007;23:305-310.

47. Sturm A, Mollard V, Cozijnsen A, Goodman CD, McFadden GI. Mitochondrial ATP synthase is dispensable in blood-stage *Plasmodium berghei* rodent malaria but essential in the mosquito phase. Proc Natl Acad Sci U S A. 2015;112:10216-10223.

48. Hino A, Hirai M, Tanaka TQ, Watanabe Y, Matsuoka H, Kita K. Critical roles of the mitochondrial complex II in oocyst formation of rodent malaria parasite *Plasmodium berghei*. J Biochem. 2012;152:259-268.

49. Boysen KE, Matuschewski K. Arrested oocyst maturation in *Plasmodium* parasites lacking type II NADH:ubiquinone dehydrogenase. J Biol Chem. 2011;286:32661-32671.

50. Klug D, Mair GR, Frischknecht F, Douglas RG. A small mitochondrial protein present in myzozoans is essential for malaria transmission. Open Biol. 2016;6:160034.

51. World Health Organization: Implications of insecticide resistance for malaria vector control. Geneva: World Health Organization; 2016: 4.

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Principal Supervisor	Jo Lines
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Exposure to deltamethrin affects development of *Plasmodium falciparum* inside wild pyrethroid resistant *Anopheles gambiae* s.s. mosquitoes in Uganda

Mojca Kristan[1*], Jo Lines[1], Anthony Nuwa[2], Charles Ntege[3], Sylvia R. Meek[4] and Tarekegn A. Abeku[4]

Abstract

Background: Pyrethroid resistance in African vector mosquitoes is a threat to malaria control. Resistant mosquitoes can survive insecticide doses that would normally be lethal. We studied effects of such doses on *Plasmodium falciparum* development inside *kdr*-resistant *Anopheles gambiae* s.s. in Uganda.

Methods: We collected *An. gambiae* s.s. homozygous for *kdr*-L1014S mutation, fed them on blood samples from 42 *P. falciparum*-infected local patients, then exposed them either to nets treated with sub-lethal doses of deltamethrin or to untreated nets. After seven days, we dissected 692 mosquitoes and examined their midguts for oocysts. Prevalence (proportion infected) and intensity of infection (number of oocysts per infected mosquito) were recorded for each group.

Results: Both prevalence and intensity of infection were significantly reduced in deltamethrin-exposed mosquitoes, compared to those exposed to untreated nets. With low doses (2.5–5.0 mg/m^2), prevalence was reduced by 59 % (95 % CI = 22 %-78 %) and intensity by 41 % (95 % CI = 25 %-54 %). With high doses (10–16.7 mg/m^2), prevalence was reduced by 80 % (95 % CI = 67 %-88 %) and intensity by 34 % (95 % CI = 20 %-46 %).

Conclusions: We showed that, with locally-sampled parasites and mosquitoes, doses of pyrethroids that are sub-lethal for resistant mosquitoes can interfere with parasite development inside mosquitoes. This mechanism could enable pyrethroid-treated nets to prevent malaria transmission despite increasing vector resistance.

Keywords: Malaria, *Anopheles gambiae*, Insecticide resistance, Pyrethroids, *Plasmodium falciparum*, Oocyst, Sporogony

Background

Increased use of insecticide-treated nets (ITNs) has contributed to substantial reductions in the global burden of malaria [1]. Unfortunately, various genes conferring resistance to pyrethroids are spreading rapidly through the main African malaria vectors [2, 3]. However, the impact of this resistance on vector control remains unclear [4]. Control failure has been associated with resistance in some areas [5], but not others [6–8].

Pyrethroid-treated nets reduce malaria transmission partly by repelling vectors, and partly by killing them [9]. As resistance increases, the proportion of the vector population surviving insecticide exposure increases. Although this is expected to reduce the effectiveness of vector control, it is possible that transmission might still be prevented by other mechanisms. One possible mechanism is that infection might restore the phenotypic susceptibility of genetically resistant mosquitoes, so they are killed by doses that they would survive without the infection. Another is through a possible effect of the insecticide on the parasite. With increasing resistance, the proportion of the vector population exposed to sub-lethal doses is also expected to increase, which in turn increases exposure of the parasite to the insecticide. Exposure of the parasite to these doses inside the mosquito might affect its development even though the insecticide fails to kill the mosquito. Either of these mechanisms could, in theory, allow insecticide

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resistance to evolve in a vector population with little impact on malaria transmission.

A number of studies have investigated potential effects of insecticides and insecticide resistance on parasite development. Resistant mosquitoes infected with *Plasmodium falciparum* have been found to be more susceptible to DDT than uninfected mosquitoes [10]. Other studies reported that *Anopheles gambiae* with knockdown resistance (*kdr*) genes exhibited increased susceptibility to *P. falciparum* [11, 12].

In one study, exposure to DDT and bendiocarb inhibited development of *P. falciparum* in insecticide-resistant *An. gambiae* s.s. [13]. Sub-lethal doses of pyrethroids were also shown to affect development of *Plasmodium* parasites in laboratory conditions [14–16]. However, other studies found no effect of organochlorines, carbamates and organophosphates on parasite development in mosquitoes [17–19].

The late Nigel Hill carried out laboratory-based research at London School of Hygiene & Tropical Medicine (LSHTM) on the effects of sub-lethal doses of pyrethroids on *Plasmodium* infection rates in *An. stephensi* mosquitoes [17]. He infected pyrethroid susceptible and resistant mosquitoes with the rodent parasite *P. yoelii nigeriensis*. Mosquitoes were exposed to deltamethrin-, permethrin- and lambda-cyhalothrin-treated nets and papers. Exposure to these pyrethroids before, during or after infective feed significantly reduced prevalence of infection under laboratory conditions. He subsequently carried out similar laboratory-based experiments using a pyrethroid resistant *An. stephensi* strain and a laboratory strain of *P. falciparum.* Again, exposure to permethrin shortly after infective feed caused a significant reduction in the infection prevalence.

Our aim was therefore to demonstrate that this phenomenon can occur in the field. We investigated the effects of deltamethrin exposure of wild, pyrethroid-resistant *An. gambiae* s.s. on the sporogonic development of *P. falciparum* parasites obtained from local patients at a health facility in a malaria endemic area of Uganda. The range of insecticide exposures was selected to resemble those that blood-seeking mosquitoes might be expected to encounter in an area where the nets are not new, and where the concentration of insecticide on nets is considerably lower than in new nets.

Methods

Study area and participants

The study was conducted in Butemba, Kyankwanzi District, mid-western Uganda, between August 2013 and June 2014. Butemba (approximately 200 km north-west of Kampala) lies at an altitude of 1000–1200 m above sea level in a moist savannah zone, with annual rainfall exceeding 1200 mm with two peaks (April–May and September–October). The study site included the catchment area of Butemba Health Centre III, which is mostly rural but includes a semi-urban village of Bukwiri. The area is highly endemic with two peaks in malaria transmission in May–July and October–December.

Forty-two gametocyte donors were recruited among outpatients at Butemba Health Centre III. Patients who fulfilled the inclusion criteria (2 years or older, *P. falciparum* positive with microscopically detectable gametocytes, no sign of severe illness, non-pregnant if adult female, and haemoglobin level of >9.9 g/dl) were recruited. Gametocytes were counted against 200 leucocytes in thick blood smears. Density was calculated assuming a standard leukocyte count of 8000/μL of blood. The experiments were conducted over three rounds (September–October 2013, November–December 2013, and May–June 2014).

Mosquito collection and rearing

Anopheles gambiae s.l. larvae were collected from breeding sites in villages around the health centre and reared at the health centre at ambient temperature and humidity. The emerging adult mosquitoes were given 10 % glucose solution until they were fed on infected blood.

Mosquito species and resistance studies

World Health Organization (WHO) susceptibility tests were conducted using different classes of insecticides to assess the phenotypic resistance levels in the study area [20]. All mosquitoes used in the transmission experiments and the WHO susceptibility tests were stored dry on silica gel for molecular analysis. Real-time polymerase chain reaction (qPCR) using TaqMan assays was used for *Anopheles* sibling species identification [21], and for detection of *kdr*-L1014F or *kdr*-L1014S mutations [22]. A further assay to detect the presence of G119S mutation in the gene *ace-1* which encodes the acetylcholinesterase enzyme was also used [23].

Experimental nets

Untreated polyester nets (Vestergaard) were treated with a range of concentrations (2.5–16.7 mg/m^2) of deltamethrin (K-Othrine SC 10B G, concentration 9.7 g/l; Bayer CropScience AG). The doses were much lower than those used on LLINs, and were chosen in an attempt to mimic the concentrations found on nets as they get older in domestic use [24].

Procedures

Approximately 9 ml blood was collected from each gametocytaemic volunteer by venepuncture. Gametocyte density ranged from 34 to 480/μl of blood (excluding one volunteer who had no microscopically detectable gametocytes but was nevertheless infectious). Blood

samples were transferred to pre-warmed membrane feeders (Hemotek Membrane Feeding System, Hemotek Ltd, UK) held at 37.5 °C. On average 217 mosquitoes were used per infective feed (range: 62–799), divided into paper cups with approximately 40 females in each, and allowed to feed through an artificial Parafilm membrane for up to 2 h. In most cases, the blood samples were offered to the mosquitoes within 10 min of being taken, but in three experiments (23, 28 and 29), they were kept for up to 1 ½ h in a water bath at 37 °C before transfer to the membrane feeders. Blood samples from the 42 volunteers were each used in separate experiments except samples from four volunteers (18, 19, 44 and 45), which were used in two insecticide exposure experiments each.

Within 1–3 h, approximately half of the blood-fed mosquitoes were exposed to a net treated with a sub-lethal dose of deltamethrin for 5 min using a wire ball frame, and the other half were exposed to an untreated net as control. After exposure, mosquitoes were kept in paper cups with access to 10 % glucose solution. Temperature and humidity were recorded every 30 min during incubation. Seven days after infection, midguts of surviving females were dissected in 0.25 % mercurochrome in phosphate buffer saline (PBS) solution and examined for oocysts.

Statistical analysis

Only data for *An. gambiae* s.s. with *kdr*-L1014S homozygous (RR) genotype were included in the statistical analyses to reduce bias due to genetic heterogeneity. Two outcome variables were studied: a) prevalence of oocyst infection and b) intensity of oocyst infection (number of oocysts) among infected mosquitoes. Mantel-Haenszel meta-analysis and forest plot were used with the *metan* procedure in Stata version 13 (StataCorp LP, College Station, Texas 77,845, USA) to study the effect of exposure to sub-lethal doses of deltamethrin on *Plasmodium* infection in *kdr*-resistant *An. gambiae* s.s. mosquitoes, stratifying by feeding experiment. Odds ratios (OR) were calculated to estimate the effect of insecticide exposure on infection prevalence, separately for each gametocyte donor or experiment. Experimental data from different insecticide doses were pooled into two exposure groups: low dose (2.5–5.0 mg/m^2) and high dose (10.0–16.7 mg/m^2), as sample sizes for some of the separate doses were inadequate for the analysis. A Mantel-Haenszel pooled OR was calculated as a summary measure of exposure effect across experiments. We used the median of ambient temperature recorded during the experiments (25.3 °C) as cut-off to plot oocyst prevalence charts under low and high temperature conditions.

The effect of deltamethrin exposure was analysed further with multi-level regression models. First, mixed-effects logistic regression was used to study the effect of the insecticide on oocyst infection rate. Secondly, mixed-effects negative binomial regression was used to study the effect of the insecticide on oocyst count in infected mosquitoes.

In both models, the main independent variable was deltamethrin dosage group, as a fixed-effect categorical variable with three levels: control, low dose and high dose, as defined above. In addition, four more fixed-effect explanatory factors were included in both models: the continuous variables average temperature during incubation, gametocyte density, and age of volunteer, and a binary categorical variable indicating whether or not the donor received medication with antimalarials in the previous seven days. To account for the correlation of mosquitoes fed on the same blood sample within each experiment, gametocyte donor volunteers were included as a random (or group) variable. In each model, the two-level random-effects models were compared with models with no random effects using log-likelihood ratio tests to confirm that the mixed-effects models were more appropriate than standard models. The *melogit* and *menbreg* procedures in Stata 13 were used to fit the mixed-effects logistic and mixed-effects negative binomial regressions, respectively.

Ethics statement

Ethical clearance was obtained from the LSHTM (reference 6454), the Vector Control Division of the Ministry of Health of Uganda (reference VCD-IRC/044), and Uganda National Council of Science and Technology (reference HS 1429). All adult subjects provided written informed consent, and a parent or guardian of any child participant provided written informed consent on their behalf.

Results

A total of 9502 *An. gambiae* s.l. up to 10 days old were offered an infective blood meal, of which 1285 fully fed. Of these, 935 survived until dissection. Midguts of 862 of the surviving mosquitoes were dissected successfully and examined. Out of these, 763 were identified by PCR as *An. gambiae* s.s. and 73 as *An. arabiensis* (26 mosquitoes could not be identified by PCR). Of the 763 *An. gambiae* s.s., 692 had *kdr*-L1014S homozygous (RR) genotype and were included in the statistical analyses involving effects of deltamethrin on infection.

Resistance gene frequencies

An. gambiae s.s. and *An. arabiensis* were found together in the study area at a ratio of approximately 10 to 1. All but one of the *An. arabiensis* mosquitoes were scored as SS (homozygote susceptible) at the *kdr*-L1014S locus, while in *An. gambiae* s.s. 95 % of the specimens were RR

resistant homozygotes (Table 1). All specimens were homozgygous susceptible at the *ace-1* locus.

Mosquito mortality rates

The WHO insecticide susceptibility tests confirmed presence of resistance against deltamethrin in the *An. gambiae* s.l. population in the study site, with 71.9 % mortality ($n = 87$). Mortality rates in the mosquitoes fed with infective blood meals were recorded after 7 days of incubation: 9.6, 32.5 and 35.5 % died in the groups exposed to untreated nets, and nets treated with the low-dose and high-dose deltamethrin, respectively (Fig. 1).

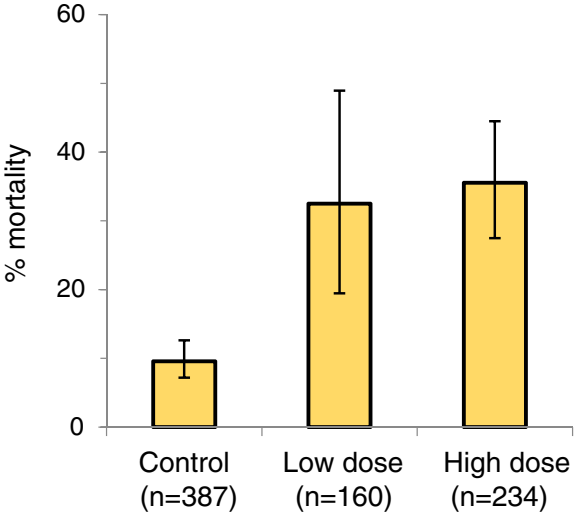

Fig. 1 Mortality rates in *kdr*-L1014S resistant homozygous (RR) *An. gambiae* s.s. exposed to different deltamethrin doses: untreated nets (control), and nets treated with low dose (2.5–5.0 mg/m^2) and high dose (10.0–16.7 mg/m^2) deltamethrin, assessed after 7 days following exposure for 5 min, at Butemba, Kyankwanzi District, Uganda

Effect of deltamethrin on infection rate

Forty-one of the 42 volunteers had detectable gametocytemia and one was found to be infectious despite a blood smear showing asexual parasites but no visible gametocytes. The mean age of these volunteers was 17 years (range: 2–56 years). Eight volunteers had taken antimalarial drugs prior to the visit to the health facility; six took artemether-lumefantrine and two took quinine.

The groups of fed females exposed to deltamethrin had lower infection rates than those exposed to untreated nets. The effect of deltamethrin on infection rates was more pronounced under low temperature conditions (Fig. 2).

A meta-analysis forest plot was constructed for 34 experiments to which the *metan* Stata procedure was applicable. The results showed a significant protective effect against infection of both low and high dose exposure to the insecticide (Fig. 3). The Mantel-Haenszel pooled OR was 0.21 for the high dose versus control, 0.47 for the low dose versus control, and 0.27 overall (see Fig. 3 for 95 % CIs). Heterogeneity tests showed a uniform effect across all experiments as indicated by the I^2 statistic (0.0 % in all cases), which is a measure of the variation of OR attributable to heterogeneity.

Mixed-effects logistic regression analysis, including the data from all experiments using samples from the 42 volunteers, produced very similar estimates of the effect of deltamethrin on infection rates (Table 2). Mosquitoes exposed to the low and high doses had 59 and 80 % lower risk of infection compared to those exposed to untreated nets, and these differences were highly significant (Table 2). This analysis also showed that the mean ambient temperature during the incubation period, which varied between 24.8 and 26.8 °C, had an independent and highly significant effect on risk of infection.

Gametocyte density, age of gametocyte donor, and prior medication with antimalarial drugs had no statistically significant effects on oocyst infection prevalence (gametocyte density data for one donor was considered an outlier and was excluded from analysis).

Table 1 Frequencies of *kdr*-L1014S allele in *An. gambiae* s.s. and *An. arabiensis*

kdr-L1014S genotype	*An. gambiae* s.s.		*An. arabiensis*	
	n	%	*n*	%
SS	4	0.5	72	98.6
RS	34	4.6	0	0.0
RR	694	94.8	1	1.4
Total	732	100	73	100
Allele frequency	97.1 %		1.4 %	

Effect of deltamethrin on oocyst counts

Oocyst-positive mosquitoes exposed to both the low and high dose deltamethrin had lower infection intensity than positive mosquitoes exposed to untreated nets. The median numbers of oocysts per infected mosquito in each experiment were compared in the control and low-dose groups, and in the control and high-dose groups, using paired scattergrams (Fig. 4). The effect of the insecticide on infection intensity was more pronounced in the low-dose group.

Negative binomial regression of oocyst count of positive mosquitoes showed that exposure to deltamethrin, mean ambient temperature during incubation period and intake of antimalarials in the previous seven days had statistically significant effects (Table 3). Compared with the control group, the number of oocysts per positive mosquito was reduced by 41 and 34 % in the low and high dose groups respectively.

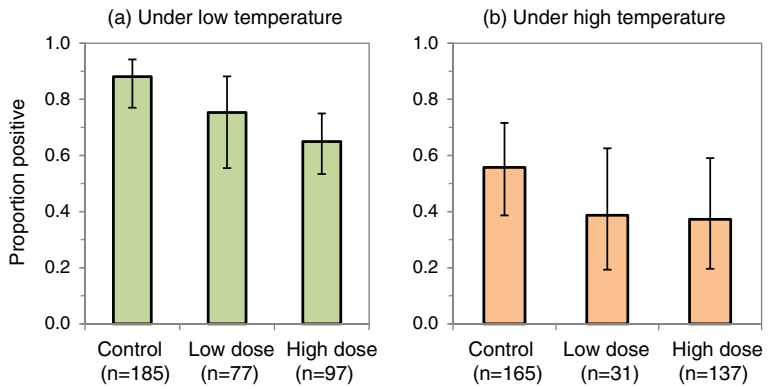

Fig. 2 Effects of deltamethrin on *P. falciparum* infection in *kdr* resistant *An. gambiae* s.s. Prevalence rates under (**a**) low temperature (<25.3 °C) and (**b**) high temperature (≥25.3 °C) conditions (control = mosquitoes exposed to untreated nets, low dose = 2.5–5.0 mg/m^2 deltamethrin and high dose = 10.0–16.7 mg/m^2 deltamethrin). Mosquitoes were exposed to nets after membrane feeding on blood samples obtained from *P. falciparum* patients (gametocyte donors) at Butemba Health Centre III, Kyankwanzi District, Uganda. Error bars indicate 95 % confidence intervals. Calculations take into account nesting of mosquito samples within gametocyte donor samples

Discussion

This study showed that deltamethrin affects development of *P. falciparum* in wild, *kdr*-L1014S resistant *An. gambiae* s.s. in a malaria endemic setting. Exposure to sub-lethal doses of the insecticide shortly after infective feeding reduced both the oocyst prevalence and intensity of infection inside the mosquito.

It is possible that the reduction could be produced by either or both of two possible mechanisms: differential insecticidal killing of infected mosquitoes (as might be seen if infection restored phenotypic susceptibility in genotypically resistant mosquitoes), and/or a direct effect of the insecticide on the parasite inside the mosquito.

Previous laboratory studies have described effects of different pyrethroids on *Plasmodium* sporogony. Deltamethrin was shown to reduce *P. yoelii yoelii* infection rates in *An. stephensi* [14, 16], whereas bioallethrin and fenvalerate affected the parasites at the sporozoite level only [15]. Hill carried out laboratory-based research on the effects of sub-lethal doses of different insecticides on malaria vectors [17]. His findings showed that exposure of insecticide resistant *An. stephensi* to pyrethroids resulted in significant inhibition of *P. yoelii nigeriensis* and *P. falciparum* sporogonic development, whereas no such effect was found with organochlorine, carbamate and organophosphate insecticides. Earlier studies also reported that non-pyrethroid insecticides have no effect on malaria infection in mosquitoes [18, 19]. However, exposure of resistant strains of *An. gambiae* s.s. to bendiocarb and DDT has been shown to reduce *P. falciparum* prevalence [13].

Higher infection rates have been reported in *kdr* resistant mosquitoes compared to susceptible ones although results from different studies were conflicting in terms of the effect on infection intensity at oocyst and sporozoite stages [11, 12]. A recent study showed that *kdr* resistant mosquitoes infected with *P. falciparum* were less able to survive DDT exposure than uninfected mosquitoes during the first seven days post infection, but there was no significant difference in mortality rates between sporozoite-infected and control groups later on [13].

Although *kdr* allele was almost fixed in the *An. gambiae* s.s. population, the susceptibility test data showed relatively high mortality (71.9 %). It is therefore likely that resistance is mediated by a combination of metabolic detoxification mechanisms and *kdr*, and that the resistance phenotype (i.e. strength of expression of resistance) differs between the mosquitoes. Metabolic resistance is a potential confounder in the effect of the insecticide on the parasite as it mediates the amount of insecticide or insecticide metabolites to which the parasite would be exposed. As mosquitoes become resistant and receive sub-lethal doses and survive, the probability of exposure of the parasites to these doses may increase. On the other hand, as detoxification becomes more powerful, most of the insecticide may be metabolised which could mean less exposure of the parasite. Nevertheless, insecticide metabolites or other resistance-related factors could still affect the parasite's development directly or through their potential effect on the mosquito's immune system indirectly [25, 26]. Further studies are needed to understand better the potential effects of resistance.

Insecticide dose, mean daily temperature, and medication were all significant variables in our models. The doses of deltamethrin used in this study were much lower than those on a standard long-lasting insecticidal net (LLIN) (e.g. 55 mg/m^2 in PermaNet® 2.0). Washing and long-term use reduce deltamethrin content of ITNs

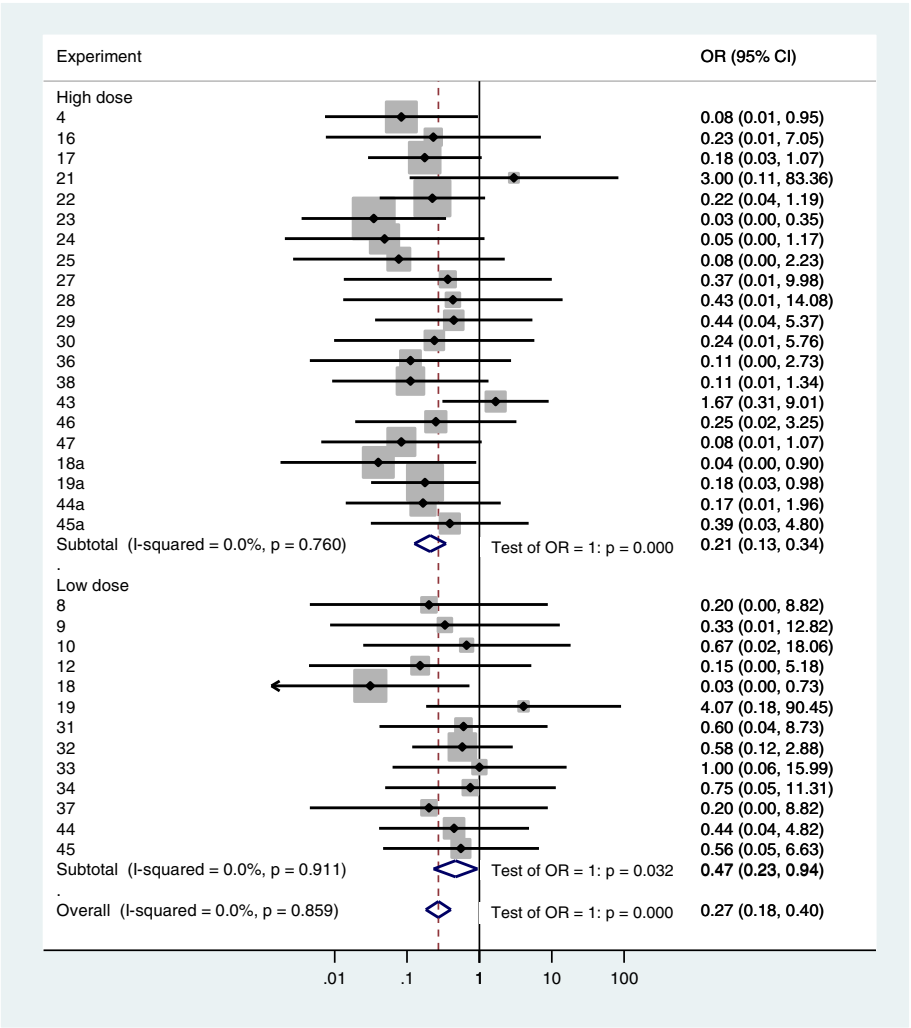

Fig. 3 Forest plot of the effects of high and low doses of deltamethrin on *P. falciparum* oocyst infection rates in *kdr*-L1014S resistant *An. gambiae* s.s. The plot shows odds ratio (OR) obtained from meta-analysis of data corresponding to 34 experiments (using blood samples from 30 of the 42 volunteers). Only experiments with sample sizes appropriate for the *metan* procedure calculation were included in the plot (12 experiments had multiple zeros in 2x2 tables and therefore were excluded from the plot). Experiment numbers represent individual volunteers, except when a suffix is used to show more than one experiment per volunteer. For each of the experiments, the OR and 95 % confidence interval (95 % CI) were computed, with OR < 1 indicating lower infection rate of deltamethrin-exposed mosquitoes compared to control. The size of each grey square represents the experiment's weight and horizontal line indicates 95 % CI. Summary (Mantel-Haenszel pooled) OR estimates for each dose and for all experiments are represented by open diamonds with their lateral tips indicating 95 % confidence limits. The dotted line indicates the overall OR

Table 2 Mixed-effects logistic regression analysis of *P. falciparum* oocyst prevalence rates

		Odds ratio	Std. Err.	Z	*p*	[95 % Confidence Interval]	
Dose category	Control	1.000	–	–	–	–	–
	Low dose	0.409	0.134	−2.71	0.007	0.215	0.780
	High dose	0.197	0.050	−6.37	<0.001	0.120	0.325
Average temperature (°C)	–	0.179	0.073	−4.23	<0.001	0.081	0.398
Variance of random intercept	–	1.889	0.672	–	–	0.941	3.793

The dependent variable is oocyst infection coded as 0 (negative) and 1 (positive)
Model $\chi^2_{3df} = 56.63$ $p < 0.001$ $n = 692$ number of groups (gametocyte donors) = 42

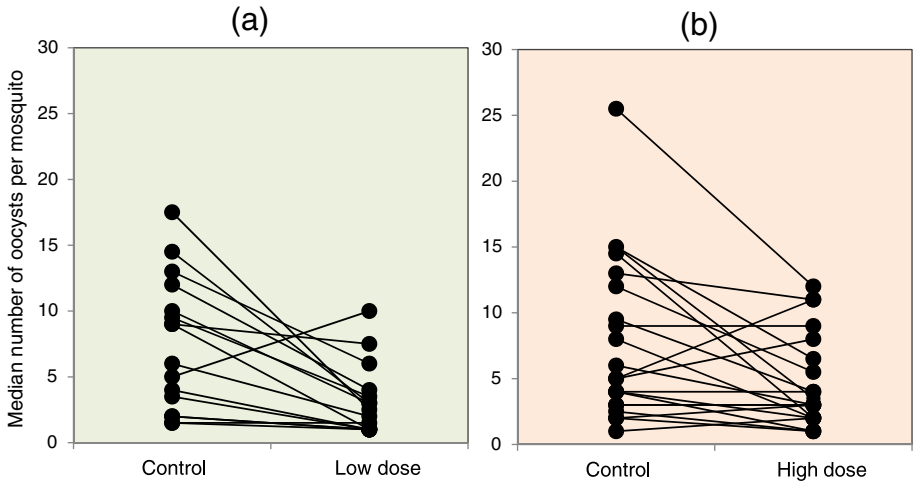

Fig. 4 Paired scattergram showing median numbers of oocysts in infected mosquitoes in each experiment for (**a**) the control and low-dose groups, and (**b**) the control and high-dose groups. Each pair of dots connected with a line represents the median numbers in the respective groups in each experiment. Only experiments with median oocyst data for both groups were included in the plot

[24]. So, effects of the kind observed here would be expected not only with new nets, but also older ones—although the effect seems to be dose-dependent, with higher insecticide doses having a bigger impact especially on infection prevalence. However, the reduction of the intensity of infection was more pronounced in the low-dose group than the high-dose group. Some studies have suggested that intensity and prevalence of infection might be regulated by different mechanisms in the mosquito, probably in relation to different immune signalling pathways [27, 28].

We showed that high ambient temperature independently reduced oocyst prevalence and intensity. Temperature affects malaria transmission by affecting the life cycles of both the vector and the parasite. Within the relevant temperature range, sporogony is shorter at higher temperatures [29]. However, higher temperatures have been shown to reduce prevalence of oocyst infection [30], may be detrimental to parasite development [31], and can affect the immune response of mosquitoes [32]. Temperature can also change the effect of insecticides on a mosquito population by modifying mortality rates [33].

Antimalarial medication reduced the intensity of oocyst infections in our study. Six of the volunteers took artemether-lumefantrine and two took quinine within seven days before providing blood samples. These drugs, especially the former, are known to have gametocytocidal properties [34]. In the present study, gametocyte density and age of the donor did not have a significant effect. This could indicate that the insecticide's effect is probably not at the gametocyte stage.

This study may have implications on the continued use of pyrethroid-based ITNs which have contributed to substantial reduction of malaria mortality in the past decade. As suggested in the study, if pyrethroids affect development of the parasite inside the mosquito, prevention tools dependent on these chemicals will continue to play a major role in malaria control despite vector resistance.

Table 3 Mixed-effects negative binomial regression analysis of number of *P. falciparum* oocysts

		Incidence-rate ratio	Std. Err.	Z	*p*	[95 % Confidence Interval]	
Dose category	Control	1.000	–	–	–	–	–
	Low dose	0.586	0.075	−4.18	<0.001	0.456	0.753
	High dose	0.655	0.068	−4.08	<0.001	0.535	0.803
Average temperature (°C)		0.522	0.086	−3.93	<0.001	0.378	0.722
Prior intake of antimalarials	Not taken	1.000	–	–	–	–	–
	Taken	0.595	0.156	−1.98	0.048	0.356	0.995
Ln(alpha[a])	–	−0.688	0.088	−7.85	<0.001	−0.861	−0.516
Variance of random intercept	–	0.243	0.0899			0.118	0.502

The dependent variable is number of oocysts
Model $\chi^2_{4df} = 47.72$ $p < 0.001$ $n = 421$ number of groups (gametocyte donors) = 40
[a]Alpha = Overdispersion parameter

The effect of pyrethroids reported here could explain, at least partly, why resistance has not always led to control failure and ITNs seem to remain effective in most situations [6, 7, 35–37]. More research will be needed to fully understand the mechanisms of interactions between the parasite, different insecticide resistance mechanisms and the insecticide in the mosquito vector, and the roles of these interactions in modulating transmission in the field. Our study suggests that the continued use of pyrethroid treated nets might be helping to prevent failure of malaria control in Africa despite the rapid evolution of insecticide resistance, and supports the efforts to maintain the use of existing effective interventions.

Conclusions

The use of nets treated with pyrethroid insecticides has contributed to the prevention of millions of deaths due to malaria, but resistance to these insecticides is spreading rapidly in the vector mosquitoes in Africa. We investigated whether the chemicals could affect malaria parasites inside resistant mosquitoes in an endemic area. The study showed that, with locally-sampled *P. falciparum* parasites and *An. gambiae* s.s., doses of pyrethroids that are sub-lethal for resistant mosquitoes can interfere with parasite development inside mosquitoes, significantly reducing both the proportion of infected mosquitoes and the intensity of infection. This mechanism could enable pyrethroid-treated nets to prevent malaria transmission despite increasing vector resistance.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MK and TAA conceived the study and developed the study design with contributions of SRM and JL. TAA, MK, and CN trained field staff. MK carried out all field experiments and coordinated the study. TAA supervised field experiments and coordinated the study. CN supervised field staff and contributed to coordination of the study. AN contributed to coordination and facilitation of the study. TAA carried out the statistical analyses with inputs from MK. MK and TAA wrote the manuscript. JL, SRM, AN and CN reviewed the manuscript and provided comments. All authors read and approved the final manuscript.

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References

1. WHO. World malaria report 2014. Geneva: World Health Organization; 2014.
2. WHO. Global plan for insecticide resistance management in malaria vectors. Geneva: World Health Organization; 2012.
3. 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(2):91–8. doi:10.1016/j.pt.2010.08.004.
4. 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 Med. 2014;11(3), e1001619. doi:10.1371/journal.pmed.1001619.
5. Maharaj R, Mthembu DJ, Sharp BL. Impact of DDT re-introduction on malaria transmission in KwaZulu-Natal. S Afr Med J. 2005;95(11):871–4.
6. Tokponnon FT, Ogouyemi AH, Sissinto Y, Sovi A, Gnanguenon V, Cornelie S, et al. Impact of long-lasting, insecticidal nets on anaemia and prevalence of *Plasmodium falciparum* among children under five years in areas with highly resistant malaria vectors. Malar J. 2014;13(1):76. doi:10.1186/1475-2875-13-76.
7. 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 U S A. 2012;109(47):19063–70. doi:10.1073/pnas.1217229109.
8. 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 U S A. 2013;110(23):9397–402. doi:10.1073/pnas.1307656110.
9. Magesa SM, Wilkes TJ, Mnzava AE, Njunwa KJ, Myamba J, Kivuyo MD, et al. Trial of pyrethroid impregnated bednets in an area of Tanzania holoendemic for malaria. Part 2. Effects on the malaria vector population. Acta Trop. 1991;49(2):97–108.
10. Alout H, Yameogo B, Djogbenou LS, Chandre F, Dabire RK, Corbel V, et al. Interplay between *Plasmodium* infection and resistance to insecticides in vector mosquitoes. J Infect Dis. 2014;210(9):1464–70. doi:10.1093/infdis/jiu276.
11. Alout H, Ndam NT, Sandeu MM, Djegbe I, Chandre F, Dabire RK, et al. Insecticide resistance alleles affect vector competence of*Anopheles gambiae* s.s. for *Plasmodium falciparum* field isolates. PLoS One. 2013;8(5), e63849. doi:10.1371/journal.pone.0063849.
12. Ndiath MO, Cailleau A, Diedhiou SM, Gaye A, Boudin C, Richard V, et al. Effects of the *kdr* resistance mutation on the susceptibility of wild *Anopheles gambiae* populations to *Plasmodium falciparum*: a hindrance for vector control. Malar J. 2014;13:340. doi:10.1186/1475-2875-13-340.
13. Alout H, Djegbe I, Chandre F, Djogbenou LS, Dabire RK, Corbel V et al. Insecticide exposure impacts vector-parasite interactions in insecticide-resistant malaria vectors. Proc Biol Sci. 2014;281 (1786). doi:10.1098/rspb.2014.0389.
14. Carle PR, Coz J, Elissa N, Gasquet M, Sannier C, Richard A, et al. Activité antiplasmodique intravectorielle d'un pyréthrinoide: la deltaméthrine. C R Acad Sci III. 1986;303(13):565–8.
15. Elissa N, Sannier C, Coz J. Étude de la réceptivité à *Plasmodium yoelii yoelii* d'*Anopheles stephensi* soumis au stade adulte à des doses toxiques de différents pyréthrinoïdes. Cah ORSTOM, ser Ent méd et Parasitol. 1987;25(3–4):187–92.
16. Elissa N, Sannier C, Richard A. [La Receptivite a *Plasmodium yoelii yoelii* d-*Anopheles* Soumis a des Subletales de Deltamethrine]. IVe Congres Sur La Protection De La Sante Humaine Et Des Cultures En Milieu Tropical; Marseille, 1986. p. 599–604.
17. Hill N. Effects of sublethal doses of pyretroids on malaria vectors [PhD thesis]. London: London School of Hygiene & Tropical Medicine, University of London; 2002.
18. Prasittisuk C, Curtis CF. Absence of effects of insecticides on susceptibility of anophelines to *Plasmodium yoelii nigeriensis*. Southeast Asian J Trop Med Public Health. 1982;13(1):127–32.

19. Rifaat MA, Khalil HM, Gad AM, Sadek S. Effect of sublethal concentrations of the insecticides DDT, abate and sevin applied to 3rd stage larvae of *Anopheles pharoensis* on malaria cycle in the adult mosquito. J Egypt Public Health Assoc. 1974;49(6):329–40.
20. WHO. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. Geneva, Switzerland, 2013.
21. Bass C, Williamson MS, Field LM. Development of a multiplex real-time PCR assay for identification of members of the *Anopheles gambiae* species complex. Acta Trop. 2008;107(1):50–3. doi:10.1016/j.actatropica.2008.04.009.
22. Bass C, Nikou D, Donnelly MJ, Williamson MS, Ranson H, Ball A, et al. Detection of knockdown resistance (*kdr*) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. Malar J. 2007;6:111. doi:10.1186/1475-2875-6-111.
23. Bass C, Nikou D, Vontas J, Williamson MS, Field LM. Development of high-throughput real-time PCR assays for the identification of insensitive acetylcholinesterase (*ace-1*^{R}) in *Anopheles gambiae*. Pestic Biochem Phys. 2010;96(2):80–5. doi:10.1016/j.pestbp.2009.09.004.
24. Kilian A, Byamukama W, Pigeon O, Gimnig J, Atieli F, Koekemoer L, et al. Evidence for a useful life of more than three years for a polyester-based long-lasting insecticidal mosquito net in western Uganda. Malar J. 2011;10:299. doi:10.1186/1475-2875-7-49.
25. McCarroll L, Paton MG, Karunaratne SH, Jayasuryia HT, Kalpage KS, Hemingway J. Insecticides and mosquito-borne disease. Nature. 2000;407(6807):961–2. doi:10.1038/35039671.
26. Stevenson BJ, Bibby J, Pignatelli P, Muangnoicharoen S, O'Neill PM, Lian LY, et al. Cytochrome P450 6 M2 from the malaria vector *Anopheles gambiae* metabolizes pyrethroids: sequential metabolism of deltamethrin revealed. Insect Biochem Mol Biol. 2011;41(7):492–502. doi:10.1016/j.ibmb.2011.02.003.
27. Garver LS, Bahia AC, Das S, Souza-Neto JA, Shiao J, Dong Y, et al. *Anopheles* Imd pathway factors and effectors in infection intensity-dependent anti-*Plasmodium* action. PLoS Pathog. 2012;8(6), e1002737. doi:10.1371/journal.ppat.1002737.
28. Harris C, Lambrechts L, Rousset F, Abate L, Nsango SE, Fontenille D, et al. Polymorphisms in *Anopheles gambiae* immune genes associated with natural resistance to *Plasmodium falciparum*. PLoS Pathog. 2010;6(9), e1001112. doi:10.1371/journal.ppat.1001112.
29. Detinova TS. Age-grouping methods in diptera of medical importance with special reference to some vectors of malaria. Monogr Ser World Health Organ. 1962;47:13–191.
30. Okech BA, Gouagna LC, Kabiru EW, Walczak E, Beier JC, Yan G, et al. Resistance of early midgut stages of natural *Plasmodium falciparum* parasites to high temperatures in experimentally infected *Anopheles gambiae* (Diptera: Culicidae). J Parasitol. 2004;90(4):764–8.
31. Mordecai EA, Paaijmans KP, Johnson LR, Balzer C, Ben-Horin T, de Moor E, et al. Optimal temperature for malaria transmission is dramatically lower than previously predicted. Ecol Lett. 2013;16(1):22–30. doi:10.1111/ele.12015.
32. Murdock CC, Paaijmans KP, Bell AS, King JG, Hillyer JF, Read AF, et al. Complex effects of temperature on mosquito immune function. Proc Biol Sci. 2012;279(1741):3357–66. doi:10.1098/rspb.2012.0638.
33. Glunt KD, Paaijmans KP, Read AF, Thomas MB. Environmental temperatures significantly change the impact of insecticides measured using WHOPES protocols. Malar J. 2014;13:350. doi:10.1186/1475-2875-13-350.
34. Makanga M. A review of the effects of artemether-lumefantrine on gametocyte carriage and disease transmission. Malar J. 2014;13:291. doi:10.1186/1475-2875-13-291.
35. Henry MC, Assi SB, Rogier C, Dossou-Yovo J, Chandre F, Guillet P, et al. Protective efficacy of lambda-cyhalothrin treated nets in *Anopheles gambiae* pyrethroid resistance areas of Cote d'Ivoire. Am J Trop Med Hyg. 2005;73(5):859–64.
36. Protopopoff N, Verhaeghen K, Van Bortel W, Roelants P, Marcotty T, Baza D, et al. A significant increase in *kdr* in *Anopheles gambiae* is associated with an intensive vector control intervention in Burundi highlands. Trop Med Int Health. 2008;13(12):1479–87. doi:10.1111/j.1365-3156.2008.02164.x.
37. Sharp BL, Ridl FC, Govender D, Kuklinski J, Kleinschmidt I. Malaria vector control by indoor residual insecticide spraying on the tropical island of Bioko, Equatorial Guinea. Malar J. 2007;6:52. doi:10.1186/1475-2875-6-52.

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Appendix 5-1

This appendix provides additional information on statistical analysis of the data presented in Figure 1 and Figure 2 of the published paper (Chapter 5).

Figure 1

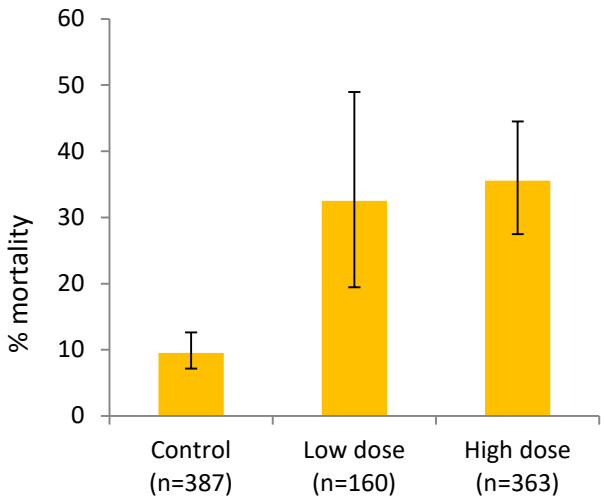

Fig. A5-1. Mortality rates in *kdr-L1014S* resistant homozygous (RR) *An. gambiae* s.s. exposed to different deltamethrin doses: untreated nets (control), and nets treated with low dose (2.5–5.0 mg/m^2) and high dose (10.0–16.7 mg/m^2) deltamethrin, assessed after 7 days following exposure for 5 min, at Butemba, Kyankwanzi District, Uganda.

Mortality rates in mosquitoes fed with infective blood meals were recorded after 7 days of incubation: 9.6, 32.5 and 35.5 % died in the groups exposed to control untreated nets, and nets treated with the low-dose and high-dose deltamethrin, respectively.

95% confidence intervals were calculated and are indicated as error bars in Fig. 1. Two-sample test of proportions was used to see if mortality was significantly different in different dose categories. Whereas mortality in the control group was significantly lower than in the low or high dose groups ($p < 0.0001$), mortality in low and high dose groups did not differ significantly ($p = 0.2505$).

Table A5-1. Number of mosquitoes in each dose group with mortality rates and 95% confidence intervals.

	n	% mortality	95% CI
Control	387	9.56	[0.0682, 0.1294]
Low dose	160	32.5	[0.2532, 0.4035]
High dose	363	35.5	[0.3061, 0.4070]

Figure 2

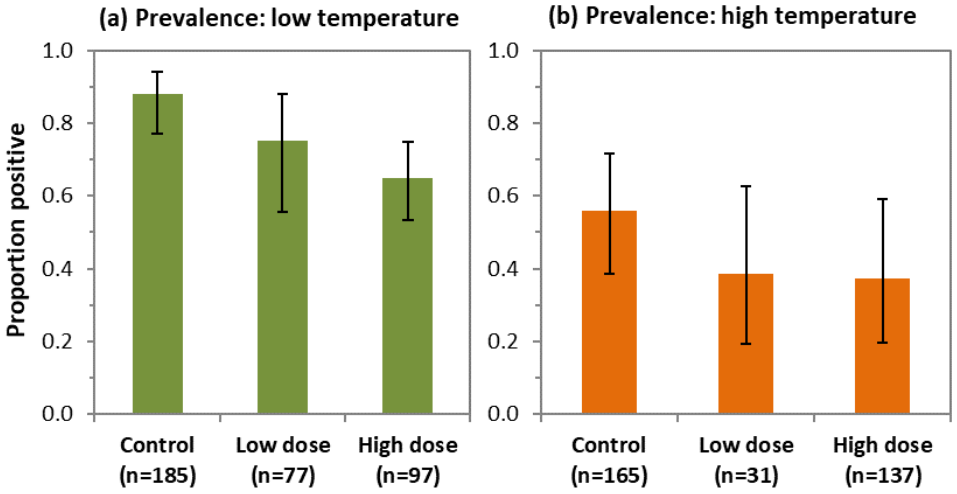

Fig. A5-2. Effects of deltamethrin on *P. falciparum* infection in *kdr* resistant *An. gambiae* s.s. Prevalence rates under (a) low temperature (<25.3 °C) and (b) high temperature (≥25.3 °C) conditions (control = mosquitoes exposed to untreated nets, low dose = 2.5–5.0 mg/m^2 deltamethrin and high dose = 10.0–16.7 mg/m^2 deltamethrin). Mosquitoes were exposed to nets after membrane feeding on blood samples obtained from *P. falciparum* patients (gametocyte donors) at Butemba Health Centre III, Kyankwanzi District, Uganda. Error bars indicate 95 % confidence intervals. Calculations take into account nesting of mosquito samples within gametocyte donor samples.

As mentioned in the Statistical Analysis section of the manuscript/chapter, the median of ambient temperature recorded during the experiments (25.3 °C) was used as cut-off to plot oocyst prevalence charts under low and high temperature conditions.

Calculations of the prevalence in different exposure groups took into account nesting of mosquito samples within gametocyte donor samples. 95% confidence intervals were calculated and are indicated as error bars in Fig. 2.

Oocyst prevalence in different dose categories was significantly different under low temperature (p = 0.0033) but was not significantly different under high temperature (p = 0.0673).

Table A5-2. Proportion of positive mosquitoes in each dose group under low and high temperature conditions, with 95% confidence intervals.

Low temperature			
	Control	Low dose	High dose
Proportion positive	0.8811	0.7532	0.6495
95% CI	[0.7701, 0.9425]	[0.5551, 0.8819]	[0.5338, 0.7499]
High temperature			
	Control	Low dose	High dose
Proportion positive	0.5576	0.3871	0.3723
95% CI	[0.3862, 0.7163]	[0.1929, 0.6254]	[0.196, 0.5906]

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SECTION A – Student Details

Student	Mojca Kristan
Principal Supervisor	Jo Lines
Thesis Title	Effects of pyrethroid exposure and insecticide resistance on the sporogonic development of Plasmodium falciparum in Anopheles gambiae s.l.

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

SECTION B – Paper already published

Where was the work published?			
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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Parasites & Vectors
Please list the paper's authors in the intended authorship order:	M. Kristan, T. A. Abeku, J. Lines
Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I designed and planned the study with TAA, with contributions of JL. I trained the field staff with TAA. I carried out all field experiments and coordinated the study, with assistance from TAA who helped with coordination of the field study, supervision of field staff and field

	experiments. I carried out statistical analyses and wrote the manuscript with inputs of co-authors.

Student Signature: [redacted] **Date:** 28/3/2018

Supervisor Signature: [redacted] **Date:** 28 · 3 · 18

Chapter 6. Effect of environmental variables and *kdr* resistance genotype on survival probability and infection rates in *Anopheles gambiae* s.s.

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Abstract

Environmental factors, especially ambient temperature and relative humidity, affect both mosquitoes and malaria parasites. The early part of sporogony is most sensitive and is affected by high temperatures and temperature fluctuation immediately following ingestion of an infectious blood meal. The aim of this study was to explore whether environmental variables such as temperature, together with the presence of the *kdr-L1014S* insecticide resistance mutation, have an impact on survival probability and infection rates in wild *An. gambiae* s.s. exposed and unexposed to a pyrethroid insecticide.

Anopheles gambiae s.s. were collected as larvae, reared to adults, and fed on blood samples from 42 *Plasmodium falciparum*-infected local patients at a health facility in mid-western Uganda, then exposed either to nets treated with sub-lethal doses of deltamethrin or to untreated nets. After seven days, surviving mosquitoes were dissected and their midguts examined for oocysts. Prevalence (proportion infected) and intensity of infection (number of oocysts per infected mosquito) were recorded for each group. Temperature and humidity were recorded every 30 minutes throughout the experiments.

Our findings indicate that apart from the effect of deltamethrin exposure, mean daily temperature during the incubation period, temperature range during the first 24 hours and on day 4 post-infectious feed had a highly significant effect on risk of infection. Deltamethrin exposure still significantly impaired survival of *kdr* homozygous mosquitoes, while mean

daily temperature and relative humidity during the incubation period independently affected mosquito mortality. Significant differences in survival of resistant genotypes were detected, with the lowest survival recorded in mosquitoes with *Re/Rw* genotype.

This study confirmed that the early part of sporogony is most affected by temperature fluctuations, while environmental factors affect mosquito survival. The impact of insecticide resistance on malaria infection and vector survival needs to be assessed separately for mosquitoes with different resistance mechanisms to fully understand its implications for currently available vector control tools and malaria transmission.

6.1 Introduction

An increasing number of people in malaria endemic countries in sub-Saharan Africa have been protected by insecticide treated nets (ITNs) or indoor residual spraying (IRS) [1]. Although the expansion of insecticide resistance in mosquitoes might endanger this progress [2, 3], there has not been a conclusive evidence of a complete failure of ITNs so far [4-8].

Longevity of vectors is one of the most important factors affecting malaria transmission [9, 10]. Vector mosquitoes must survive long enough to become infectious and transmit the disease to a new host. Environmental factors, especially ambient temperature and relative humidity, affect *Anopheles* mosquitoes and parasite development. Temperature affects mosquito biting rates, blood meal digestion, duration of the gonotrophic cycle, fecundity, development of larval stages, and survival of larvae and adults [11]. It can also affect immune system of mosquitoes [12-14] and consequently parasite development.

Duration of the sporogonic cycle is also temperature-dependent, with permissive range for *P. falciparum* sporogony being between 16^{o}C and 35^{o}C [15, 16]. Very high temperatures are lethal to parasites, while sporogony at low temperatures is lengthened to an extent that mosquitoes may not survive long enough to be able to transmit the parasites [17, 18]. The early part of sporogony is thought to be the most sensitive to temperature [12, 13, 18-24]. Ookinetes are the key transitional stage affecting the probability of vector infectivity, and also define thermal limits for parasite development. Once the parasites complete early sporogony and oocysts are formed, the effect of temperature is thought to be less damaging [19]. Both high and low temperatures have an effect, but the parasites are especially sensitive to high temperatures above 30^{o}C [19], and to temperature fluctuation immediately following the infectious blood meal [22].

Some studies have shown that the efficacy of insecticides against mosquitoes is to some extent temperature-dependent [25-28]. Furthermore, environmental factors such as temperature [25, 26, 29, 30], larval food sources and availability of blood meals [31-35], infection by various parasites [36, 37], and insect microflora [38, 39] can all influence susceptibility to insecticides or expression of resistance.

When vectors are exposed to treated nets or sprayed surfaces, genetically resistant insects may survive doses that would have killed susceptible ones but might still be affected by them. Sub-lethal doses of pyrethroids were shown to reduce vector longevity and spontaneous flight activity, and to affect host seeking and probing responses [40, 41]. Such doses could potentially also affect mosquito physiology or trigger immune response of mosquitoes [42]. Furthermore, sub-lethal doses of pyrethroids were shown to affect sporogonic development of *Plasmodium* parasites in laboratory conditions [40, 43-45], and also in the field [46]. The observed effects on parasite development could be caused by direct or indirect effect of insecticides, or through differential insecticidal killing of infected mosquitoes, as might be seen if infection restored phenotypic susceptibility in genotypically resistant mosquitoes.

Our aim in this study was to explore whether environmental variables such as temperature, together with the presence of the *kdr-L1014S* mutation, have an impact on survival probability and infection rates in wild *An. gambiae* s.s. exposed and unexposed to a pyrethroid insecticide.

6.2 Materials and Methods

6.2.1 Study area and participants

The study was conducted in Butemba, Kyankwanzi District, mid-western Uganda, between August 2013 and June 2014. Butemba is located at an altitude of 1,000-1,200m above sea level in a moist savannah zone, with annual rainfall exceeding 1,200mm with two peaks (April-May and September-October). The area is highly endemic with two peaks of malaria transmission in May-July and October-December.

Forty-two gametocyte carriers were recruited among outpatients at Butemba Health Centre III. Volunteer patients who fulfilled the inclusion criteria (2 years or older, *P. falciparum* positive with microscopically detectable gametocytes, no sign of severe illness, non-pregnant if adult

female, and with a haemoglobin level of >9.9 g/dl) were recruited. Gametocytes were counted against 200 leucocytes in thick blood smears. Density was calculated assuming a standard leukocyte count of 8000/μL of blood [47].

The experiments were carried out over three rounds (September-October 2013, November-December 2013, and May-June 2014).

6.2.2 Mosquito collection and rearing

Anopheles gambiae s.l. larvae were collected from breeding sites in villages around the Health Centre and reared at the Health Centre at ambient temperature and humidity, in water from the breeding sites. The emerging adult mosquitoes were given 10% glucose solution until they were fed on infected blood.

6.2.3 Experimental nets

Untreated polyester nets (Vestergaard) were treated with a range of concentrations (2.5-16.7mg/m^2) of deltamethrin (K-Othrine SC 10B G, concentration 9.7g/l; Bayer CropScience AG). The doses were chosen in an attempt to mimic the concentrations found on nets as they get older in domestic use [48] and were much lower than those used on LLINs.

6.2.4 Procedures

Standard membrane feeding experiments were carried out as previously described [46]. Briefly, blood samples collected from gametocytaemic volunteers by venepuncture were transferred to pre-warmed membrane feeders (Hemotek Membrane Feeding System, Hemotek Ltd, UK) held at 37.5^{o}C. Approximately 40 female mosquitoes were placed in each paper cup and allowed to feed through an artificial Parafilm membrane for up to 2 hours. Within 1-3 hours following the feed, some of the blood-fed mosquitoes were exposed to a net treated with a sub-lethal dose of deltamethrin for 5 minutes using a wire ball frame, while others were exposed to an untreated net as control. After exposure, mosquitoes were kept in paper cups with access to 10% glucose solution. Seven days after infection, midguts of surviving females were dissected in 0.25% mercurochrome in phosphate buffered saline (PBS) solution and examined for oocysts. Daily mortality of control and insecticide exposed mosquitoes was recorded.

6.2.5 Mosquito processing

All mosquitoes were stored dry on silica gel in individual microtubes for molecular analysis.

Real-time polymerase chain reaction (qPCR) using TaqMan assays was used for *Anopheles* sibling species identification [49], and for detection of *kdr-L1014F* (*Rw*) or *kdr-L1014S* (*Re*) mutations [50]. A further assay to detect the presence of *G119S* mutation in the gene *ace-1* which encodes the acetylcholinesterase enzyme was also used [51].

6.2.6 Temperature and relative humidity

Temperature and humidity were recorded every 30 minutes throughout the experiments, using EL-USB-2 data loggers (Lascar Electronics) placed next to the mosquito cages and pots in the laboratory.

6.2.7 Statistical analysis

Software

Statistical analysis was carried out using Stata version 14 (StataCorp LP, College Station, Texas 77845, USA). Excel 2016 (Microsoft Corp) and Prism 7 (GraphPad Software Inc., 7825 Fay Avenue, Suite 230, La Jolla, CA 92037 USA) were used for data management and presentation of graphics.

Analysis of temperature and relative humidity variations between study rounds

One-way ANOVA with Tukey-Kramer post-hoc test [52] was used to compare temperature and relative humidity parameters between the three study rounds.

Effects of temperature and insecticide exposure on infection prevalence

Average daily temperatures, daily maximum and minimum temperatures, and daily temperature ranges (i.e. daily maximum minus minimum, indicating variation within a day) for each feed were obtained from the temperature records, providing 42 time-points in total for the feeds included in the analyses. Based on these, averages were also calculated for the period following the first 24 hours post infective blood meal until dissection day (i.e. day 7).

Sample sizes for some of the separate deltamethrin doses were inadequate for further analysis, so data from different insecticide doses were pooled into two exposure groups: low dose (2.5–5.0 mg/m^2) and high dose (10.0–16.7 mg/m^2).

The effect on oocyst infection rates of temperature in the first 24 hours post-feeding compared with subsequent days, together with deltamethrin exposure, was studied using mixed-effects logistic regression with backward elimination. Prevalence of oocyst infection among *An. gambiae* s.s. mosquitoes with *kdr-L1014S* homozygous (*ReRe*) genotype (692 mosquitoes) was studied as an outcome variable. Deltamethrin dosage group was entered as a categorical variable with three levels: control (untreated nets), low dose and high dose. In addition, different temperature-related variables were entered during model development but as they are derivatives of one another only temperature range on days 1 - 7 post-feeding on infective blood meal were kept during development of the final model. To account for the correlation of mosquitoes fed on the same blood sample within each experiment, gametocyte donor volunteers were included as a random (or group) variable. Model predictions were obtained using a *margins* command in Stata and were plotted in Excel.

Mosquito survival

Mosquito survival following the transmission experiments and insecticide exposure was studied among the *An. gambiae* s.s. with *kdr-L1014S* homozygous (*ReRe*) genotype, including the 692 mosquitoes which survived following the transmission experiments until day 7 and were successfully dissected, 13 mosquitoes which survived the period but were not successfully dissected, and 187 mosquitoes that died before day 7.

The influence of insecticide exposure and environmental variables on mosquito survival was studied using Kaplan-Meier survival curves, Log-Rank test and Cox proportional hazards model (*stcox* command in Stata).

The effect of the temperature, relative humidity and deltamethrin exposure on mosquito survival through the seven days of incubation was studied using mixed-effects logistic regression with backward elimination. Mosquito mortality among *An. gambiae* s.s. mosquitoes with *kdr-L1014S* homozygous (*ReRe*) genotype was studied as an outcome variable.

To account for the correlation of mosquitoes fed on the same blood sample within each experiment, gametocyte donor volunteers were included as a random (or group) variable.

Model predictions were obtained using a *margins* command in Stata and were plotted in Excel.

In addition, mosquito survival during the transmission experiments was studied among different *kdr* genotypes of *An. gambiae* s.s., including 771 mosquitoes which survived following the transmission experiments until day 7, and 243 mosquitoes that died before day 7. Mortality of different *kdr* genotypes in *An. gambiae* s.s. mosquitoes used in transmission experiments was also compared using the Log-Rank Statistic test.

6.2.8 Ethics statement

Ethical clearance was obtained from the London School of Hygiene & Tropical Medicine (reference 6454), Vector Control Division of the Ministry of Health of Uganda (reference VCD-IRC/044), and Uganda National Council of Science and Technology (reference HS 1429). All adult subjects provided written informed consent, and a parent or guardian of any child participant provided written informed consent on their behalf.

6.3 Results

6.3.1 Oocyst prevalence and intensity variations between study rounds

Significant variation in oocyst prevalence and oocyst intensity was observed between the rounds (see Appendix 6-1, Table A6-1). The lowest infection prevalence and intensity values were recorded in round 2 in all three insecticide dose categories. Within each round, both values were higher in mosquitoes that were not exposed to insecticides compared to those exposed.

6.3.2 Temperature and relative humidity variations between study rounds

There was significant variation in mean daily temperature (T) ($F_{2,21}$ = 47.003, p < 0.0001) and maximum daily T ($F_{2,21}$ = 21.587, p < 0.0001) during the 7-day incubation period, and in daily T range during the same period ($F_{2,21}$ = 26.746, p < 0.0001). However, the mean minimum T during the incubation period were not significantly different between the three rounds ($F_{2,21}$ = 1.558, p = 0.234). Round 2 was on average the warmest, with the largest daily T variations.

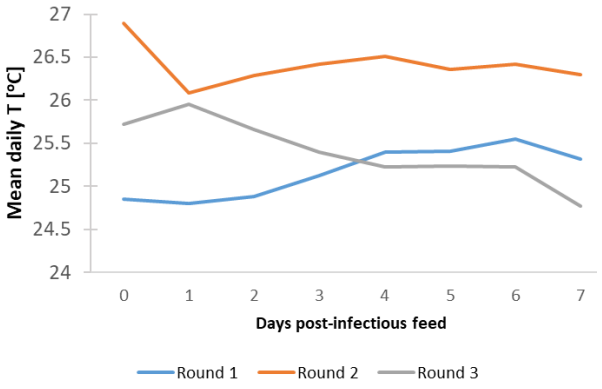
Rounds 1 and 3 were similar, but round 3 had slightly higher mean daily T during the incubation period (Fig. 6-1 and Table 6-1).

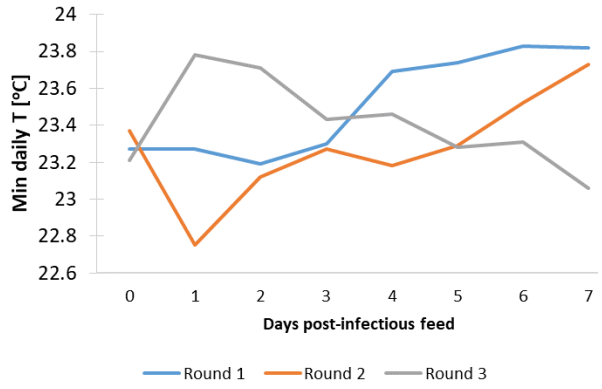
There was also significant variation in all the relative humidity (RH) parameters: mean daily RH ($F_{2,21}$ = 216.85, p < 0.0001), minimum daily RH ($F_{2,21}$ = 97.334, p < 0.0001), maximum daily RH ($F_{2,21}$ = 132.1, p < 0.0001) and daily RH range ($F_{2,21}$ = 15.005, p < 0.0001) during the incubation period between the three rounds. The highest mean daily RH during the incubation period was measured in round 1, while RH in round 2 was the lowest (Fig. 6-2).

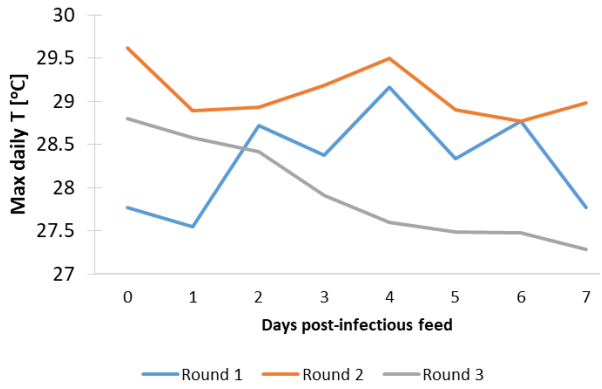
Table 6-1. Means of daily temperature (T), maximum and minimum temperature and daily temperature variation during the seven day incubation period, recorded during the three study rounds.

	Mean daily T [^{o}C]	Max T [^{o}C]	Min T [^{o}C]	T range [^{o}C]
Round 1	25.2	27.8	23.4	4.3
Round 2	26.4	28.9	23.2	5.7
Round 3	25.4	28.0	23.4	4.6

Study rounds: 1 = September-October 2013, 2 = November-December 2013, and 3 = May-June 2014.







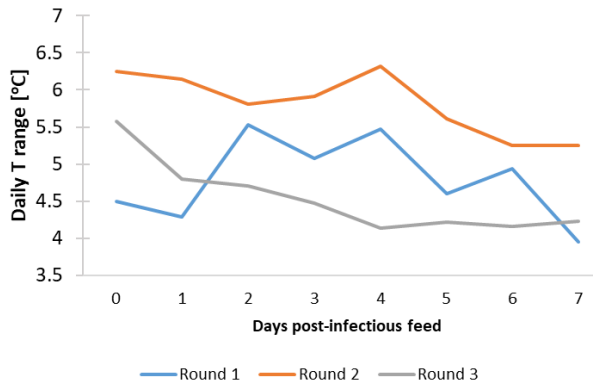
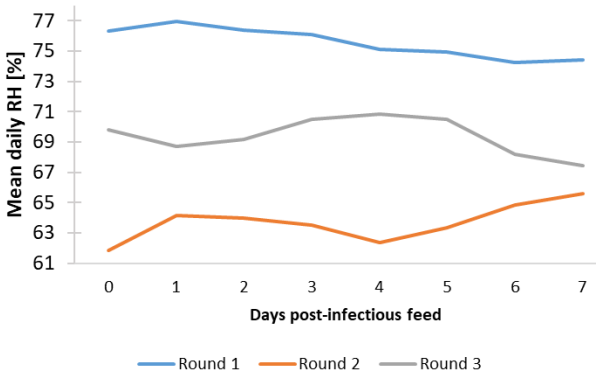
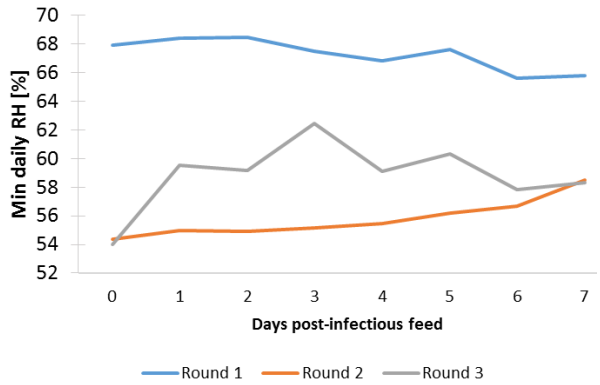
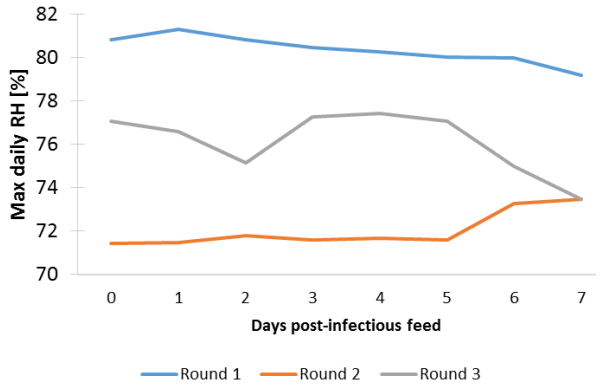


Fig. 6-1. Averages of temperature measurements during the incubation period (from day of feed, which is day 0, to day of dissection) for the three study rounds (round 1 = September-October 2013, round 2 = November-December 2013, and round 3 = May-June 2014).







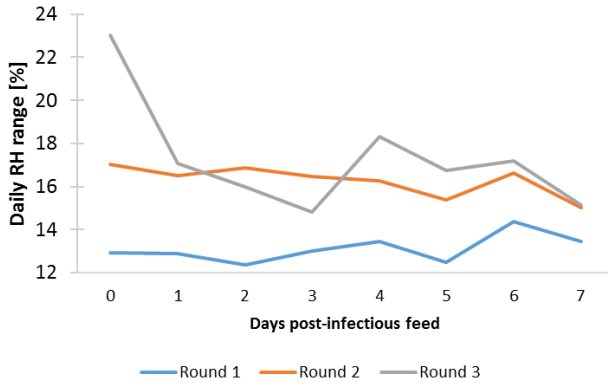


Fig. 6-2. Averages of relative humidity (RH) measurements during the incubation period (from day of feed, which is day 0, to day of dissection) for the three study rounds (round 1 = September-October 2013, round 2 = November-December 2013, and round 3 = May-June 2014).

6.3.3 Temperature variations during first 24 hours post-infectious feed

Because the early part of sporogony, especially transition from zygotes into ookinetes and their passage through the midgut wall, is thought to be sensitive to temperature, the effects of temperature variables during the first 24 hours post-infectious feed in each study round were studied in comparison with values in subsequent days. There was significant variation in all the temperature parameters during the first 24 hours post-infectious feed between the three rounds (Fig 6-3).

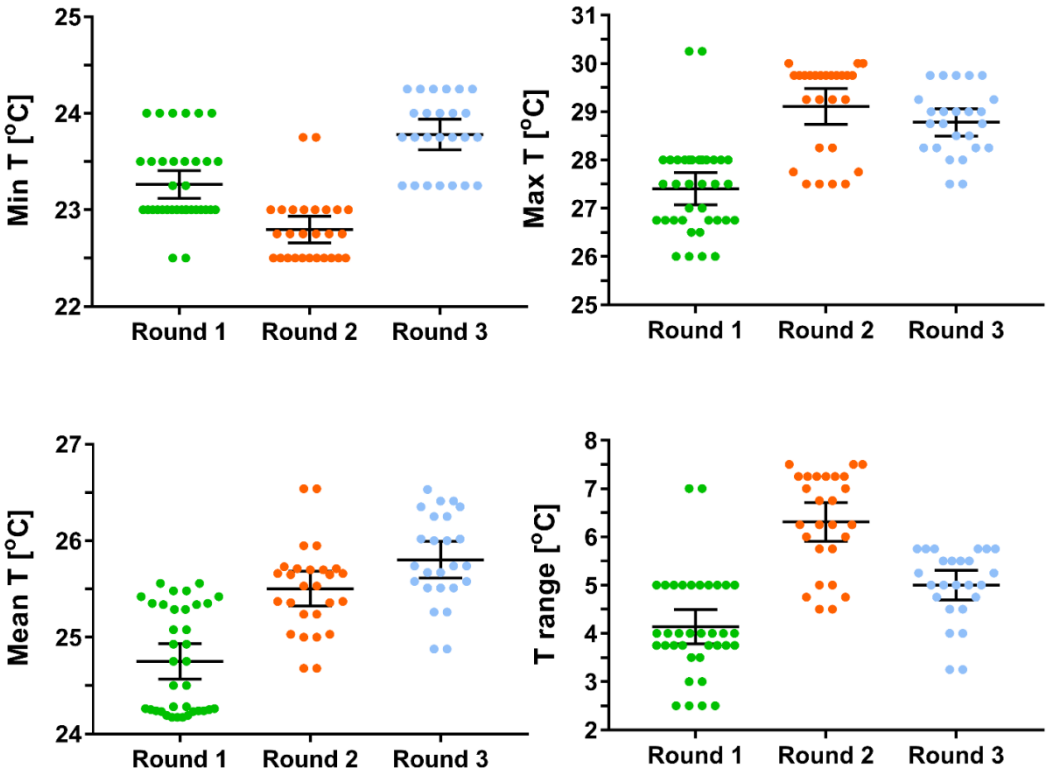


Fig. 6-3. Temperature measurements during the first 24 hours post-infectious feed recorded in each transmission experiment, in the three study rounds. There was significant variation in all temperature parameters during the first 24 hours post-infectious feed between the three rounds: mean temperature ($F_{2,85} = 39.328$, $p < 0.0001$), minimum temperature ($F_{2,85} = 41.749$, $p < 0.0001$), maximum temperature ($F_{2,85} = 32.861$, $p < 0.0001$) and the temperature range ($F_{2,85} = 36.57$, $p < 0.0001$). Error bars show 95% confidence intervals of the means.

6.3.4 Effect of temperature on oocyst prevalence

The effect of different temperature variables during the first 24 hours post-infective feed and deltamethrin exposure on oocyst prevalence was investigated using mixed-effects

logistic regression. A total of 692 *An. gambiae* s.s. homozygous for *ReRe* genotype from experiments that used blood samples from 42 gametocyte volunteers were included in the analysis. The results showed that apart from the effect of deltamethrin on infection rates, temperature range during the first 24 hours post-infectious feed (i.e. the difference between maximum and minimum temperature on day 1) and temperature range on day 4 post-infectious feed had a highly significant effect on risk of infection (Table 6-2). The results indicate that an increase in temperature range was associated with lower infection, after controlling for the effects of insecticide exposure. Figure 6-4 shows the model predictions, confirming that with increasing insecticide dose and increasingly large temperature variations during (a) the first 24 hours and (b) on day 4 post-infectious blood meal, prevalence of infection in mosquitoes will decrease.

Table 6-2. Mixed-effects logistic regression analysis of *P. falciparum* oocyst prevalence rates.

		Odds ratio	Std. Err.	Z	*p*	[95% Confidence Interval]	
Dose category	Control	1.000	-	-	-	-	-
	Low dose	0.423	0.139	-2.61	0.009	0.221	0.807
	High dose	0.190	0.048	-6.51	<0.0001	0.115	0.313
Temp range day 1 (^{o}C)		0.631	0.133	-2.18	0.029	0.417	0.954
Temp range day 4 (^{o}C)		0.606	0.137	-2.21	0.027	0.389	0.944
Variance of random intercept		2.092	0.729			1.057	4.143

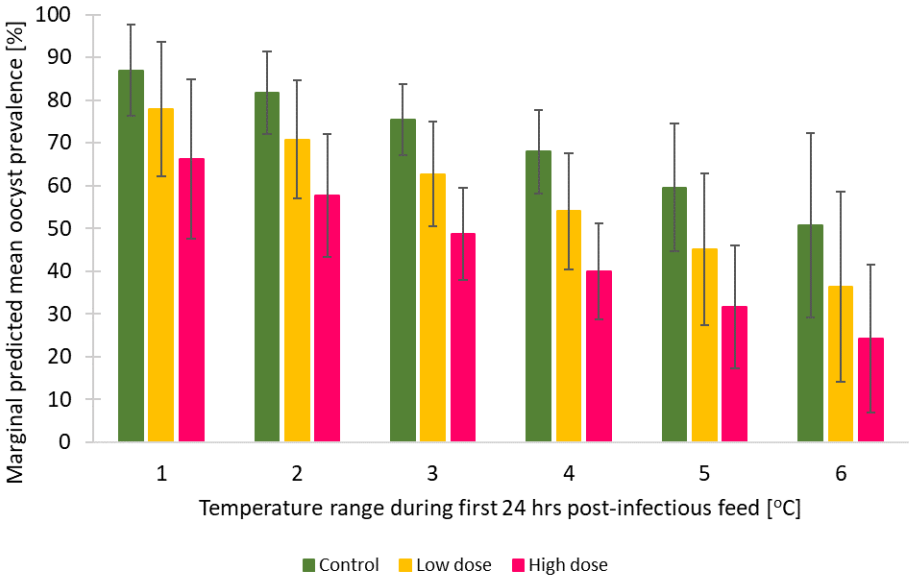
Model χ^2_{3df} = 54.33, p < 0.001; n = 692; number of groups (gametocyte donors) = 42.

Note: The dependent variable is oocyst infection coded as 0 (negative) and 1 (positive).

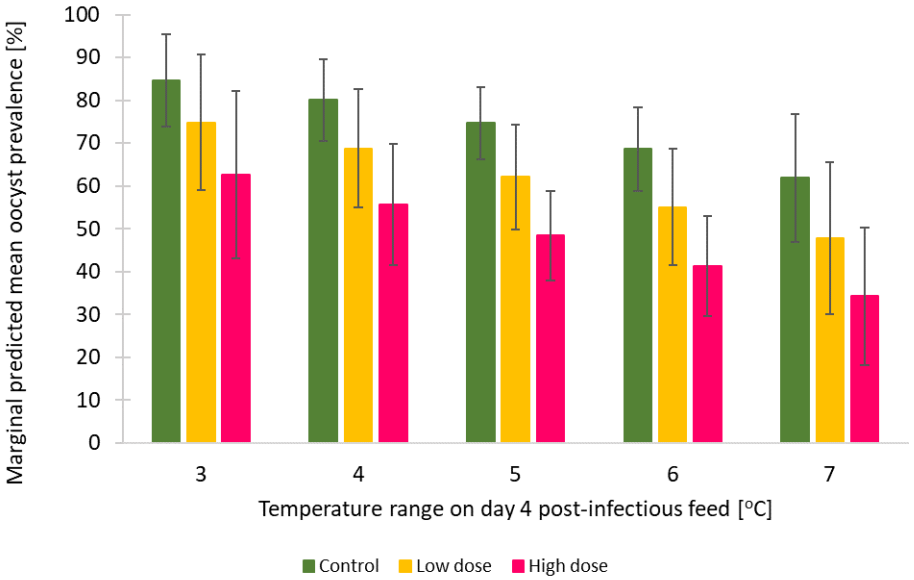
6.3.5 Mosquito survival in relation to temperature and humidity

Following the standard membrane feeds and insecticide exposure, fed mosquitoes were kept for seven days until dissection for the presence of oocysts. Mosquito mortality was recorded daily.

Figure 6-5 shows Kaplan-Meier survival curves for the three insecticide exposure groups within each study round. The survival curves showed the influence of insecticide exposure on mosquito survival, together with the influence of environmental variables. The survival distributions were significantly different between the insecticide exposure groups within each study round, showing that insecticide exposure impaired survival of *kdr* homozygous mosquitoes.



(a)



(b)

Fig. 6-4. Plot of predictive margins of deltamethrin exposure group (Control, Low dose = 2.5-5.0 mg/m^2, High dose = 10.0-16.7 mg/m^2) on infection rates in mosquitoes with 95% confidence intervals (a) Showing the effect of insecticide exposure and variations in temperature during the first 24 hours post-infectious feed; (b) Showing the effect of insecticide exposure and variations in temperature on day 4 post-infectious feed.

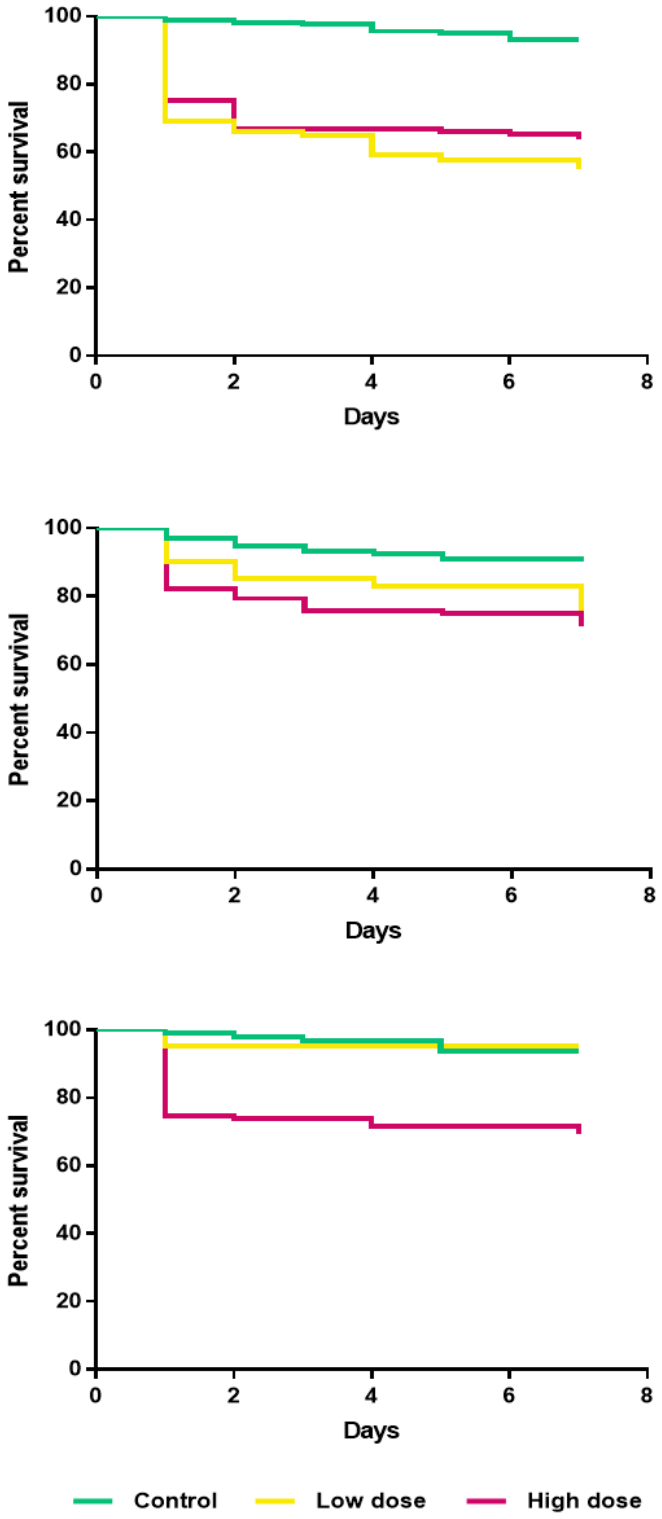


Fig. 6-5. The Kaplan-Meier survival curves and estimates for mosquito survival in each of the three experimental rounds per each insecticide exposure dose. Only *An. gambiae* s.s. mosquitoes homozygous for *kdr-L1014S* mutation were included in the analysis (control = untreated netting; low dose = 2.5-5.0 mg/m^2 deltamethrin; high dose = 10.0-16.7 mg/m^2 deltamethrin).

Table 6-3. Cox proportional hazards model analysis of mosquito survival in the three rounds following exposure to untreated or treated netting.

		Hazard ratio	Std. Err.	Z	*p*	[95% Confidence Interval]	
Dose category	Control	1.000	-	-	-	-	-
	Low dose	4.063	0.963	5.91	<0.0001	2.553	6.466
	High dose	4.725	0.975	7.53	<0.0001	3.154	7.078
Round	1	1.000	-	-	-	-	-
	2	0.735	0.123	-1.84	0.066	0.529	1.019
	3	0.584	0.115	-2.74	0.006	0.397	0.858
Variance of random intercept		2.092	0.729			1.057	4.143

Model χ^2_{4df} = 82.64 $p < 0.0001$; n = 892 *Re/Re* mosquitoes; number of failures (death before dissection) = 186

Cox proportional hazards model (Table 6-3) shows that compared to control untreated nets, mosquitoes exposed to low dose deltamethrin had 4.1 times the chance of dying, whereas mosquitoes exposed to high dose had 4.7 times chance of dying before dissection. Moreover, the rate of death decreased by 26.5% in round 2 compared to round 1, and by 41.6% in round 3 compared to round 1, if dose category was held constant.

To graphically assess the proportional-hazards assumption, separate Cox models were fitted to each insecticide dose, while adjusting for study round. The lines are roughly parallel, implying that the proportional-hazards assumption was not violated (Fig. 6-6).

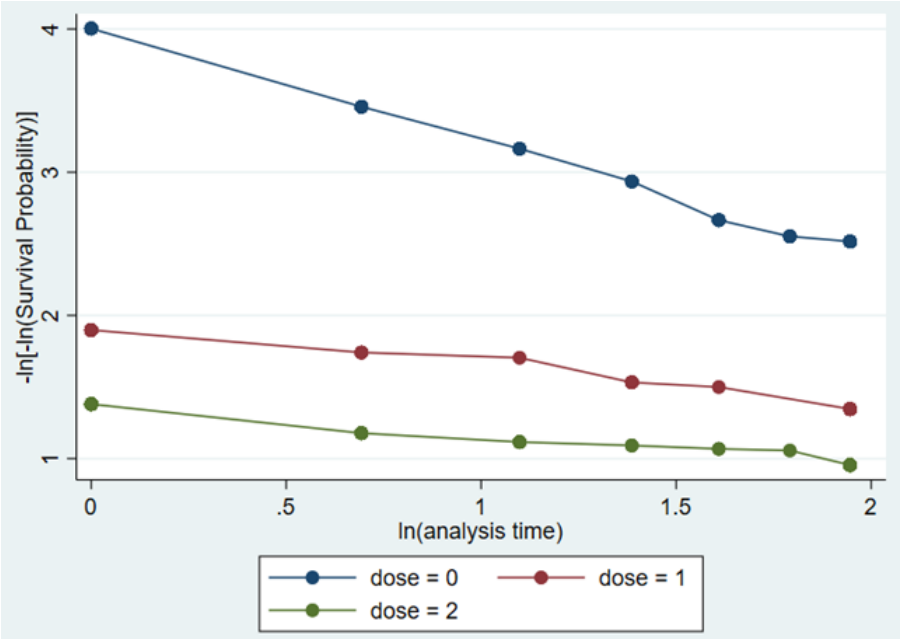


Fig. 6-6. A log-log plot to test the proportionality assumption of the Cox proportional hazards model.

Mixed-effects regression analysis showed that apart from the effect of deltamethrin, average daily temperature and average daily relative humidity during the seven day incubation period had an independent and highly significant effect on mosquito mortality (Table 6-4).

Table 6-4. Mixed-effects logistic regression analysis of mortality rates of *An. gambiae* s.s. homozygous for *kdr-L1014S.*

		Odds ratio	Std. Err.	Z	*p*	[95% Confidence Interval]	
Dose category	Control	1.000	-	-	-	-	-
	Low dose	5.144	1.588	5.31	<0.0001	2.809	9.422
	High dose	5.069	1.299	6.34	<0.0001	3.068	8.376
Average temp (^{o}C)		8.472	5.133	3.53	<0.0001	2.583	27.780
Average relative humidity [%]		1.248	0.073	3.80	<0.0001	1.113	1.399
Variance of random intercept		0.105	0.122			0.011	1.029

Model χ^2_{3df} = 57.97, p < 0.0001; n = 837; number of groups (gametocyte donors) = 42.

Note: The dependent variable dead is mosquito death coded as 0 (alive) and 1 (dead).

Interactions of temperature, relative humidity and insecticide exposure and their effect on mosquito survival are shown in Figure 6-7. Increased temperature and relative humidity resulted in increased mortality of deltamethrin-exposed mosquitoes compared with unexposed mosquitoes.

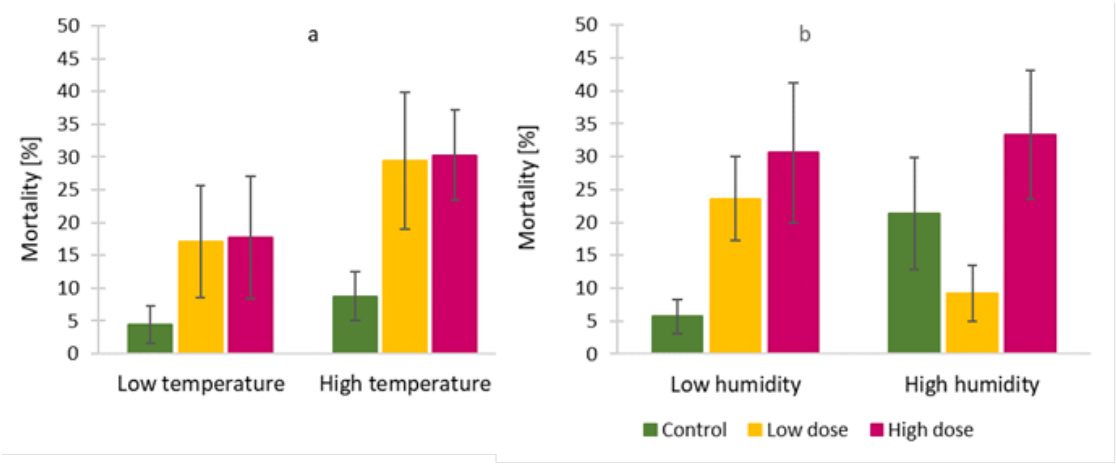


Fig. 6-7. The predictive margins effect of (a) variations in temperature (under low (<25.3°C) and high temperature (≥25.3°C) conditions) and insecticide exposure; (b) variations in relative humidity (under low (<69.7%) and high relative humidity (≥69.7%) conditions) and insecticide exposure on mosquito mortality in *kdr-L1014S* homozygous resistant *An. gambiae* s.s. mosquitoes with 95% confidence intervals Predictions are based on the mortality of mosquitoes exposed to control untreated nets, nets treated with low dose (2.5-5.0 mg/m^2 deltamethrin) or high dose (10.0-16.7 mg/m^2 deltamethrin) after feeding on blood samples from gametocytaemic volunteers. The median of ambient temperature recorded during the experiments (25.3°C) and ambient relative humidity (69.7%) was used as a cut-off to plot mosquito mortality charts.

6.3.6 Mosquito survival in relation to different *kdr* genotypes

Survival of mosquitoes with different *kdr* genotypes was compared following the membrane feeds and insecticide exposure (Fig. 6-8). No wild type susceptible mosquitoes (*S/S*) or heterozygotes (*Re/S*) survived exposure to high doses of deltamethrin, although these two genotypes were present among the tested mosquitoes. The low numbers of *S/S* and *S/Re* mosquitoes did not allow for a detailed analysis. Statistical tests of the effect of genotype on mortality did not provide a clear or consistent pattern among the three insecticide exposure groups. Survival of resistant *Re/Re, Rw/Rw* and *Re/Rw* genotypes was significantly different in control (Fisher's exact, p = 0.001), and high dose groups (Fisher's exact, p = 0.007), but not in a low dose group (Fisher's exact, p = 0.084). In all three insecticide exposure groups, a

higher proportion of *Rw/Rw* genotype mosquitoes survived than of *Re/Re* mosquitoes, whereas *Re/Rw* genotype had the lowest survival of the three genotypes.

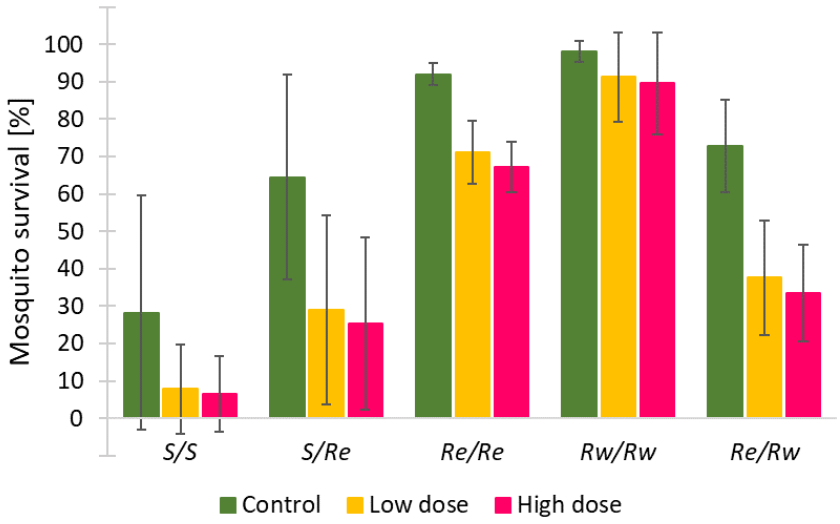


Fig. 6-8. The predicted effect of deltamethrin exposure on mosquito survival in different *kdr* genotypes of *An. gambiae* s.s. mosquitoes with 95% confidence intervals. Predictions are based on survival of mosquitoes with different *kdr* genotypes following the membrane feeds and exposure to treated or untreated nets, at the end of the seven day incubation period and compared per each exposure dose. Mosquitoes were exposed for 5 minutes using a wire ball frame to control untreated nets, nets treated with low dose (2.5-5.0 mg/m^2 deltamethrin) or high dose (10.0-16.7 mg/m^2 deltamethrin) after feeding on blood samples from gametocytaemic volunteers.

6.4 Discussion

In order to determine whether environmental variables such as temperature and relative humidity, together with the presence of *kdr-L1014S* mutation, have an impact on survival probability and malaria infection, we compared daily survival and *Plasmodium* infection rates in wild insecticide resistant *An. gambiae* s.s. fed on infective blood from gametocytaemic volunteers and exposed to untreated or deltamethrin-treated nets.

We have previously shown that average ambient temperature during the seven days of incubation, together with insecticide exposure, had a highly significant effect on the risk of infection in mosquitoes and on the parasite load [46]. In the present study, we wanted to further explore any possible effects of different environmental variables on parasite development and vector survival, in the presence or absence of insecticide exposure and in different *kdr* genotypes. Apart from insecticide dose, temperature range on day 1 (i.e. within

the first 24 hours) and on day 4 post-infective blood meal had significant effects on parasite development.

The period of the first 24 hours following the infective feed is the time of blood meal digestion and early sporogony, with ookinete densities reaching peak numbers [20] while the peritrophic matrix, which the ookinetes must traverse, reaches its maximal thickness [53]. This part of sporogony is particularly sensitive to both temperature [12, 13, 18, 19, 20, 21, 22, 23, 24] and exposure of infected mosquitoes to sub-lethal doses of pyrethroids [40]. Blood meal digestion in mosquitoes is temperature-dependent [54], while the speed of digestion also affects the sporogony, especially formation of ookinetes and their migration through the peritrophic matrix and the midgut wall [55]. During study round 2, temperature variations in the first 24 hours post-feed were significantly larger than in rounds 1 or 3, whereas parasite prevalence and intensity of infection were significantly lower, regardless of insecticide exposure.

Young oocysts can be seen from day 2 post infection [56]. During this period, mitotic divisions start taking place, forming a multinucleate oocyst, and circumsporozoite protein (CSP) must be produced for formation and budding of the sporozoites [57, 58]. Although it is possible that some of the processes taking place during sporozoite development in the oocysts are temperature-sensitive, previous studies show that oocysts, once formed, are no longer sensitive to changes in ambient temperature [19].

Exposure to insecticides, ambient temperature and relative humidity, malaria infection and insecticide resistance all interact in nature and can affect vector competence in differing ways, but their combined effect on mosquito survival is not well understood. Increase in environmental temperature has been shown to be associated with reduced adult survival [59, 60]. Temperature also affects the extent to which insecticides kill mosquitoes [25, 26], possibly because mosquito immune responses [12], nervous-system sensitivity [61], and metabolic activity [62] are all temperature-dependent. Apart from its effect on mosquito survival in combination with ambient temperature [11], humidity was shown to have a strong impact on insecticide resistance phenotype [63].

Insecticide resistance mechanisms can also exert a wide range of effects on vector longevity, competence and behavior and could in principle affect malaria transmission in either a positive or negative manner [64]. Moreover, different resistance alleles can interact to influence the fitness of mosquitoes [65]. Extensive comparison of survival between different

kdr genotypes and the wild type was not possible due to low numbers of mosquitoes with a wild type *S* allele. However, survival over the seven day incubation period (during which the oocysts developed) of mosquitoes with resistant genotypes (i.e. with at least one resistant allele, *Re* or *Rw*) was higher than survival of wild susceptible *S/S* type. There were also significant differences in survival over the incubation period of resistant *Re/Re, Rw/Rw* and *Re/Rw* genotypes in control and high dose groups; in both instances, survival was the lowest in mosquitoes with *Re/Rw* genotype, which could be due to combination of resistant alleles exerting a fitness cost on mosquitoes. While a study from Cameroon showed that *Re/Rw* heterozygotes were significantly less resistant to permethrin than *Rw/Rw* homozygotes, this was not observed with deltamethrin [66]. Several properties of infected blood can impair mosquito fitness, even in the absence of actual mosquito infection, while survival of infected mosquitoes is also affected by environmental stress [67]. Furthermore, survival of uninfected *kdr* resistant mosquitoes was shown to be higher than that of the susceptible strain, while their survival was similar when exposed to *P. falciparum* infection [68].

This study allowed us to examine the relationships between environmental variables and insecticide exposure on survival probability and infection rates in wild *An. gambiae* s.s. in the presence of *kdr-L1014S* mutation. As previously observed, early sporogony was most sensitive to temperature, especially to temperature variation, regardless of the insecticide exposure. We also show that temperature and relative humidity, together with insecticide exposure, impact mosquito survival following infected feeds. From a vector control perspective, it was encouraging to find that deltamethrin exposure still significantly impaired survival of *kdr* homozygous mosquitoes. The impact of insecticide resistance on malaria infection and vector survival needs to be assessed separately for mosquitoes carrying target site or metabolic resistance mechanisms before we will be able to fully understand the impact of resistance on currently available vector control tools and on malaria transmission.

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6.5 References

1. 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;526:207-211.

2. 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.

3. Ranson H, Lissenden N. Insecticide resistance in African *Anopheles* mosquitoes: a worsening situation that needs urgent action to maintain malaria control. Trends Parasitol. 2016;32:187-196.

4. 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 Med. 2014;11:e1001619.

5. Lindblade KA, Mwandama D, Mzilahowa T, Steinhardt L, Gimnig J, Shah M*, et al*. A cohort study of the effectiveness of insecticide-treated bed nets to prevent malaria in an area of moderate pyrethroid resistance, Malawi. Malar J. 2015;14:31.

6. World Health Organization: Implications of insecticide resistance for malaria vector control. Geneva: World Health Organization; 2016: 4.

7. Ochomo E, Chahilu M, Cook J, Kinyari T, Bayoh NM, West P*, et al*. Insecticide-treated nets and protection against insecticide-resistant malaria vectors in western Kenya. Emerg Infect Dis. 2017;23:758-764.

8. World Health Organization. World Malaria Report 2017. Geneva: World Health Organization; 2017.

9. Garrett-Jones C, Shidrawi GR. Malaria vectorial capacity of a population of *Anopheles gambiae*: an exercise in epidemiological entomology. Bull World Health Organ. 1969;40:531-545.

10. Macdonald G. Epidemiological basis of malaria control. Bull World Health Organ. 1956;15:613-626.

11. Afrane YA, Githeko AK, Yan G. The ecology of *Anopheles* mosquitoes under climate change: case studies from the effects of deforestation in East African highlands. Ann N Y Acad Sci. 2012;1249:204-210.

12. Murdock CC, Paaijmans KP, Bell AS, King JG, Hillyer JF, Read AF*, et al*. Complex effects of temperature on mosquito immune function. Proc Biol Sci. 2012;279:3357-3366.

13. Murdock CC, Paaijmans KP, Cox-Foster D, Read AF, Thomas MB. Rethinking vector immunology: the role of environmental temperature in shaping resistance. Nat Rev Microbiol. 2012;10:869-876.

14. Murdock CC, Moller-Jacobs LL, Thomas MB. Complex environmental drivers of immunity and resistance in malaria mosquitoes. Proc Biol Sci. 2013;280:20132030.

15. Detinova TS. Age-grouping methods in Diptera of medical importance with special reference to some vectors of malaria. Monogr Ser World Health Organ. 1962;47:13-191.

16. Ohm JR, Baldini F, Barreaux P, Lefevre T, Lynch PA, Suh E*, et al*. Rethinking the extrinsic incubation period of malaria parasites. Parasit Vectors. 2018;11:178.

17. Macdonald G. The analysis of the sporozoite rate. Trop Dis Bull. 1952;49:569-586.

18. Boyd MF. Malariology: A Comprehensive Survey of All Aspects of this Group of Diseases from a Global Standpoint. Philadelphia and London: W. B. Saunders & Co.; 1949.

19. Eling W, Hooghof J, van de Vegte-Bolmer M, Sauerwein R, van Gemert G-J. Tropical temperatures can inhibit development of the human malaria parasite *Plasmodium falciparum* in the mosquito. Proc Exper Appl Entomol. 2001;12:151-156.

20. Noden BH, Kent MD, Beier JC. The impact of variations in temperature on early *Plasmodium falciparum* development in *Anopheles stephensi*. Parasitology. 1995;111 (Pt 5):539-545.

21. Okech BA, Gouagna LC, Kabiru EW, Walczak E, Beier JC, Yan G*, et al*. Resistance of early midgut stages of natural *Plasmodium falciparum* parasites to high temperatures in experimentally infected *Anopheles gambiae* (Diptera: Culicidae). J Parasitol. 2004;90:764-768.

22. Paaijmans KP, Blanford S, Bell AS, Blanford JI, Read AF, Thomas MB. Influence of climate on malaria transmission depends on daily temperature variation. Proc Natl Acad Sci U S A. 2010;107:15135-15139.

23. Rastogi M, Pal NL, Sen AB. Effect of variation in temperature on development of *Plasmodium berghei* (NK 65 strain) in *Anopheles stephensi*. Folia Parasitol. 1987;34:289-297.

24. Vanderberg JP, Yoeli M. Effects of temperature on sporogonic development of *Plasmodium berghei*. J Parasitol. 1966;52:559-564.

25. Glunt KD, Paaijmans KP, Read AF, Thomas MB. Environmental temperatures significantly change the impact of insecticides measured using WHOPES protocols. Malar J. 2014;13:350.

26. Hodjati MH, Curtis CF. Effects of permethrin at different temperatures on pyrethroid-resistant and susceptible strains of *Anopheles*. Med Vet Entomol. 1999;13:415-422.

27. Cutkomp LK, Subramanyam B. Toxicity of pyrethroids to *Aedes aegypti* larvae in relation to temperature. J Am Mosq Control Assoc. 1986;2:347-349.

28. Harwood AD, You J, Lydy MJ. Temperature as a toxicity identification evaluation tool for pyrethroid insecticides: toxicokinetic confirmation. Environ Toxicol Chem. 2009;28:1051-1058.

29. Polson KA, Brogdon WG, Rawlins SC, Chadee DD. Impact of environmental temperatures on resistance to organophosphate insecticides in *Aedes aegypti* from Trinidad. Rev Panam Salud Publica. 2012;32:1-8.

30. Glunt KD, Oliver SV, Hunt RH, Paaijmans KP. The impact of temperature on insecticide toxicity against the malaria vectors *Anopheles arabiensis* and *Anopheles funestus*. Malar J. 2018;17:131.

31. Bourguet D, Guillemaud T, Chevillon C, Raymond M. Fitness costs of insecticide resistance in natural breeding sites of the mosquito *Culex pipiens*. Evolution. 2004;58:128-135.

32. Oliver SV, Brooke BD. The effect of larval nutritional deprivation on the life history and DDT resistance phenotype in laboratory strains of the malaria vector *Anopheles arabiensis*. Malar J. 2013;12:44.

33. Oliver SV, Brooke BD. The effect of multiple blood-feeding on the longevity and insecticide resistant phenotype in the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). Parasit Vectors. 2014;7:390.

34. Kulma K, Saddler A, Koella JC. Effects of age and larval nutrition on phenotypic expression of insecticide-resistance in *Anopheles* mosquitoes. PLoS One. 2013;8:e58322.

35. Owusu HF, Chitnis N, Muller P. Insecticide susceptibility of *Anopheles* mosquitoes changes in response to variations in the larval environment. Sci Rep. 2017;7:3667.

36. Farenhorst M, Mouatcho JC, Kikankie CK, Brooke BD, Hunt RH, Thomas MB*, et al*. Fungal infection counters insecticide resistance in African malaria mosquitoes. Proc Natl Acad Sci U S A. 2009;106:17443-17447.

37. Koella JC, Saddler A, Karacs TP. Blocking the evolution of insecticide-resistant malaria vectors with a microsporidian. Evol Appl. 2012;5:283-292.

38. Kikuchi Y, Hayatsu M, Hosokawa T, Nagayama A, Tago K, Fukatsu T. Symbiont-mediated insecticide resistance. Proc Natl Acad Sci U S A. 2012;109:8618-8622.

39. Patil CD, Borase HP, Salunke BK, Patil SV. Alteration in *Bacillus thuringiensis* toxicity by curing gut flora: novel approach for mosquito resistance management. Parasitol Res. 2013;112:3283-3288.

40. Hill N. Effects of sublethal doses of pyrethroids on malaria vectors. *PhD Thesis.* London: London School of Hygiene & Tropical Medicine, University of London; 2002.

41. Glunt KD, Coetzee M, Huijben S, Koffi AA, Lynch PA, N'Guessan R*, et al*. Empirical and theoretical investigation into the potential impacts of insecticide resistance on the effectiveness of insecticide-treated bed nets. Evol Appl. 2017;11:431-441.

42. James RR, Xu J. Mechanisms by which pesticides affect insect immunity. J Invertebr Pathol. 2012;109:175-182.

43. Carle PR, Coz J, Elissa N, Gasquet M, Sannier C, Richard A*, et al*. [Intravector anti-*Plasmodium* activity of a pyrethrinoid: deltamethrin]. C R Acad Sci III. 1986;303:565-568.

44. Elissa N, Sannier C, Coz J. [Étude de la réceptivité à *Plasmodium yoelii yoelii* d'*Anopheles stephensi* soumis au stade adulte à des doses toxiques de différents pyréthrinoïdes]. Cah ORSTOM, ser Ent méd et Parasitol. 1987;25:187-192.

45. Elissa N, Sannier C, Richard A: [La Receptivite a *Plasmodium yoelii yoelii* d'*Anopheles* Soumis a des Subletales de Deltamethrine]. In: *IVe Congres Sur La Protection De La Sante Humaine Et Des Cultures En Milieu Tropical*. Marseille, France; 1986: 599-604.

46. Kristan M, Lines J, Nuwa A, Ntege C, Meek SR, Abeku TA. Exposure to deltamethrin affects development of *Plasmodium falciparum* inside wild pyrethroid resistant *Anopheles gambiae* s.s. mosquitoes in Uganda. Parasit Vectors. 2016;9:100.

47. World Health Organization: Malaria parasite counting. Malaria microscopy standard operating procedure - MM-SOP-09. Geneva: World Health Organization; 2016: 5.

48. Kilian A, Byamukama W, Pigeon O, Gimnig J, Atieli F, Koekemoer L*, et al*. Evidence for a useful life of more than three years for a polyester-based long-lasting insecticidal mosquito net in Western Uganda. Malar J. 2011;10:299.

49. Bass C, Williamson MS, Field LM. Development of a multiplex real-time PCR assay for identification of members of the *Anopheles gambiae* species complex. Acta Trop. 2008;107:50-53.

50. Bass C, Nikou D, Donnelly MJ, Williamson MS, Ranson H, Ball A*, et al*. Detection of knockdown resistance (*kdr*) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. Malar J. 2007;6:111.

51. Bass C, Nikou D, Vontas J, Williamson MS, Field LM. Development of high-throughput real-time PCR assays for the identification of insensitive acetylcholinesterase (*ace-1*^{R}) in *Anopheles gambiae*. Pestic Biochem Phys. 2010;96:80-85.

52. McDonald JH. Handbook of Biological Statistics. 3rd edn. Baltimore, Maryland: Sparky House Publishing; 2014.

53. Dinglasan RR, Devenport M, Florens L, Johnson JR, McHugh CA, Donnelly-Doman M*, et al*. The *Anopheles gambiae* adult midgut peritrophic matrix proteome. Insect Biochem Mol Biol. 2009;39:125-134.

54. O'Gower AK. The rate of digestion of human blood by certain species of mosquitoes. Aust J Biol Sci. 1956;9:125-129.

55. Feldmann AM, Billingsley PF, Savelkoul E. Bloodmeal digestion by strains of *Anopheles stephensi* liston (Diptera: Culicidae) of differing susceptibility to *Plasmodium falciparum*. Parasitology. 1990;101 Pt 2:193-200.

56. Lensen AH, Van Gemert GJ, Bolmer MG, Meis JF, Kaslow D, Meuwissen JH*, et al*. Transmission blocking antibody of the *Plasmodium falciparum* zygote/ookinete surface protein Pfs25 also influences sporozoite development. Parasite Immunol. 1992;14:471-479.

57. Aly AS, Vaughan AM, Kappe SH. Malaria parasite development in the mosquito and infection of the mammalian host. Annu Rev Microbiol. 2009;63:195-221.

58. Thathy V, Fujioka H, Gantt S, Nussenzweig R, Nussenzweig V, Menard R. Levels of circumsporozoite protein in the *Plasmodium* oocyst determine sporozoite morphology. EMBO J. 2002;21:1586-1596.

59. Afrane YA, Zhou G, Lawson BW, Githeko AK, Yan G. Effects of microclimatic changes caused by deforestation on the survivorship and reproductive fitness of *Anopheles gambiae* in western Kenya highlands. Am J Trop Med Hyg. 2006;74:772-778.

60. Christiansen-Jucht C, Parham PE, Saddler A, Koella JC, Basanez MG. Temperature during larval development and adult maintenance influences the survival of *Anopheles gambiae* s.s. Parasit Vectors. 2014;7:489.

61. Montgomery JC, Macdonald JA. Effects of temperature on nervous system: implications for behavioral performance. Am J Physiol. 1990;259:R191-196.

62. Gillooly JF, Brown JH, West GB, Savage VM, Charnov EL. Effects of size and temperature on metabolic rate. Science. 2001;293:2248-2251.

63. Weetman D, Wilding CS, Neafsey DE, Muller P, Ochomo E, Isaacs AT*, et al*. Candidate-gene based GWAS identifies reproducible DNA markers for metabolic pyrethroid resistance from standing genetic variation in East African *Anopheles gambiae*. Sci Rep. 2018;8:2920.

64. Rivero A, Vezilier J, Weill M, Read AF, Gandon S. Insecticide control of vector-borne diseases: when is insecticide resistance a problem? PLoS Pathog. 2010;6.

65. Berticat C, Bonnet J, Duchon S, Agnew P, Weill M, Corbel V. Costs and benefits of multiple resistance to insecticides for *Culex quinquefasciatus* mosquitoes. BMC Evol Biol. 2008;8:104.

66. Reimer L, Fondjo E, Patchoke S, Diallo B, Lee Y, Ng A*, et al*. Relationship between *kdr* mutation and resistance to pyrethroid and DDT insecticides in natural populations of *Anopheles gambiae*. J Med Entomol. 2008;45:260-266.

67. Ferguson HM, Mackinnon MJ, Chan BH, Read AF. Mosquito mortality and the evolution of malaria virulence. Evolution. 2003;57:2792-2804.

68. Alout H, Dabire RK, Djogbenou LS, Abate L, Corbel V, Chandre F*, et al*. Interactive cost of *Plasmodium* infection and insecticide resistance in the malaria vector *Anopheles gambiae*. Sci Rep. 2016;6:29755.

Appendix 6-1

Table A6-1. Oocyst prevalence and mean oocyst intensity (number of oocysts/midgut in infected mosquitoes) variation between the study rounds and doses of deltamethrin the mosquitoes were exposed to after infective feeds.

Deltamethrin dose*	Study round**	Oocyst prevalence [%] (95% CI)	Comparison of prevalence between rounds	Mean number of oocysts/midgut (95% CI)	Analysis of variance
Control	1	92.7 (84.2, 96.8)		12.45 (10.00, 14.89)	
	2	41.2 (23.3, 61.8)	$\chi^2 = 94.14$	2.96 (2.33, 3.59)	$F_{2,252} = 12.32$
	3	82.7 (67.3, 91.8)	$p < 0.0001$	8.78 (6.75, 10.80)	$p < 0.0001$
Low dose	1	79.5 (44.8, 94.9)		8.48 (5.95, 11.01)	
	2	25.8 (18.2, 35.2)	$\chi^2 = 29.05$	1.00 (1, 1)	$F_{2,67} = 6.42$
	3	81.6 (72.8, 87.9)	p = 0.0020	5.03 (3.18, 6.88)	$p = 0.0028$
High dose	1	71.4 (52.9, 84.7)		8.55 (6.58, 10.51)	
	2	23.7 (7.0, 56.1)	$\chi^2 = 43.23$	2.17 (1.37, 2.97)	$F_{2,111} = 11.93$
	3	60.0 (64.5, 82.9)	p = 0.0069	4.86 (3.55, 6.17)	$p < 0.0001$

* Deltamethrin dose: Low dose = 2.5-5.0 mg/m^2 , high dose = 10.0-16.7 mg/m^2.

** Study rounds: 1 = September-October 2013, 2 = November-December 2013, and 3 = May-June 2014.

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Student	Mojca Kristan
Principal Supervisor	Jo Lines
Thesis Title	Effects of pyrethroid exposure and insecticide resistance on the sporogonic development of Plasmodium falciparum in Anopheles gambiae s.l.

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

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Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I designed and planned the study with TAA, with contributions of JL. I trained the field staff with TAA. I carried out all field experiments and coordinated the study, with assistance from TAA who helped with coordination of the field study, supervision of field staff and field experiments. I carried out

	statistical analyses with assistance from TAA. I wrote the manuscript with inputs of co-authors.

Student Signature: [redacted] **Date:** 28/3/2018

Supervisor Signature [redacted] **Date:** 28.3.18

Chapter 7. Effect of *kdr* resistance genotype on oocyst and sporozoite infection rates in *Anopheles gambiae* s.s.

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Abstract

Insecticide resistance mechanisms may affect vector competence through modifications of the environment the pathogens are exposed to within vectors. Parasite survival and transmission may therefore be directly affected by insecticide resistance status of mosquitoes. In this study the association of *kdr* and infection rates in *Anopheles gambiae* s.l. was assessed to explore the effect of insecticide resistance on *Plasmodium falciparum* sporogony and better understand the impact of resistance in the field.

Anopheles gambiae s.l. were collected in a study site in Kyankwanzi District, Uganda, fed on blood samples from 42 *P. falciparum*-infected local patients, then exposed either to nets treated with sub-lethal doses of deltamethrin or to untreated nets. After seven days, surviving mosquitoes were dissected and their midguts examined for oocysts. Prevalence and intensity of infection were recorded for each group. Adult mosquitoes were also collected during entomological surveys in the study area and sporozoite rates were determined.

Oocyst prevalence rates and infection intensity in *An. gambiae* s.s. and *An. arabiensis* were not significantly different between *kdr* genotypes nor did they vary between the two vector species. Due to the predominance of the *Re* allele in *An. gambiae* s.s. we were not able to compare oocyst burdens in resistant and susceptible mosquitoes. Similarly, due to lack of wild type mosquitoes with the homozygous susceptible allele, only *Re/Re* and *Re/Rw*

resistant mosquitoes were found to be sporozoite-positive, but the sporozoite rates were not significantly different between the two genotypes.

Further studies will be needed using mosquitoes collected from areas with moderate *kdr* frequencies to fully understand the impact of the mutation on *Plasmodium* infection and malaria transmission.

7.1 Introduction

Insecticide resistance in malaria vectors is widespread, potentially posing a threat to malaria control and elimination efforts [1, 2, 3, 4]. In particular, most endemic countries in Africa have reported resistance of *Anopheles gambiae* s.l. to pyrethroids that are used in insecticide-treated nets (ITN).

Pyrethroids and DDT target voltage-gated sodium channels (VGSC), causing repetitive nerve discharges, paralysis and death [5]. In *An. gambiae* s.l., three knockdown resistance (*kdr*) mutations have been identified within the VGSC that prevent insecticides from binding, and result in target site resistance: *L1014S* (*Re*), *L1014F* (*Rw*) and *N1575Y* [6, 7, 8]. Laboratory studies show that *kdr* mutations confer a fitness cost in the absence of insecticide pressure [9]. Mosquitoes carrying the *kdr* mutation have a decreased neuronal and behavioural excitability, since *kdr* mutation enhances closed-state inactivation of nerves, and more stimulation is required to make the nerves fire impulses and release acetylcholine [10]. The presence of *kdr* resistance mechanisms also negatively impacts the mating competitiveness of male *An. gambiae* mosquitoes [11].

Mosquitoes with the *kdr* mutation might react differently to pathogen infections than mosquitoes without the mutation [12]. Potential effects of *kdr* on vector competence of *An. gambiae* s.l. to transmit *Plasmodium falciparum*, and the effect of infection in resistant mosquitoes on insecticide susceptibility, have been investigated in several studies but are still not well understood [13, 14, 15, 16, 17]. This could be partly due to the *kdr* allele being a part of an extended haplotype, in which other genes with strong immune function – and not *kdr* itself – might affect infection levels in mosquitoes [18].

In Bioko Island, *An. gambiae* homozygous for *kdr* appeared less likely to transmit malaria as their sporozoite rates were lower than those of heterozygous and homozygous non-*kdr* mosquitoes [19]. On the contrary, a more recent report from Tanzania showed that

significantly higher sporozoite rates were detected in *kdr*-homozygous mosquitoes compared to the those without *kdr* mutation, with heterozygotes showing intermediate sporozoite rates [20]. These studies show interactions between insecticide resistance and *Plasmodium* infection in mosquitoes, although the impact on malaria transmission remains unclear [21].

The aim of this study was to explore whether the presence of the *kdr-L1014S* mutation has an impact on oocyst prevalence and sporozoite rates in wild *An. gambiae* s.s., and to discuss the impact of resistance on effectiveness of ITNs.

7.2 Methods

7.2.1 Study site

Field work was carried out in Butemba, Kyankwanzi District in mid-western Uganda in three rounds: August-October 2013, November-December 2013, and May-June 2014. Larval and adult mosquito collections were carried out in the catchment area of Butemba Health Centre III (N 1^{o} 8' 33.86'', E 31^{o} 36' 8.79''). The study area lies at an altitude of 1,000 - 1,200 m above sea level in a moist savannah zone, with annual rainfall exceeding 1,200mm with two peaks (April-May and September-October). The area is highly malaria endemic with two peaks in transmission in May-July and October-December.

7.2.2 Mosquito collection and rearing

Anopheles gambiae s.l. larvae were collected from breeding sites in villages around Butemba Health Centre III. They were reared in a laboratory at the health centre in water brought from their original breeding sites, at ambient temperature and humidity. Adult mosquitoes were used in direct membrane feeding assays. Additionally, entomological surveys were carried out twice (2013 and 2014) in 18 randomly selected households in Kakifulukwa village, which is within the catchment area of the Butemba Health Centre III. Mosquitoes were collected using CDC light traps (LTC), pyrethrum spray collection method (PSC) and human landing collection (HLC) to gather data on entomological indicators.

Temperature and humidity measurements were recorded in the laboratory at Butemba HC III at 30-minute intervals during all experiments using data loggers (Lascar Electronics).

7.2.3 Direct membrane feeding assays

Blood samples from gametocytaemic volunteers recruited from among the outpatients at Butemba Health Centre III were used in direct membrane feeding assays to test transmission blocking properties of sub-lethal doses of deltamethrin, as previously described [22].

Blood samples were transferred to pre-warmed membrane feeders (Hemotek Membrane Feeding System, Hemotek Ltd, UK), which were held at 37.5^{o}C throughout the feed. On average 40 *An. gambiae* s.s. females were placed in paper containers and allowed to feed through an artificial Parafilm membrane for up to 2 hours.

An hour after the end of the feed, all fully fed mosquitoes were separated from the unfed ones and randomly divided into two groups. Approximately half of the fed mosquitoes were exposed to a net treated with a sub-lethal dose of deltamethrin (2.5-16.7mg/m^2) for 5 minutes using a wire ball frame, while others were exposed to an untreated net as a control. After exposure, mosquitoes were kept in paper containers with access to 10% glucose solution. Mortality of infected mosquitoes was recorded daily.

7.2.4 *Plasmodium* detection in mosquitoes

Midguts of the surviving females were dissected and stained with 0.25% mercurochrome in physiological buffer solution (PBS) and examined for the presence of oocysts on day 7 post infectious blood meal. Oocyst prevalence and the number of oocysts per midgut were recorded.

Enzyme-linked immunosorbent assay (ELISA) was used to detect *P. falciparum* circumsporozoite protein (CSP) and hence sporozoites in all the specimens collected during entomological surveys [23], using the head and upper part of the thorax [24].

7.2.5 Species identification

Mosquitoes were identified to species level using molecular methods. Genomic DNA was extracted from mosquito tissue using Chelex-100 with heat application [25]. Real time polymerase chain reaction (rtPCR) (Stratagene MX 3005P, Agilent Technologies) using TaqMan assays with three probes was used for *An. gambiae s.l.* sibling species identification to distinguish between *An. gambiae* s.s., *An. arabiensis* and other members of the complex [26].

7.2.6 *Kdr* and *ace-1* detection

Genetic resistance against different insecticides in collected mosquitoes was determined with TaqMan molecular assays. Two separate assays were used for the detection of *kdr-L1014F* (*Rw*) or *kdr-L1014S* (*Re*) mutations [27]. A further assay to detect the presence of the *G119S* mutation in the gene *ace-1* which encodes the AChE enzyme was also used [28]. Adult mosquitoes collected during entomological surveys were also tested for the presence of the *N1575Y* mutation using TaqMan rtPCR [8].

7.2.7 Data entry and statistical analysis

Microsoft Excel (Microsoft Corporation), Stata version 14 (StataCorp LP, College Station, Texas 77845, USA), and Prism 7 (GraphPad Software, Inc.) were used for data entry, cleaning and statistical analysis.

To study the effect of *kdr* genotypes on (a) oocyst prevalence rate, and (b) number of oocysts per infected mosquito (oocyst intensity), we used mixed-effects logistic regression for the former and mixed-effects negative binomial regression for the latter [22], using *kdr* as a fixed-effect categorical variable with five levels for *S/S, S/Re, Re/Re, Rw/Rw* and *Re/Rw* genotypes, and gametocyte donor volunteers as a random group variable. Sample sizes for some of the separate deltamethrin doses were inadequate for further analysis, so data from different insecticide doses were pooled into two exposure groups: low dose (2.5–5.0 mg/m^2) and high dose (10.0–16.7 mg/m^2). Deltamethrin dosage group was then entered as a categorical variable with three levels: control (untreated nets), low dose and high dose. A mixed-effects logistic regression model was also used to study the effect of *kdr* genotypes on sporozoite rates, using *kdr* as a fixed-effect categorical variable and study round as a random group variable.

Oocyst prevalence and sporozoite rates in different mosquito genotypes were additionally compared using Fisher's exact test.

To compare the association between resistant *kdr* alleles and *P. falciparum* infection, an odds ratio was calculated based on allelic data.

7.2.8 Ethics

Ethical clearance was obtained from the LSHTM (reference 6454), the Vector Control Division of the Ministry of Health of Uganda (reference VCD-IRC/044), and Uganda National Council of Science and Technology (reference HS 1429).

All adult subjects, including staff who assisted with human landing catches, provided written informed consent, and a parent or guardian of any child participant provided written informed consent on their behalf.

7.3 Results

Following direct membrane feeding assays, 763 of successfully dissected mosquitoes were identified by PCR as *An. gambiae* s.s. and 73 were identified as *An. arabiensis*. *Kdr* genotype frequencies of both species were examined, together with infection prevalence and intensity.

7.3.1 Oocyst prevalence and intensity of infection in different *kdr* genotypes

Kdr genotype of 0.7 % (5/763) of dissected *An. gambiae* s.s. could not be confirmed, so these mosquitoes were excluded from the analysis. *Re/Re* was a predominant genotype (91%), followed by *Re/Rw* (3.8%) and *Rw/Rw* (3.7%), while genotypes with the wild type *S* allele were the least frequent: *S/S* (0.3%), *Re/S* (0.7%). No *ace-1* or *N1575Y* mutations were detected in the samples.

Among *An. arabiensis*, wild type homozygous genotype (*S/S*) was predominant (63/73) (86.3%), followed by heterozygous *Rw/S* (11.0%), while single specimens with *Re/Re* (1.4%) and *Rw/Rw* (1.4%) were also found.

The mixed-effects logistic regression model showed that while insecticide exposure and mean ambient temperature had a significant effect on the risk of infection, *kdr* genotype had no significant effect on oocyst infection rate (Table 7-1).

Table 7-1. Mixed-effects logistic regression analysis of *P. falciparum* oocyst prevalence rates.

		Odds ratio	Std. Err.	Z	*p*	[95% Confidence Interval]	
Dose category	Control	1.000	-	-	-	-	-
	Low dose	0.384	0.120	-3.06	0.002	0.208	0.709
	High dose	0.189	0.047	-6.64	<0.0001	0.115	0.308
Average temp (^{o}C)		0.169	0.069	-4.37	<0.0001	0.076	0.375
Kdr	*S/S*	1	(empty)				
	S/Re	0.767	0.988	-0.21	0.837	0.061	9.574
	Re/Re	1.404	0.728	0.65	0.516	0.506	3.878
	Rw/Rw	1.911	1.405	0.88	0.379	0.452	8.070
	Re/Rw	1	(omitted)				
Variance of random intercept		1.98	0.693			0.997	3.933

Model χ^2_{6df} = 61.20; $p < 0.001$; n = 754; number of groups (gametocyte donors) = 42.

Note: The dependent variable is oocyst infection coded as 0 (negative) and 1 (positive).

Analysed across all three study rounds and insecticide exposures, oocyst prevalence rates were not significantly different between different genotypes of *An. gambiae* s.s. (p = 0.494), nor were they different between genotypes of *An. arabiensis* (p = 0.504), confirming the results of regression models. No significant difference in oocyst prevalence rates between the two mosquito species was observed (Fig. 7-1).

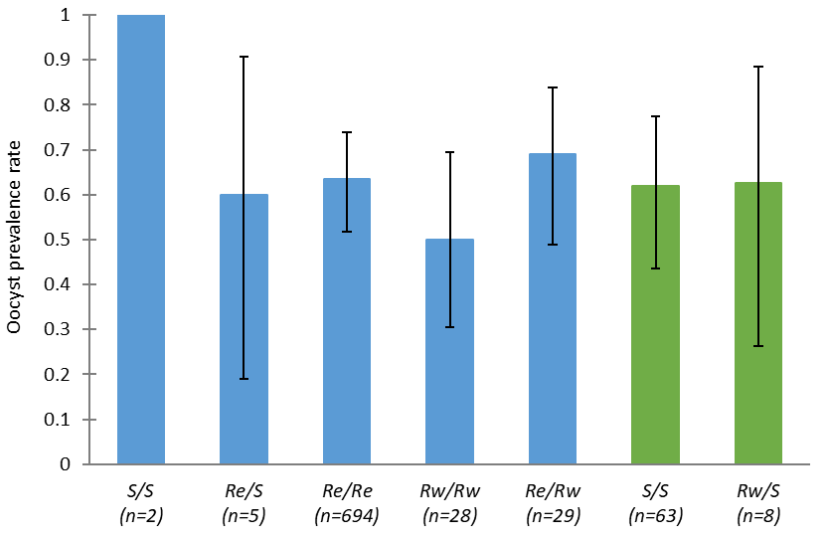

Fig. 7-1. Oocyst prevalence rates in *An. gambiae* s.s. (in blue) and *An. arabiensis* (in green) with different *kdr* genotypes. Mosquitoes were membrane-fed using infectious blood provided by gametocytaemic volunteers, then either exposed to untreated nets or deltamethrin-treated nets. Error bars indicate 95% confidence intervals.

The mixed-effects negative binomial regression model showed that while insecticide exposure and mean ambient temperature had a significant effect on oocyst burden, *kdr* genotype had no significant effect on the number of oocysts per infected mosquito (Table 7-2).

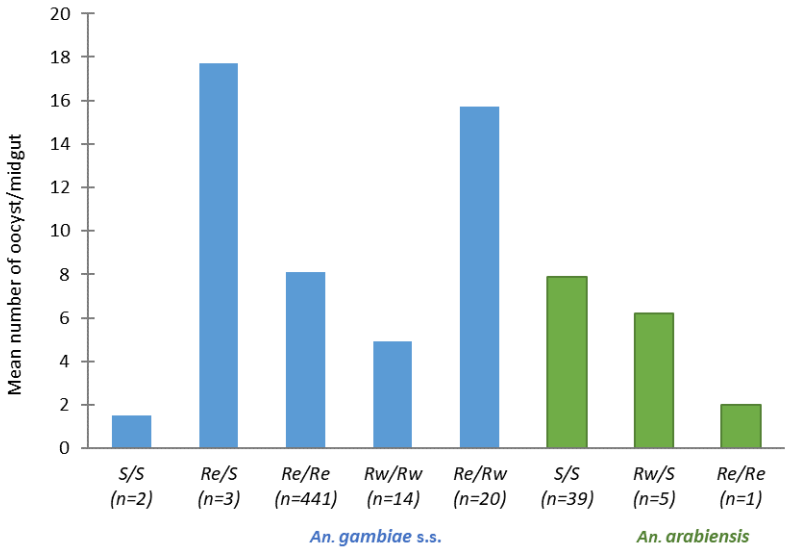

Fig. 7-2. Oocyst intensity (mean number of oocysts/midgut) in *P. falciparum*-positive *An. gambiae* s.s. and *An. arabiensis* with different *kdr* genotypes. Mosquitoes were membrane-fed using infectious blood provided by gametocytaemic volunteers, then either exposed to control or deltamethrin-treated nets.

Table 7-2. Mixed-effects negative binomial regression analysis of *P. falciparum* oocyst intensity.

		Incidence-rate ratio	Std. Err.	Z	*p*	[95% Confidence Interval]	
Dose category	Control	1.000	-	-	-	-	-
	Low dose	0.592	0.073	-4.28	<0.0001	0.465	0.753
	High dose	0.629	0.063	-4.61	<0.0001	0.517	0.767
Average temp (^{o}C)		0.503	0.083	-4.15	<0.0001	0.364	0.696
Prior intake of antimalarials		0.592	0.156	-1.98	0.047	0.353	0.994
Kdr	*S/Re*	3.361	3.123	1.30	0.192	0.544	20.763
	Re/Re	2.296	1.869	1.02	0.307	0.465	11.324
	Rw/Rw	2.045	3.140	0.84	0.403	0.382	10.929
	Re/Rw	3.736	3.140	1.57	0.117	0.719	19.403
Variance of random intercept		0.260	0.093			0.129	0.524

Model χ^2_{8df} = 60.24, $p < 0.001$; n = 456; number of groups (gametocyte donors) = 40.

Note: The dependent variable is number of oocysts.

Data showing mean numbers of oocysts in *P. falciparum*-positive *An. gambiae* s.s. and *An. arabiensis* are plotted for illustration purpose only (Fig. 7-2). As *Re* allele is fixed in the area, sample size for majority of the genotypes is too small for meaningful comparisons.

The association between different *kdr* alleles and *P. falciparum* infection was further explored. An odds ratio of 1.825 indicated that *P. falciparum* infection was more likely to occur when *Re* allele is present compared to *Rw* allele but the result was not significant (p = 0.0774).

7.3.2 Sporozoite rates in different *kdr* genotypes of *An. gambiae* s.s.

Two hundred and fifty-two mosquitoes were collected in the entomological surveys; 97 % (n = 245) were *An. gambiae* s.s., 0.8% (n= 2) were *An. arabiensis,* whereas 1.2% (n = 3) could not be identified to species. CSP ELISA was carried out on all mosquitoes.

All positive mosquitoes were *An. gambiae* s.s. Sporozoite rates in 2014 (13.8%) were higher than in 2013 (5.7%); however, the difference between the years was not statistically significant (p = 0.069).

Only *Re/Re* and *Re/Rw* mosquitoes were positive. Although sporozoite rates were higher in the *Re/Rw* genotype in both years they were not significantly different from those in *Re/Re* mosquitoes (2013: p = 0.307; 2014: p = 0.710); differences within genotypes between the years are also not significant (*Re/Re*: p = 0.06; *Re/Rw*: p = 1.00).

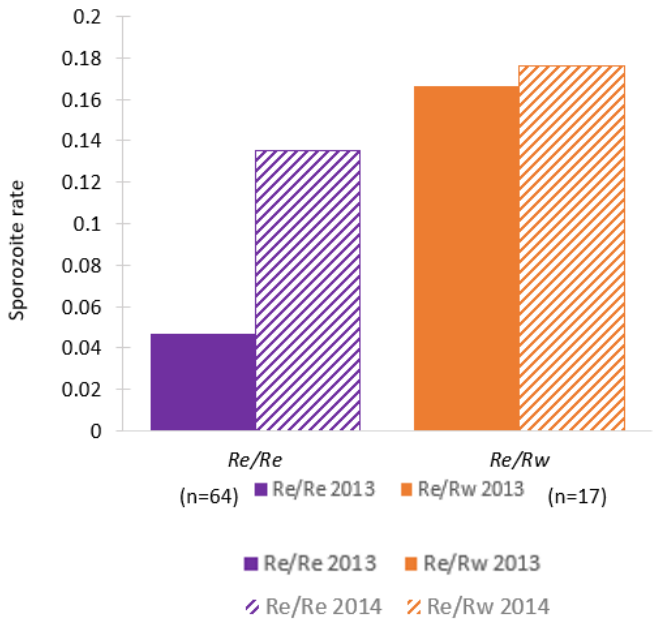

Fig. 7-3. Sporozoite rates in *Re/Re* and *Re/Rw* genotypes of *An. gambiae* s.s. in 2013 and 2014. Sporozoite rates between the years and between the genotypes were not significantly different (p > 0.05).

The mixed-effects logistic regression model confirmed that *kdr* genotype had no significant effect on sporozoite rates (Table 7-3).

Table 7-3. Mixed-effects logistic regression analysis of *P. falciparum* sporozoite rates in mosquitoes collected during entomological surveys.

		Odds ratio	Std. Err.	Z	*p*	[95% Confidence Interval]	
Kdr	*S/S*	1	-	-	-	-	-
	S/Re	1	(empty)				
	Re/Re	0.589	0.349	-0.89	0.371	0.184	1.882
	Rw/Rw	1	(empty)				
	Re/Rw	1	(omitted)				
Variance of random intercept		0.067	0.212			0.0001	32.292

Model χ^2_{1df} = 0.80; *p* < 0.3714; *n* = 242; number of groups (rounds of entomological survey) = 2.

Note: The dependent variable is sporozoite infection coded as 0 (negative) and 1 (positive).

7.4 Discussion

In this study, the effects of *kdr* resistance genotype on oocyst and sporozoite infection rates in *An. gambiae* s.s. were examined. All three *L1014-kdr* alleles were found at the study site in both sibling species. While *Re/Re* was the predominant genotype among *An. gambiae* s.s., with the *Re* allele practically reaching fixation and reducing the frequency of other alleles, *An. arabiensis* were mainly wild susceptible type (*S/S*). Oocyst prevalence rates in *An. gambiae* s.s. and *An. arabiensis* were not significantly different between *kdr* genotypes, nor did they vary between the two species. Similarly, infection intensity in *An. gambiae* s.s. was not significantly different between genotypes, and the same outcome was seen in *An. arabiensis*. Furthermore, sporozoite rates in *An. gambiae* s.s. were also not significantly different between *kdr* genotypes.

The association between insecticide resistance and disease transmission, and practical implications of resistance for malaria control have remained only partially understood despite recognition of the problem since at least the 1960s [29]. Resistant mosquitoes

survive longer than susceptible ones following exposure to insecticides and may be able to infect new hosts, maintaining the transmission cycle and leading to the reduced efficacy of vector control measures [29, 30, 31]. However, there is one widely accepted example of metabolic-based pyrethroid resistance which led to control failure and resurgence in malaria cases [30, 32]. In a systematic review of the cases of malaria resurgence, only 19% were attributed to insecticide resistance and in all of these, other factors such as resource constraints or war might have played a significant role [33]. Moreover, a meta-analysis showed that ITNs are still more effective than untreated nets, regardless of insecticide resistance [34]. Modelling studies on the other hand show that recent gains in malaria control may be jeopardized due to insecticide resistance [4, 35, 36]. Results of a recent trial in Tanzania suggest that ITNs co-treated with pyrethroids and a synergist piperonyl butoxide (PBO) may have additional public health value when compared to pyrethroid-only long-lasting insecticidal nets (LLINs) in areas with pyrethroid resistance [37, 38]. Findings showed that areas with PBO nets had significantly lower malaria infection prevalence than areas with standard LLINs, which indirectly indicates the impact of resistance.

Apart from the protection insecticide resistance offers to insecticide-exposed mosquitoes, other factors which can affect transmission should be considered [36, 39]. Several potential effects of insecticide resistance mechanisms on disease transmission have been described [40]. *Kdr* is a target site resistance mechanism providing protection against pyrethroids and DDT, and potential interactions between *Plasmodium* infection and target site resistance mechanisms have previously been described, at times with contradictory findings [13, 14, 15, 16, 17].

Studies with wild mosquitoes and parasites and natural mosquito-parasite combinations are more realistic in their outcome than studies using laboratory-adapted strains [41, 42], but suffer from factors which are beyond our control, such as lack of susceptible wild type mosquitoes, predominance of one genotype and the possible presence of mixed resistance mechanisms in mosquitoes. These factors can affect the strength of the study conclusions and have to be taken into account as potential confounders [22]. Sample size directly affects the power of a study to detect differences between groups. Due to the lack of wild type homozygotes (*S/S*), the predominance of *Re* allele and *Re/Re* genotype, we were unable to compare infection rates in resistant and susceptible genotypes and draw definitive conclusions. Although Alout *et al* observed that a *kdr* resistant strain is more susceptible to

infection, with higher parasite prevalence rates, the difference was less pronounced at the sporozoite stage, while parasite burden was lower in resistant mosquitoes [15].

Other field studies similarly showed no conclusive results, with sporozoite rates in *kdr*-homozygous mosquitoes being either lower than in susceptible wild type and heterozygotes [19], or on the contrary higher in *kdr*-homozygotes than in other mosquitoes, demonstrating a significant association between *Re/Re* genotype and *P. falciparum* infection [20]. We collected adult mosquitoes during entomological surveys, but only mosquitoes of *Re/Re* and *Re/Rw* genotypes were sporozoite-positive. Although sporozoite rates were higher in the *Re/Rw* genotype in both survey rounds they did not significantly differ between the genotypes. However, the number of *Re/Re* mosquitoes caught was much higher than those of *Re/Rw* genotype, again affecting the power to detect any significant differences. Sporozoite rates were higher in June 2014 than September 2013, which is not completely unexpected since May, June and July are peak malaria transmission months, with the highest number of confirmed malaria cases seen at the health facility at the study site. Sporozoite rates previously recorded in the area were much lower than those we detected [43]. This could be due to the timing of our entomological surveys, during which older mosquitoes were caught that were more likely to harbour sporozoites. Similarly, sporozoite rate might be higher in a certain genotype simply because the small sample caught (eg. *Re/Rw*) contained older females, whereas the larger *Re/Re* sample contained mosquitoes of different ages.

Malaria transmission is a result of complex interactions between vectors, parasites and vertebrate hosts, the environment, and numerous other factors – including insecticide resistance mechanisms and the presence of insecticides [36, 39]. Sublethal doses of pyrethroids have been shown to affect resistant vectors [36, 44], and parasites developing in them [22, 44, 45, 46, 47]. The internal mosquito environment can change when vectors become resistant to insecticides and this may affect the parasites developing in them [40, 48, 49, 50, 51, 52, 53]. However, to what extent insecticide resistance affects mosquito biological traits and vector competence remains largely unknown. Moreover, pathogen infection of vectors induces physiological changes, triggers immune reactions and is thought to reduce vector fitness, which could in turn increase vector susceptibility to environmental stress factors, including insecticides [54]. Exposure of mosquitoes to parasites and to insecticides could result in changes in expression of detoxification enzymes leading to a

trade-off between the necessary activation of the immune system in response to infection and the elimination of insecticides, increasing sensitivity to insecticides [16, 55, 56].

In summary, oocyst prevalence rates and infection intensity in *An. gambiae* s.s. and *An. arabiensis* were not significantly different between *kdr* genotypes, nor did they vary between the two species. Due to predominance of *Re* allele in *An. gambiae* s.s. we were not able to compare oocyst burdens in resistant and susceptible mosquitoes. Similarly, due to lack of wild type mosquitoes, only *Re/Re* and *Re/Rw* resistant mosquitoes were found to be sporozoite-positive, but the sporozoite rates were not significantly different between the two genotypes.

Further studies will be needed using mosquitoes collected from areas with moderate levels of *kdr* resistance gene frequencies to determine the impact of the mutation on *Plasmodium* infection and malaria transmission.

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7.5 References

1. Ranson H, Lissenden N. Insecticide Resistance in African *Anopheles* Mosquitoes: A worsening situation that needs urgent action to maintain malaria control. Trends Parasitol. 2016;32:187-196.

2. 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;27:91-98.

3. World Health Organization. World Malaria Report 2017. Geneva: World Health Organization; 2017.

4. 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.

5. Soderlund DM, Bloomquist JR. Neurotoxic actions of pyrethroid insecticides. Annu Rev Entomol. 1989;34:77-96.

6. Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Berge JB, Devonshire AL*, et al*. Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae* s.s. Insect Mol Biol. 1998;7:179-184.

7. Ranson H, Jensen B, Vulule JM, Wang X, Hemingway J, Collins FH. Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. Insect Mol Biol. 2000;9:491-497.

8. Jones CM, Liyanapathirana M, Agossa FR, Weetman D, Ranson H, Donnelly MJ*, et al*. Footprints of positive selection associated with a mutation (*N1575Y*) in the voltage-gated sodium channel of *Anopheles gambiae*. Proc Natl Acad Sci U S A. 2012;109:6614-6619.

9. Chen L, Zhong D, Zhang D, Shi L, Zhou G, Gong M*, et al*. Molecular ecology of pyrethroid knockdown resistance in *Culex pipiens pallens* mosquitoes. PLoS One. 2010;5:e11681.

10. Diop MM, Moiroux N, Chandre F, Martin-Herrou H, Milesi P, Boussari O*, et al*. Behavioral cost and overdominance in *Anopheles gambiae*. PLoS One. 2015;10:e0121755.

11. Platt N, Kwiatkowska RM, Irving H, Diabate A, Dabire R, Wondji CS. Target-site resistance mutations (*kdr* and *RDL*), but not metabolic resistance, negatively impact male mating competiveness in the malaria vector *Anopheles gambiae*. Heredity (Edinb). 2015;115:243-252.

12. Howard AF, Koenraadt CJ, Farenhorst M, Knols BG, Takken W. Pyrethroid resistance in *Anopheles gambiae* leads to increased susceptibility to the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*. Malar J. 2010;9:168.

13. Alout H, Dabire RK, Djogbenou LS, Abate L, Corbel V, Chandre F*, et al*. Interactive cost of *Plasmodium* infection and insecticide resistance in the malaria vector *Anopheles gambiae*. Sci Rep. 2016;6:29755.

14. Alout H, Djegbe I, Chandre F, Djogbenou LS, Dabire RK, Corbel V*, et al*. Insecticide exposure impacts vector-parasite interactions in insecticide-resistant malaria vectors. Proc Biol Sci. 2014;281.

15. Alout H, Ndam NT, Sandeu MM, Djegbe I, Chandre F, Dabire RK*, et al*. Insecticide resistance alleles affect vector competence of *Anopheles gambiae* s.s. for *Plasmodium falciparum* field isolates. PLoS One. 2013;8:e63849.

16. Alout H, Yameogo B, Djogbenou LS, Chandre F, Dabire RK, Corbel V*, et al*. Interplay between *Plasmodium* infection and resistance to insecticides in vector mosquitoes. J Infect Dis. 2014;210:1464-1470.

17. Ndiath MO, Cailleau A, Diedhiou SM, Gaye A, Boudin C, Richard V*, et al*. Effects of the *kdr* resistance mutation on the susceptibility of wild *Anopheles gambiae* populations to *Plasmodium falciparum*: a hindrance for vector control. Malar J. 2014;13:340.

18. Mitri C, Markianos K, Guelbeogo WM, Bischoff E, Gneme A, Eiglmeier K*, et al*. The *kdr*-bearing haplotype and susceptibility to *Plasmodium falciparum* in *Anopheles gambiae*: genetic correlation and functional testing. Malar J. 2015;14:391.

19. 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 U S A. 2013;110:9397-9402.

20. Kabula B, Tungu P, Rippon EJ, Steen K, Kisinza W, Magesa S*, et al*. A significant association between deltamethrin resistance, *Plasmodium falciparum* infection and the

Vgsc-1014S resistance mutation in *Anopheles gambiae* highlights the epidemiological importance of resistance markers. Malar J. 2016;15:1-5.

21. World Health Organization: Implications of insecticide resistance for malaria vector control. Geneva: World Health Organization; 2016: 4.

22. Kristan M, Lines J, Nuwa A, Ntege C, Meek SR, Abeku TA. Exposure to deltamethrin affects development of *Plasmodium falciparum* inside wild pyrethroid resistant *Anopheles gambiae* s.s. mosquitoes in Uganda. Parasit Vectors. 2016;9:100.

23. Wirtz RA, Zavala F, Charoenvit Y, Campbell GH, Burkot TR, Schneider I*, et al*. Comparative testing of monoclonal antibodies against *Plasmodium falciparum* sporozoites for ELISA development. Bull World Health Organ. 1987;65:39-45.

24. Foley DH, Harrison G, Murphy JR, Dowler M, Rueda LM, Wilkerson RC. Mosquito bisection as a variable in estimates of PCR-derived malaria sporozoite rates. Malar J. 2012;11:145.

25. Lardeux F, Tejerina R, Aliaga C, Ursic-Bedoya R, Lowenberger C, Chavez T. Optimization of a semi-nested multiplex PCR to identify *Plasmodium* parasites in wild-caught *Anopheles* in Bolivia, and its application to field epidemiological studies. Trans R Soc Trop Med Hyg. 2008;102:485-492.

26. Bass C, Williamson MS, Field LM. Development of a multiplex real-time PCR assay for identification of members of the *Anopheles gambiae* species complex. Acta Trop. 2008;107:50-53.

27. Bass C, Nikou D, Donnelly MJ, Williamson MS, Ranson H, Ball A*, et al*. Detection of knockdown resistance (*kdr*) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. Malar J. 2007;6:111.

28. Bass C, Nikou D, Vontas J, Williamson MS, Field LM. Development of high-throughput real-time PCR assays for the identification of insensitive acetylcholinesterase (*ace-1*^{R}) in *Anopheles gambiae*. Pestic Biochem Phys. 2010;96:80-85.

29. Busvine JR, Pal R. The impact of insecticide-resistance on control of vectors and vector-borne diseases. Bull World Health Organ. 1969;40:731-744.

30. 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;14:181-189.

31. N'Guessan R, Corbel V, Akogbeto M, Rowland M. Reduced efficacy of insecticide-treated nets and indoor residual spraying for malaria control in pyrethroid resistance area, Benin. Emerg Infect Dis. 2007;13:199-206.

32. Maharaj R, Mthembu DJ, Sharp BL. Impact of DDT re-introduction on malaria transmission in KwaZulu-Natal. S Afr Med J. 2005;95:871-874.

33. Cohen JM, Smith DL, Cotter C, Ward A, Yamey G, Sabot OJ*, et al*. Malaria resurgence: a systematic review and assessment of its causes. Malar J. 2012;11:122.

34. 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 Med. 2014;11:e1001619.

35. World Health Organization. Global Plan for Insecticide Resistance Management in Malaria Vectors. Geneva: World Health Organization; 2012.

36. Glunt KD, Coetzee M, Huijben S, Koffi AA, Lynch PA, N'Guessan R*, et al*. Empirical and theoretical investigation into the potential impacts of insecticide resistance on the effectiveness of insecticide-treated bed nets. Evol Appl. 2017;11:431-441.

37. World Health Organization: Conditions for deployment of mosquito nets treated with a pyrethroid and piperonyl butoxide. Geneva: World Health Organization; 2017: 5.

38. Protopopoff N, Mosha JF, Lukole E, Charlwood JD, Wright A, Mwalimu CD*, et al*. 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 factorial design trial. Lancet. 2018;391:1577-1588.

39. Thomas MB, Read AF. The threat (or not) of insecticide resistance for malaria control. Proc Natl Acad Sci U S A. 2016;113:8900-8902.

40. Rivero A, Vezilier J, Weill M, Read AF, Gandon S. Insecticide control of vector-borne diseases: when is insecticide resistance a problem? PLoS Pathog. 2010;6.

41. Aguilar R, Dong Y, Warr E, Dimopoulos G. *Anopheles* infection responses; laboratory models versus field malaria transmission systems. Acta Trop. 2005;95:285-291.

42. Cohuet A, Osta MA, Morlais I, Awono-Ambene PH, Michel K, Simard F*, et al*. *Anopheles* and *Plasmodium*: from laboratory models to natural systems in the field. EMBO Rep. 2006;7:1285-1289.

43. Helinski M, Nuwa A, Protopopoff N, Feldman M, Ojuka P, Oguttu DW*, et al*. Entomological surveillance following a long-lasting insecticidal net universal coverage campaign in Midwestern Uganda. Parasit Vectors. 2015;8:458.

44. Hill N. Effects of sublethal doses of pyrethroids on malaria vectors. *PhD Thesis.* London: London School of Hygiene & Tropical Medicine, University of London; 2002.

45. Carle PR, Coz J, Elissa N, Gasquet M, Sannier C, Richard A*, et al*. [Intravector anti-*Plasmodium* activity of a pyrethrinoid: deltamethrin]. C R Acad Sci III. 1986;303:565-568.

46. Elissa N, Sannier C, Coz J. [Étude de la réceptivité à *Plasmodium yoelii yoelii* d'*Anopheles stephensi* soumis au stade adulte à des doses toxiques de différents pyréthrinoïdes]. Cah ORSTOM, ser Ent méd et Parasitol. 1987;25:187-192.

47. Elissa N, Sannier C, Richard A: [La Receptivite a *Plasmodium yoelii yoelii* d'*Anopheles* Soumis a des Subletales de Deltamethrine]. In: *IVe Congres Sur La Protection De La Sante Humaine Et Des Cultures En Milieu Tropical*. Marseille, France; 1986: 599-604.

48. Vontas J, Blass C, Koutsos AC, David JP, Kafatos FC, Louis C*, et al*. Gene expression in insecticide resistant and susceptible *Anopheles gambiae* strains constitutively or after insecticide exposure. Insect Mol Biol. 2005;14:509-521.

49. Vontas J, David JP, Nikou D, Hemingway J, Christophides GK, Louis C*, et al*. Transcriptional analysis of insecticide resistance in *Anopheles stephensi* using cross-species microarray hybridization. Insect Mol Biol. 2007;16:315-324.

50. Reid WR, Zhang L, Gong Y, Li T, Liu N. Gene expression profiles of the Southern house mosquito *Culex quinquefasciatus* during exposure to permethrin. Insect Sci. 2017.

51. Gong Y, Li T, Zhang L, Gao X, Liu N. Permethrin induction of multiple cytochrome P450 genes in insecticide resistant mosquitoes, *Culex quinquefasciatus*. Int J Biol Sci. 2013;9:863-871.

52. Yang T, Liu N. Genome analysis of cytochrome P450s and their expression profiles in insecticide resistant mosquitoes, *Culex quinquefasciatus*. PLoS One. 2011;6:e29418.

53. McCarroll L, Hemingway J. Can insecticide resistance status affect parasite transmission in mosquitoes? Insect Biochem Mol Biol. 2002;32:1345-1351.

54. Alout H, Labbe P, Chandre F, Cohuet A. Malaria vector control still matters despite insecticide resistance. Trends Parasitol. 2017.

55. Farenhorst M, Mouatcho JC, Kikankie CK, Brooke BD, Hunt RH, Thomas MB*, et al*. Fungal infection counters insecticide resistance in African malaria mosquitoes. Proc Natl Acad Sci U S A. 2009;106:17443-17447.

56. Koella JC, Saddler A, Karacs TP. Blocking the evolution of insecticide-resistant malaria vectors with a microsporidian. Evol Appl. 2012;5:283-292.

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SECTION A – Student Details

Student	Mojca Kristan
Principal Supervisor	Jo Lines
Thesis Title	Effects of pyrethroid exposure and insecticide resistance on the sporogonic development of Plasmodium falciparum in Anopheles gambiae s.l.

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

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Where is the work intended to be published?	Malaria Journal
Please list the paper's authors in the intended authorship order:	M. Kristan, T. A. Abeku, A. Nuwa, C. Ntege, J. Lines
Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I designed and planned the study with TAA, with contributions of JL. I trained the field staff with TAA and CN. I carried out all field experiments and coordinated the study. TAA, CN and AN helped with coordination of the field study. TAA supervised field experiments. CN supervised

	field staff. TAA carried out the statistical analyses with my inputs. I and TAA wrote the manuscript. JL, AN and CN reviewed the manuscript and provided comments.

Student Signature: [redacted] **Date:** 28/3/2018

Supervisor Signature: [redacted] **Date:** 28.3.18

Chapter 8. Variations in phenotypic and genetic resistance against commonly used insecticides among *An. gambiae* s.s. and *An. arabiensis* in mid-western Uganda

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Abstract

Insecticide resistance in malaria vectors is usually monitored by using the World Health Organization (WHO) susceptibility tests. These tests are used to detect the presence and assess the strength of insecticide resistance phenotypes in a vector population. Molecular tools are also used to detect mutations such as *kdr*, but they do not automatically indicate the presence of resistance and are most commonly used in addition to the WHO susceptibility tests. However, both are useful as the presence of *kdr* genotype might explain only a portion of variation in resistance due to the role additional resistance mechanisms may play. This study examined *kdr*-associated insecticide resistance in *Anopheles gambiae* s.s. and *An. arabiensis* in a site located in Kyankwanzi District, mid-western Uganda, and explored the relationship between bioassay test survival and genotype frequency at the *kdr* locus.

Anopheles gambiae s.l. were collected either as larvae and reared to adults, or as adults during entomological surveys. Adult female mosquitoes were used in the WHO susceptibility tests with discriminating doses of DDT, deltamethrin, permethrin, lambda-cyhalothrin, bendiocarb and pirimiphos-methyl.

Both *kdr* mutations, *L1014S (Re)* and *L1014F (Rw)* were found in the two sibling species, but the frequencies of *Re* and *Rw* resistant alleles were significantly higher in *An. gambiae* s.s. than in *An. arabiensis.* The *Re* mutation was present at high frequencies in *An. gambiae* s.s., approaching fixation, and a double resistant heterozygote genotype (*Re/Rw*) was also detected. In *An. arabiensis* the wild type *S* allele remained dominant. Results of WHO susceptibility tests indicated presence of resistance to pyrethroids and DDT in *An. gambiae* s.l. However, bioassay mortality was only weakly associated with *kdr* genotype in both sibling species, implying a possible role of other metabolic resistance mechanisms.

The findings of this study add some further information to the wider picture on insecticide resistance in Uganda and show that *kdr* resistance in the two sibling vector species, *An. gambiae* s.s. and *An. arabiensis*, is significantly different. Continued entomological surveillance, including phenotypic and genetic resistance profiling of local vector populations, will be essential for resistance management and implementation of evidence-based control strategies.

8.1 Introduction

Substantial reductions in malaria incidence and prevalence have followed a massive scale-up in the access and use of vector control interventions in sub-Saharan Africa since 2000, and especially since 2010 [1, 2, 3]. Vector control is primarily based on the use of synthetically produced insecticides. Insecticide-treated nets (ITNs) and indoor residual spraying (IRS) are the two most commonly used methods of malaria prevention and control. Whereas four classes of insecticides (organochlorines, organophosphates, carbamates and pyrethroids) can be used for IRS, pyrethroids are currently used on all ITNs, due to their low mammalian toxicity and rapid insecticidal activity [4, 5]. As a response to increasing pyrethroid resistance, a new generation of ITNs treated with a combination of a pyrethroid alpha-cypermethrin and chlorfenapyr, a pyrrole, and ITNs treated with a combination of pyrethroids and a synergist piperonyl butoxide (PBO) are now available [6].

Insecticide resistance has become widespread and globally, 61 out of 76 malaria-endemic countries providing data to World Health Organization (WHO) for the period 2010 – 2016, reported resistance to at least one insecticide, with pyrethroid resistance the most commonly reported [3]. The distribution and intensity of pyrethroid resistance has increased significantly in recent years, as has the number of reports of resistance to other insecticide

classes [7, 8]. Increases in resistance have been attributed mainly to selection pressure caused by the scale up of vector control interventions [9, 10] but also by the use of related insecticides in agriculture [11].

Resistance is caused by a number of mechanisms [10, 12]. One of these mechanisms is target-site knockdown resistance (*kdr*), which is closely associated with pyrethroid and DDT resistance in the major malaria vector *An. gambiae* s.l. These insecticides target voltage-gated sodium channels (VGSC), where *kdr* mutations can occur and impair insecticide binding [13]. Two mutations in the VGSC have been associated with *kdr:* the West African mutation *L1014F* or *Rw* [14], and the East African mutation *L1014S* or *Re* [15]. The degree of resistance caused by *kdr L1014F* or *L1014S* was found to vary with insecticide treatment, but *L1014F* is thought to provide more protection against pyrethroids [16], especially when paired with *L1014S* in heterozygote form (*Re/Rw*) [17]. An additional asparagine-to-tyrosine mutation, *N1575Y*, within VGSC has been identified more recently, occurring on a single haplotype also bearing *L1014F* mutation [18].

Determination of resistance in vector populations is usually assessed by WHO susceptibility tests using discriminating concentrations of insecticides which are used to discriminate between susceptible and resistant phenotypes in a given mosquito population [19]. Molecular tools which detect mutations such as the genotype at the *kdr* locus do not automatically indicate the presence of resistance, i.e. the resistance phenotype, as measured by the WHO susceptibility tests, and are most commonly used in addition to those tests [20]. There is a strong causal relationship between *kdr* genotype and pyrethroid/DDT resistance, but the presence of *kdr* genotype might explain only a portion of variation in resistance due to the role additional resistance mechanisms may play [21]. Other mechanisms, such as increased production of detoxification enzymes causing metabolic resistance (eg. cytochrome P450-dependent monooxygenases or CYP450s), might be needed to produce a measurable resistance phenotype that might be affecting control interventions [22].

Insecticide resistance is widespread in Uganda in the main malaria vectors (*An. gambiae* s.s., *An. arabiensis,* and *An. funestus*). Decreasing susceptibility to pyrethroids and DDT in *An. gambiae* s.l. and increasingly in *An. funestus* have been observed over the years in different parts of the country. DDT resistance is common and has been reported in the central and eastern parts [23, 24, 25], but also in southwestern Uganda [26]. Resistance to permethrin and deltamethrin is found throughout the country [23, 24, 25, 27, 28, 29, 30, 31]. *Kdr-L1014S* allele frequency varies from moderate to near fixation in *An. gambiae* s.s. populations, while

kdr-L1014F mutation is also present but at much lower frequency. *Kdr-L1014S* and *L1014F* alleles are present at lower frequencies in some *An. arabiensis* populations, but in many locations, these genes are still absent or at very low frequency. Results of the WHO tests indicate that other resistance mechanisms such as metabolic resistance based on CYP450s are involved in conferring resistance [23, 25, 31, 32, 33].

Our aim was to study the *kdr*-associated resistance in the two sibling vector species, *An. gambiae* s.s. and *An. arabiensis,* in Kyankwanzi in mid-western Uganda, and explore associations between bioassay mortality and genetic resistance.

8.2 Methods

8.2.1 Study site

Field work was carried out in Butemba, Kyankwanzi District in mid-western Uganda in three rounds: August-October 2013, November-December 2013, and May-June 2014. Larval and adult mosquito collections were carried out in the catchment area of Butemba Health Centre III (N 1° 8′ 33.86″, E 31° 36′ 8.79″). The study area lies at an altitude of 1,000 - 1,200 m above sea level in a moist savannah zone, with annual rainfall exceeding 1,200 mm with two peaks (April-May and September-October). The area is highly endemic with two peaks in malaria transmission in May-July and October-December.

8.2.2 Mosquito collection

Anopheles gambiae s.l. larvae were collected from breeding sites in villages around Butemba Health Centre III during all three rounds of field work (August - October 2013, November - December 2013, May - June 2014), and reared to adults in the laboratory in water brought from their original breeding sites, at ambient temperature and humidity. Emerged adult mosquitoes were used in transmission studies [32] and for WHO susceptibility tests. All emerging adult mosquitoes were used for species identification and *kdr* resistance profiling.

Additionally, entomological surveys were carried out twice (August-September 2013 and June 2014) in 18 randomly selected households in Kakifulukwa village, within the catchment area of Butemba Health Centre III. Mosquitoes were collected using CDC light traps (LTC), pyrethrum spray collection method (PSC) and human landing collection (HLC) for species identification, resistance profiling and to gather data on entomological indicators.

Temperature and humidity measurements were recorded in the laboratory at the health centre at 30-minute intervals during all experiments using data loggers (Lascar Electronics).

All collected mosquitoes were stored dry on silica gel for further processing, which was carried out at the Pan-African Malaria Vector Research Consortium (PAMVERC) laboratory in Moshi, Tanzania, and at LSHTM.

8.2.3 WHO susceptibility tests

WHO susceptibility tests were conducted according to WHO guidelines [34]. The following insecticides with discriminating concentrations were used: deltamethrin (0.05%), DDT (4%), bendiocarb (0.1%), pirimiphos-methyl (0.25%), permethrin (0.75%), and lambda-cyhalothrin (0.05%).

8.2.4 Species identification

Mosquitoes were identified to species level using molecular methods. Genomic DNA was extracted from mosquito tissue using Chelex-100 with heat application [35]. Real-time polymerase chain reaction (rtPCR) (Stratagene MX 3005P, Agilent Technologies, with MxPro-Mx3005P v4.00 Build 367 software) using TaqMan assays with three probes was used for *Anopheles* sibling species identification to distinguish between *An. gambiae* s.s., *An. arabiensis* and other members of the complex [36].

8.2.5 *Kdr* and *Ace1* detection

The resistance genotype of collected mosquitoes was determined using molecular methods. Separate TaqMan assays were used for the detection of *kdr-L1014F* and *kdr-L1014S* mutations [37] and the wild-type allele. A further assay was used to detect the presence of *G119S* mutation in the gene *ace-1* which encodes the acetycholinesterase (AChE) enzyme [38] (Stratagene MX 3005P, Agilent Technologies, with MxPro-Mx3005P v4.00 Build 367 software). Adult mosquitoes collected during entomological surveys were also tested for the presence of the *N1575Y* mutation [18].

8.2.6 Data entry and statistical analysis

Microsoft Excel (Microsoft Corporation) was used for data entry. Excel, Stata version 14 (StataCorp LP, College Station, Texas 77845, USA), SPSS version 24 (IBM Corporation) and Prism 7 (GraphPad Software, Inc.) were used for data cleaning and statistical analysis.

Following the WHO susceptibility test procedures, mortality was calculated as the percentage of mosquitoes that died within 24 hours of exposure. If the control mortality was between 5-20%, the observed mortality was corrected using Abbott's formula [19]. Levels of resistance were classified according to WHO guidelines [19]: mortality in the range of 98-100% indicates susceptibility; mortality between 90% and 97% is suggestive of the existence of resistance requiring confirmation by additional tests; mortality <90% confirms the existence of resistance in the population.

Genotype and allele frequencies for the *kdr* locus were calculated using GENEPOP software (M. Raymond & F. Rousset, Laboratiore de Genetique et Environment, Montpellier, France) [39] (version 4.2.), Option 5. For analysis of changes in the frequency of the three *kdr* alleles (*S*, *Re* and *Rw*) between the three study rounds and between the two sibling species, mosquitoes collected during each round were treated as a separate subpopulation. For each of these subpopulations, genotype frequencies were compared to Hardy-Weinberg expected frequencies using GENEPOP, Option 1 (Hardy-Weinberg Exact Tests), Sub-option 3 (Probability test). Expected genotype frequencies were obtained using GENEPOP, Option 5, Sub-option 1 (Basic information). For populations not in Hardy-Weinberg equilibrium, tests for heterozygote deficiency and excess were carried out (Option 1, Sub-options 1 and 2, respectively). GENEPOP (Option 1, Sub-option 3) was also used to estimate Wright's inbreeding coefficient (F_{IS}) [40]. Because of multiple tests, Bonferroni correction was applied to adjust the level of significance accordingly. For the overall population estimates of Wright's inbreeding coefficient (F_{IS}) and the fixation index (F_{ST}) GENEPOP (Option 6, Sub-option 1) was used.

Relative frequencies of sibling species were compared using Pearson's Chi-squared with Bonferroni correction. Logistic regression was used to check for a trend over time in the relative proportions of the sibling species present and in *kdr* allele frequencies between the three collection rounds. Mortalities were compared using Chi-square tests and when values were small Fisher's exact tests.

8.3 Results

8.3.1 Vector composition

A total of 2,496 *An. gambiae* complex mosquitoes were processed for species identification and insecticide resistance profiling.

Relative frequencies of the two sibling species were significantly different during all three collection periods ($p < 0.0001$) (Table 8-1).

Table 8-1. Species composition of *An. gambiae* s.l. mosquitoes collected during three rounds of field work.

		Collection period		
	Total n	Aug-Oct 2013 % (n)	Nov-Dec 2013 % (n)	May-Jun 2014 % (n)
An. gambiae s.s.	2,131	95.44 (941)	80.89 (508)	77.32 (682)
An. arabiensis	267	2.94 (29)	10.99 (69)	19.16 (169)
Unidentified	98	1.62 (16)	8.12 (51)	3.51 (31)

Almost four percent of all processed mosquitoes could not be identified to species level, despite morphological identification as *An. gambiae* s.l. Results of rtPCR repeatedly showed 2.7% of them belonging to the "other *Anopheles*" group, which is meant to specifically amplify DNA of *An. quadriannulatus/An. merus/An. melas*; the rest did not produce any results.

Anopheles gambiae s.s. was the predominant species. The proportion of *An. gambiae* s.s., among those identified to species, significantly decreased between collection rounds, while the proportion of *An. arabiensis* significantly increased from one round to the next (LR χ^2 = 143.78, $p < 0.0001$) with a significant increase in odds that a mosquito will be *An. arabiensis* from round 1 to round 3 (OR = 4.41 for round 2 vs round 1, $p < 0.0001$; OR = 8.04 for round 3 vs round 1, $p < 0.0001$). These changes were detected in adults reared from collected larvae but were not observed in adults collected during entomological surveys.

8.3.2 *Kdr* genotype frequencies

Kdr genotypes were successfully determined for 2,098 *An. gambiae* s.s. and 267 *An. arabiensis* mosquitoes.

L1014S (*Re*) and *L1014F* (*Rw*) *kdr* mutations were detected in both sibling species but at much lower frequencies in *An. arabiensis* (Table 8-2). There was a significant difference in genotype frequencies between the two species ($p < 0.0001$). The *N1575Y* and *G119S* (a*ce-1R*) mutations were not detected.

An increase in *Re/Re* homozygotes was observed in *An. gambiae* s.s. between the three study rounds from 80.4% to 90.6% as the *Re* allele frequency significantly increased from 88.4% to 95.2% (LR $\chi^2 = 34.11$, $p < 0.0001$), while the frequency of the *S* allele decreased from 5.4% to 0.2% (LR $\chi^2 = 89.19$, $p < 0.0001$) (Table 8-2).

In *An. arabiensis*, an increase in the presence of homozygous wild type (*S/S*) was seen between the three study rounds as the frequency of the *S* allele increased from 82.8% to 93.2% (LR $\chi^2 = 5.02$, $p = 0.081$) (Table 8-2). The *Re* allele was only detected in the second study round at low frequency (0.02), while the *Rw* allele was mostly present in heterozygote *S/Rw* form.

While no *Re/Rw* resistant heterozygotes were observed in *An. arabiensis*, they were found in *An. gambiae* s.s. in all three rounds and presented between 4.5 and 8.8% of all genotypes.

Table 8-2. *Kdr* status of *An. gambiae* s.s. and *An. arabiensis* mosquitoes collected in the study.

An. gambiae s.s.															
		Number of *kdr* genotypes						Number of alleles			Allele frequency^			*Kdr* homozygosity	
Round	N	*S/S*	*S/Re*	*S/Rw*	*Re/Re*	*Rw/Rw*	*Re/Rw*	*S*	*Re*	*Rw*	*S*	*Re*	*Rw*	*Re/Re*	*Rw/Rw*
1	927	3	93	1	745	29	56	100	1639	115	0.054	0.884	0.062	0.804	0.031
2	491	14	10	0	423	22	22	38	878	66	0.039	0.894	0.067	0.862	0.045
3	680	0	3	0	616	1	60	3	1295	62	0.002	0.952	0.046	0.906	0.001
An. arabiensis															
1	29	21	0	6	0	2	0	48	0	10	0.828	0.000	0.172	0.000	0.069
2	69	62	1	5	1	0	0	130	3	5	0.942	0.022	0.036	0.014	0.000
3	169	146	0	23	0	0	0	315	0	23	0.932	0.000	0.068	0.000	0.000

^ Logistic regression: Allele frequencies differ significantly between rounds 1 and 3 for *An. gambiae* s.s. *S* and *Re* alleles, and between rounds 1 and 3 for *An. arabiensis S* allele. *An. gambiae* s.s. *Re* allele frequency significantly increased (OR for the presence of homozygotes between round 1 to round 2 = 1.43, p = 0.021; OR for the presence of homozygotes between round 1 to round 3 = 2.37, p < 0.0001), while the frequency of the *S* allele decreased (OR for the presence of homozygotes between round 1 to round 2 = 0.46, p = 0.001; OR for the presence of homozygotes between round 1 to round 3 = 0.04, p < 0.0001). *An. arabiensis S* allele frequency increased (OR for the presence of homozygotes between round 1 to round 2 = 3.56, p = 0.028; OR for the presence of homozygotes between round 1 to round 3 = 2.61, p = 0.043).

Table 8-3. Observed and expected values for *kdr* genotypes of *An. gambiae* s.s. and *An. arabiensis* mosquitoes collected in the study, together with Hardy Weinberg equilibrium calculations.

An. gambiae s.s.														
Round	Number of kdr genotypes												p(HW)[1]	F_{IS}[2]
	S/S		*S/Re*		*S/Rw*		*Re/Re*		*Rw/Rw*		*Re/Rw*			
	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp		
1	3	2.67	93	88.4	1	6.2	745	724.42	29	3.54	56	101.72	<0.0001*	0.2363
2	14	0.72	10	34.01	0	2.56	423	392.46	22	2.19	22	59.07	<0.0001*	0.6656
3	0	0	3	2.86	0	0.14	616	616.53	1	1.39	60	59.08	1.0000	-0.015
An. arabiensis														
Round	Number of kdr genotypes												p(HW)[1]	F_{IS}[2]
	S/S		*S/Re*		*S/Rw*		*Re/Re*		*Rw/Rw*		*Re/Rw*			
	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp		
1	21	19.79	/	/	6	8.42	/	/	2	0.79	/	/	0.1620	0.2911
2	62	61.2	1	2.85	5	4.74	1	0.02	0	0.07	0	0.11	0.0354*	0.2221
3	146	146.75	/	/	23	21.49	/	/	0	0.75	/	/	1.0000	-0.0701

[1]P(HW): Probability of the exact test for goodness of fit to Hardy-Weinberg equilibrium

[2]F_{IS}: Wright's inbreeding coefficient (calculated according to Weir and Cockerham)

* These values remain significant after a Bonferroni correction for multiple tests

Genotype frequencies differed significantly from expected values in rounds 1 and 2 in *An. gambiae* s.s., and in round 2 in *An. arabiensis* according to exact tests for Hardy Weinberg equilibrium. Positive estimates of the Wright's inbreeding coefficient (F_{IS}) in rounds 1 and 2 indicate a deficit of heterozygotes, while negative F_{IS} values for round 3 indicate a slight excess of heterozygotes (Table 8-3). Overall, genotype frequencies differed significantly from the Hardy-Weinberg equilibrium for *An. gambiae* s.s. population with a deficit of heterozygotes (F_{IS} = 0.308) and a low value fixation index F_{ST} = 0.013, which can be expected when the frequency of the most frequent allele (i.e. *Re*) is high. Similar results were obtained for *An. arabiensis* population (F_{IS} = 0.069; F_{ST} = 0.028), where the wild type *S* allele was dominant.

8.3.3 WHO susceptibility test results and phenotypic resistance

In total, six WHO susceptibility tests were carried out, testing 737 *An. gambiae* s.l. mosquitoes (Table 8-4). Phenotypic resistance of mosquitoes varied between different insecticides. *An. gambiae* s.l. populations remained fully susceptible to bendiocarb and pirimiphos-methyl but were resistant to pyrethroids and DDT.

Table 8-4. WHO susceptibility test results for *An. gambiae* s.l. collected in Kyankwanzi, Uganda.

Date of test	Insecticide	% mortality	Number tested	Resistance status**
7 October 2013	Deltamethrin	71.9*	87	R
8 October 2013	DDT	62.5*	80	R
9 October 2013	Bendiocarb	100.0	31	S
9 December 2013	Pirimiphos-methyl	100.0*	96	S
20 June 2014	Permethrin	63.0	100	R
23 June 2013	Lambda-cyhalothrin	33.4*	92	R

* Corrected using Abbott's formula

^ R = resistant (mortality < 90%); SR = suspected resistant (mortality 90 – 97%); S = susceptible (mortality >=98%) [19]

Mortality after exposure to different types of pyrethroids differed significantly (χ^2 = 22.98, df = 2; p < 0.0001). The lowest mortality was seen with lambda-cyhalothrin, followed by permethrin and deltamethrin.

Following the tests, 680 mosquitoes were genotyped and identified to species level using rtPCR. Results were re-calculated and are only shown if the number of analysed mosquitoes was greater than 10 (Table 8-5). WHO susceptibility test results indicate that both sibling species are resistant to pyrethroids. Mortalities caused by exposure to permethrin were significantly different between *An. gambiae* s.s. and *An. arabiensis* (Fisher's exact test, p = 0.0007), as were mortalities following exposure to lambda-cyhalothrin (Fisher's exact test, p = 0.0009).

Table 8-5. WHO susceptibility test results for *An. gambiae* s.s. and *An. arabiensis* collected in Kyankwanzi, Uganda.

Insecticide	*An. gambiae* s.s.			*An. arabiensis*		
	% mortality	n	Resistance status^	% mortality	n	Resistance status^
Deltamethrin	75.3	81	R	-		
DDT	62.3	77	R	-		
Bendiocarb	100.0	31	S	-		
Pirimiphos-methyl	100.0	71	S	100.0	11	S
Permethrin	50.0	56	R	83.3	42	R
Lambda-cyhalothrin	25.4	55	R	61.8	34	R

^ R = resistant (mortality < 90%); SR = suspected resistant (mortality 90 – 97%); S = susceptible (mortality >=98%) [19]

8.3.4 Correlation between phenotypic and genetic resistance frequencies

All the resistant *An. gambiae* s.s. individuals that survived in pyrethroid and DDT bioassays carried at least one copy of either the *Re* or the *Rw kdr* alleles (Table 8-6 and Fig.8-1). There was a significant difference in *kdr* genotypes between surviving and dead *An. gambiae* s.s. mosquitoes following exposure to DDT only showing there was a correlation between phenotypic and genotypic frequencies (Fisher's exact test, p < 0.0001; Kendall's tau p < 0.0001), but not following pyrethroid bioassays where a large proportion of *kdr*-resistant mosquitoes died after insecticide exposure (Fisher's exact test, p > 0.05; Kendall's tau p > 0.05).

When pooled, *Re* alleles were found in *An. gambiae* s.s. used in bioassays at frequencies of 90.7% in survivors and 83.5% in dead (z = 2.36; p = 0.018), and *Rw* at 8.8% in survivors and

3.9% in dead (z = 2.33; p = 0.020), respectively. There was a significant difference between mosquitoes with none, one or two *kdr* alleles (i.e. *SS; S/Re and S/Rw; and Re/Re, Rw/Rw and Re/Rw* genotypes) that either survived or died during the exposure (Fisher's exact test, p < 0.0001).In *An. arabiensis* used in bioassays, only the *Rw* allele was present (frequency 13% in survivors, 6% in dead) (z = 1.00; p = 0.318) but never as the homozygous genotype, whereas the majority of mosquitoes were homozygous susceptible (Table 8-7 and Fig. 8-2). There was no significant difference in *kdr* genotypes between surviving and dead *An. arabiensis* mosquitoes (Fisher's exact test, p = 0.282).

Moreover, resistance was observed in 23.1% of *An. arabiensis* homozygous for the susceptible *S* allele, whereas 48.9% of homozygous resistant (*Re/Re*, *Rw/Rw* and *Rw/Re*) *An. gambiae* s.s. were susceptible in bioassays using pyrethroids and DDT.

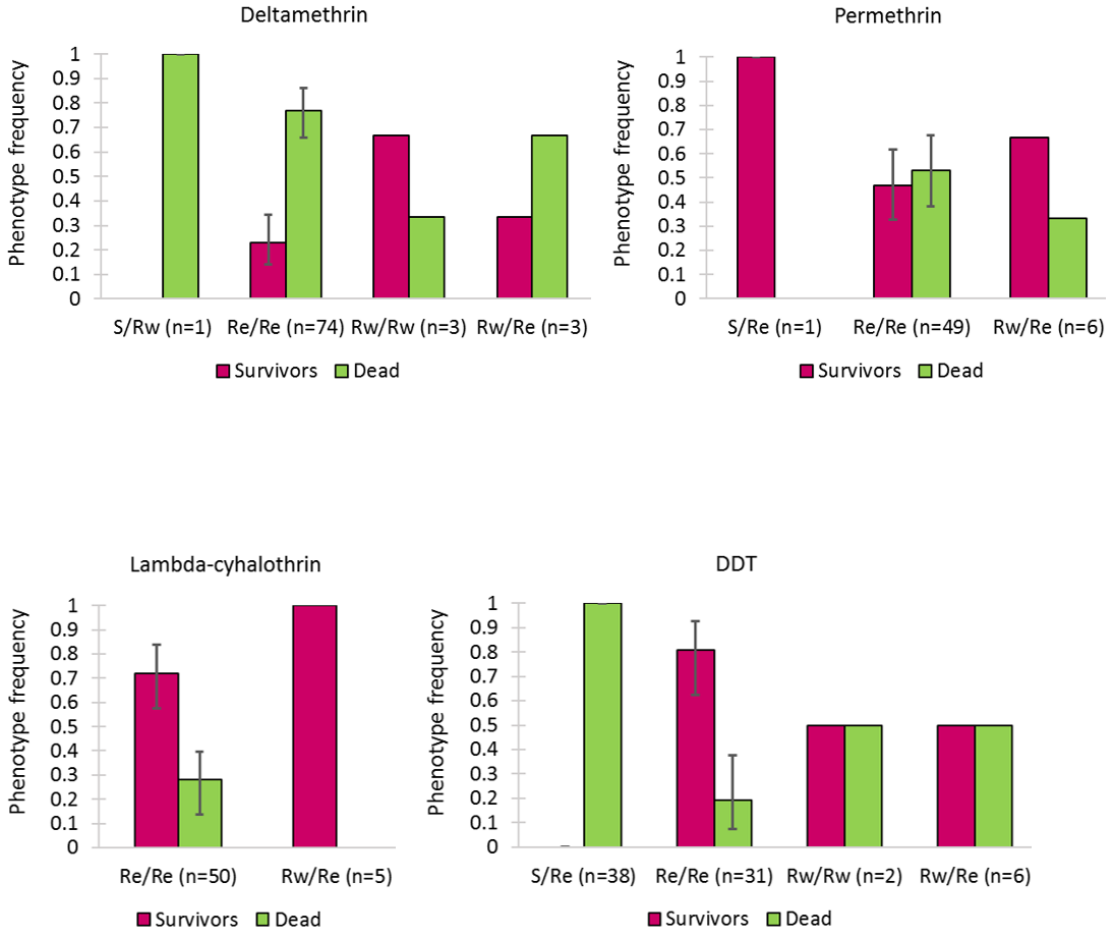

Fig. 8-1. Correlation between *kdr* genotypes and associated resistance phenotypes in female *An. gambiae* s.s. Genotypes were determined for mosquitoes following exposure to insecticides in WHO bioassays: 0.05% deltamethrin (*N* = 81); 0.75% permethrin (*N* = 56); 0.05% lambda-cyhalothrin (*N* = 55); 4% DDT (*N* = 77). Differences in survival were analysed using Fisher's exact test (see Table 8-6). Error bars indicate 95% confidence intervals and are shown only where n>10 for a given genotype.

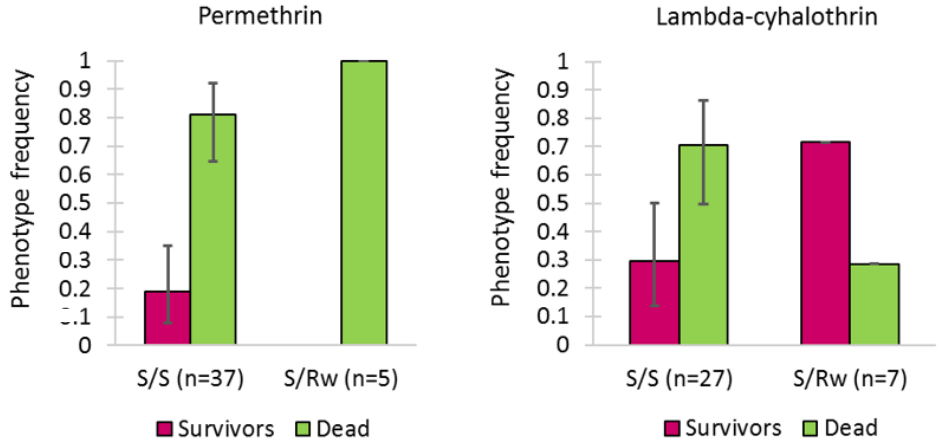

Fig. 8-2. Correlation between *kdr* genotypes and associated resistance phenotypes in female *An. arabiensis*. Genotypes were determined for mosquitoes following exposure to insecticides in WHO bioassays: 0.75% permethrin (*N* = 42); 0.05% lambda-cyhalothrin (*N* = 34). Differences in survival were analysed using Fisher's exact test (see Table 8-7). Error bars indicate 95% confidence intervals and are shown only where n>10 for a given genotype.

Table 8-6. *Kdr* mutation genotypes and allelic frequencies among dead and surviving *An. gambiae* s.s. mosquitoes following WHO susceptibility tests.

			Number of *kdr* genotypes						Fisher's exact	Allele frequency			Fisher's exact
Insecticide	Phenotype	N	*S/S*	*S/Re*	*S/Rw*	*Re/Re*	*Rw/Rw*	*Re/Rw*	test *p*	Freq. (*S*)	Freq. (*Re*)	Freq. (*Rw*)	test *p*
Deltamethrin	Survivors	20	0	0	0	17	2	1		0.00	0.88	0.13	
	Dead	61	0	0	1	57	1	2	0.23	0.01	0.95	0.04	0.155
DDT	Survivors	29	0	0	0	25	1	3		0.00	0.91	0.09	
	Dead	48	0	38	0	6	1	3	**< 0.001**	0.40	0.55	0.05	**< 0.001**
Permethrin	Survivors	28	0	1	0	23	0	4		0.02	0.91	0.07	
	Dead	28	0	0	0	26	0	2	0.422	0.00	0.96	0.04	0.438
Lambda-cyhalothrin	Survivors	41	0	0	0	36	0	5		0.00	0.94	0.06	
	Dead	14	0	0	0	14	0	0	0.314	0.00	1.00	0.00	0.326

Table 8-7. *Kdr* mutation genotypes and allelic frequencies among dead and surviving *An. arabiensis* mosquitoes following WHO susceptibility tests.

			Number of *kdr* genotypes						Fisher's exact	Allele frequency			Fisher's exact
Insecticide	Phenotype	N	*S/S*	*S/Re*	*S/Rw*	*Re/Re*	*Rw/Rw*	*Re/Rw*	test *p*	Freq. (*S*)	Freq. (*Re*)	Freq. (*Rw*)	test *p*
Permethrin	Survivors	7	7	0	0	0	0	0		1.00	0.00	0.00	
	Dead	35	30	0	5	0	0	0	0.569	0.93	0.00	0.07	0.584
Lambda-cyhalothrin	Survivors	13	8	0	5	0	0	0		0.81	0.00	0.19	
	Dead	21	19	0	2	0	0	0	0.079	0.95	0.00	0.05	0.097

8.4 Discussion

Correct identification and incrimination of the vectors present in an area, and determination of their insecticide resistance status, are required to guide malaria vector control programmes in their choice of control measures and insecticides used. This study examined *kdr*-associated insecticide resistance in the two sibling vector species, *An. gambiae* s.s. and *An. arabiensis*, in Kyankwanzi, mid-western Uganda, and explored the relationship between bioassay-survival and genotype at the *kdr* locus.

Anopheles gambiae s.s. and *An. arabiensis* were confirmed to be the main vector species in the area, and during our study no *An. funestus* s.l. were found, confirming findings of a previous study [31]. However, we also observed a shift in species composition, with a significant decrease in *An. gambiae* s.s. relative abundance and a corresponding increase in the frequency of *An. arabiensis*. Shifts in vector sibling species have been described previously, usually as a result of the extensive use of vector control interventions [41, 42, 43, 44, 45]. The first mass net distribution campaign in Uganda – including in Kyankwanzi - took place between 2007–2010, targeting women and children only, while the first Universal Coverage Campaign took place in 2013-2014, between Rounds 2 and 3 of our project. However, a significant change in abundance already started occurring from Round 1 to Round 2, i.e. before the distribution of ITNs, and hence this is an unlikely cause of the change in species abundances. Changes in the presence of different vector species are often seasonal and follow changes in climate. Further sampling in the area would be necessary to see whether this shift was permanent and has affected malaria transmission in the area or whether the abundance of *An. gambiae* s.s. increased again during the latter parts of 2014. As the differences in species composition of mosquitoes were only detected in mosquitoes collected as larvae and reared to adults, but not in adults collected during entomological surveys, the method of collection represents a bias. Whereas collection of adult mosquitoes is random, collection of larvae is not as the larvae collected in a single breeding site are very likely to be offspring of the same mother, therefore not giving a true representation of the species makeup in the area.

Ninety-nine (3.97%) of all processed mosquitoes could not be identified to species level. Most of these were identified by rtPCR as “other *Anopheles*”, i.e. either *An. quadriannulatus, An. bwambae* or *An. merus* or *An. melas*, none of which have been previously reported in the study area. *Kdr* results were obtained for 88 out of 99 of unidentified specimens, using the

probes that work with *An. gambiae* complex. One possible explanation could be a presence of a cryptic subgroup in the area. *An. gambiae* populations are still undergoing speciation, are genetically divided into subgroups which are sometimes reproductively isolated from each other and can only be differentiated using very specific markers [46]. These subgroups can display significantly differing phenotypes and great plasticity in adjusting to diverse ecological conditions. Differences such as choice of hosts, feeding and resting places, and parasite susceptibility might need to be taken into account when planning control measures due to possible epidemiological consequences [46, 47, 48]. The presence of these "other *Anopheles*" warrants further investigation as they may play an important part in the local malaria epidemiology. Keeping mosquitoes collected from different larval sites apart as separate batches of emerged adults might reveal further differences on a local scale. However, due to the nature of the experiments carried out we were not able to keep emerged adults separate and are unable to tell whether most of them are somehow associated.

Insecticide resistance is widespread in Uganda. Previous studies showed that both *kdr* mutations, *L1014S* and *L1014F*, were already present in *An. gambiae* s.s. samples collected between 2001-2002 throughout Uganda, while only *L1014S* was found in *An. arabiensis* [23, 27]. U.S. President's Malaria Initiative (PMI) have been monitoring insecticide resistance in Uganda since 2009 in a number of locations, including Hoima district (adjacent to our study district Kyankwanzi). During 2016, CDC intensity bioassays showed high intensity resistance to both permethrin and deltamethrin in *An. gambiae* s.l. in Hoima, while CDC bottle synergist bioassays using piperonyl butoxide (PBO) showed increased mortality of *An. gambiae* s.l., indicating the presence of oxidase activity in detoxification of deltamethrin and permethrin [45, 49]. Resistance was previously also detected in Kyankwanzi where both *L1014S* and *L1014F kdr* mutations were found in the two sibling species [30, 31].

In Kyankwanzi, the frequencies of *Re* and *Rw* resistant alleles were significantly higher in *An. gambiae* s.s. than in *An. arabiensis.* This species tends to be more endophilic and endophagic than *An. arabiensis* and is therefore more likely to be exposed to insecticides in ITNs.

In *An. gambiae* s.s. *L1014S* (*Re*) was present at high frequencies, approaching fixation, with the frequency of this allele increasing significantly between round 1 (88%) and round 3 (95%), and the frequency of *Re/Re* homozygotes increasing from 80% to 90%. These increases could partly be due to the LLIN distribution that took place between rounds 2 and 3. *Kdr* mutations are recessive and are functionally more significant in the homozygous state,

with the bioassay mortality rates of *S/S* wild type mosquitoes and *kdr* heterozygotes (*S/Re* or *S/Rw*) being similar when no other insecticide resistance mechanisms are involved [15, 50, 51]. The *L1014*F (*Rw*) mutation was present at much lower frequencies (< 10%) which did not differ significantly between the rounds. *L1014F* is thought to confer a greater degree of resistance than *L1014S*, but it is likely there is also some fitness cost associated with this allele [51, 52], which might limit its spread. Few homozygous susceptible wild type (*S/S*) mosquitoes were collected in the first two rounds whereas none were found in the third round, with *S* allele almost disappearing.

A double resistant heterozygote genotype (*Re/Rw*) was detected in all three rounds, reaching 9% frequency in round 3. Such "simultaneous *kdr* resistant heterozygotes" were previously found in Gabon [53], Cameroon [17] and in Uganda where they occurred at lower frequencies [23, 27]. However, apart from round 3, these *Re/Rw* heterozygotes do not appear in excess (as they did in Gabon) and it is likely they do not confer a selective advantage over homozygotes under insecticide pressure.

In *An. arabiensis, Re* and *Rw* alleles were present at much lower frequencies while the wild type *S* allele remained dominant (frequencies between 83% and 94%). This was previously observed in Uganda [25, 31] and Kenya [54], whereas insecticide resistance and the *L1014F* mutation are widespread and the *L1014S* mutation is absent in *An. arabiensis* populations in Ethiopia [55, 56, 57].

The *N1575Y* mutation has so far only been in detected in West Africa [18, 58, 59] and was not detected in this study. Mosquitoes were found to be fully susceptible to carbamates and organophosphates and correspondingly the *G119S* (a*ce-1R*) mutation was also not detected. These insecticides might therefore represent useful alternatives to pyrethroids and DDT, although they cannot be used on nets.

Survival after bioassay exposure to pyrethroids and DDT in *An. gambiae* s.l., as detected in our study, has been described in Uganda previously [24, 25, 28, 29, 45, 49, 60]. Observed survival rates were the highest to lambda-cyhalothrin (67%), followed by DDT (38%), permethrin (37%), and deltamethrin (28%), indicating resistance to all of these insecticides. Knockdown resistance limits the effectiveness of all pyrethroids, pyrethrins and DDT [52]. Studies of comparative performances of different pyrethroids using susceptible and resistant mosquito strains have shown they differ significantly in knock-down effects, mortality, irritancy and inhibition of blood feeding [61].

The use of bioassays, followed by genotyping of *kdr* mutation, can be used to test whether the presence of *kdr* mutations is correlated with the resistant phenotype [62]. All resistant *An. gambiae* s.s. which survived exposure to pyrethroids or DDT carried at least one copy of either *Re* or *Rw* alleles. However, we only detected a significant association between the *kdr L1014S* (*Re*) mutation and DDT resistance, but not resistance to any of the three pyrethroids. There was also a significant difference in the frequency of the *Rw* allele between mosquitoes which were resistant and susceptible to deltamethrin, and generally frequencies of the *Rw* allele were higher in resistant than susceptible mosquitoes. A strong association between *L1014S* and DDT resistance, and some association between *L1014S* and permethrin resistance were previously detected in eastern Uganda [24], but no such association was detected more recently in Jinja [25]. Although *kdr* alleles are present in *An. arabiensis* in low frequencies, high levels of bioassay survival were observed with both permethrin (mortality 83%) and lambda-cyhalothrin (mortality 62%). Based on our findings, phenotypic resistance in both sibling species is most likely not only caused by target site mechanisms but also by metabolic resistance mechanisms, and possibly cuticular resistance, as suggested by Mawejje *et al.* [25]. Lack of data on metabolic resistance represents a limitation of our study. With the advent of DNA markers for metabolic pyrethroid resistance such studies will be more easily carried out in the future [63].

The observed differences between resistance allele frequencies between the two sibling species could be due to *An. gambiae* s.s. being a more anthropophilic, endophagic and endophilic species than *An. arabiensis*, which has a wide range of feeding and resting patterns and may therefore be generally less exposed to insecticide-based vector control interventions [64].

Our study shows that the problem of *kdr* resistance in the two sibling vector species, *An. gambiae* s.s. and *An. arabiensis*, is significantly different. Whereas the two *kdr* mutations, *L1014S* and *L1014F*, have been detected in both sibling species, the *Re* (*L1014S*) allele has become almost fixed in *An. gambiae* s.s., whereas the wild type *S* allele remains dominant in *An. arabiensis*. The *Rw* (*L1014F*) is present in both species at low frequencies. The fact that bioassay mortality is only weakly associated with genotypic resistance suggests that other metabolic mechanisms are probably an additional major factor influencing bioassay survival.

Continued surveillance of vector populations is needed to detect any additional changes in vector species composition and their insecticide resistance status, which might have an impact on the effectiveness of LLINs and on malaria transmission in the area.

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8.5 References

1. 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;526:207-211.

2. World Health Organization. World Malaria Report 2016. Geneva: World Health Organization; 2016.

3. World Health Organization. World Malaria Report 2017. Geneva: World Health Organization; 2017.

4. World Health Organization: Pesticide evaluation scheme (WHOPES). Geneva, Switzerland: 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. World Health Organization: Report of the twentieth WHOPES working group meeting, WHO/HQ, Geneva, 20-24 March 2017: review of Interceptor G2 LN, DawaPlus 3.0 LN, DawaPlus 4.0 LN, SumiLarv 2 MR, Chlorfenapyr 240 SC. Geneva: World Health Organization; 2017: 122.

7. Ranson H, Lissenden N. Insecticide Resistance in African *Anopheles* Mosquitoes: A Worsening Situation that Needs Urgent Action to Maintain Malaria Control. Trends Parasitol. 2016;32:187-196.

8. 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 Vectors. 2014;7:76.

9. Barnes KG, Weedall GD, Ndula M, Irving H, Mzihalowa T, Hemingway J*, et al*. Genomic Footprints of Selective Sweeps from Metabolic Resistance to Pyrethroids in African Malaria Vectors Are Driven by Scale up of Insecticide-Based Vector Control. PLoS Genet. 2017;13:e1006539.

10. 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;27:91-98.

11. Hien AS, Soma DD, Hema O, Bayili B, Namountougou M, Gnankine O*, et al*. Evidence that agricultural use of pesticides selects pyrethroid resistance within *Anopheles gambiae* s.l. populations from cotton growing areas in Burkina Faso, West Africa. PLoS One. 2017;12:e0173098.

12. Hemingway J, Hawkes NJ, McCarroll L, Ranson H. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem Mol Biol. 2004;34:653-665.

13. Chandre F, Manguin S, Brengues C, Dossou Yovo J, Darriet F, Diabate A*, et al*. Current distribution of a pyrethroid resistance gene (*kdr*) in *Anopheles gambiae* complex from west Africa and further evidence for reproductive isolation of the Mopti form. Parassitologia. 1999;41:319-322.

14. Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Berge JB, Devonshire AL*, et al*. Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae* s.s. Insect Mol Biol. 1998;7:179-184.

15. Ranson H, Jensen B, Vulule JM, Wang X, Hemingway J, Collins FH. Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. Insect Mol Biol. 2000;9:491-497.

16. Reddy MR, Godoy A, Dion K, Matias A, Callender K, Kiszewski AE*, et al*. Insecticide resistance allele frequencies in *Anopheles gambiae* before and after anti-vector interventions in continental Equatorial Guinea. Am J Trop Med Hyg. 2013;88:897-907.

17. Reimer L, Fondjo E, Patchoke S, Diallo B, Lee Y, Ng A*, et al*. Relationship between *kdr* mutation and resistance to pyrethroid and DDT insecticides in natural populations of *Anopheles gambiae*. J Med Entomol. 2008;45:260-266.

18. Jones CM, Liyanapathirana M, Agossa FR, Weetman D, Ranson H, Donnelly MJ*, et al*. Footprints of positive selection associated with a mutation (*N1575Y*) in the voltage-gated sodium channel of *Anopheles gambiae*. Proc Natl Acad Sci U S A. 2012;109:6614-6619.

19. World Health Organization: Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. 2nd edn. Geneva, Switzerland: World Health Organization; 2016.

20. Weetman D, Donnelly MJ. Evolution of insecticide resistance diagnostics in malaria vectors. Trans R Soc Trop Med Hyg. 2015;109.

21. Donnelly MJ, Corbel V, Weetman D, Wilding CS, Williamson MS, Black WCt. Does *kdr* genotype predict insecticide-resistance phenotype in mosquitoes? Trends Parasitol. 2009;25:213-219.

22. Brooke BD. *Kdr*: can a single mutation produce an entire insecticide resistance phenotype? Trans R Soc Trop Med Hyg. 2008;102:524-525.

23. Verhaeghen K, Bortel WV, Roelants P, Okello PE, Talisuna A, Coosemans M. Spatio-temporal patterns in *kdr* frequency in permethrin and DDT resistant *Anopheles gambiae* s.s. from Uganda. Am J Trop Med Hyg. 2010;82:566-573.

24. Ramphul U, Boase T, Bass C, Okedi LM, Donnelly MJ, Muller P. Insecticide resistance and its association with target-site mutations in natural populations of *Anopheles gambiae* from eastern Uganda. Trans R Soc Trop Med Hyg. 2009;103:1121-1126.

25. Mawejje HD, Wilding CS, Rippon EJ, Hughes A, Weetman D, Donnelly MJ. Insecticide resistance monitoring of field-collected *Anopheles gambiae* s.l. populations from Jinja, eastern Uganda, identifies high levels of pyrethroid resistance. Med Vet Entomol. 2013;27:276-283.

26. Ojuka P, Boum Y, 2nd, Denoeud-Ndam L, Nabasumba C, Muller Y, Okia M*, et al*. Early biting and insecticide resistance in the malaria vector *Anopheles* might compromise the effectiveness of vector control intervention in Southwestern Uganda. Malaria journal. 2015;14:148.

27. Verhaeghen K, Van Bortel W, Roelants P, Backeljau T, Coosemans M. Detection of the East and West African *kdr* mutation in *Anopheles gambiae* and *Anopheles arabiensis* from Uganda using a new assay based on FRET/Melt Curve analysis. Malar J. 2006;5:16.

28. Rubaihayo J, Ephraim T, Andrew A. Reduced susceptibility to pyrethroid insecticide treated nets by the malaria vector *Anopheles gambiae* s.l. in western Uganda. Malar J. 2008;7:92.

29. Okia M, Ndyomugyenyi R, Kirunda J, Byaruhanga A, Adibaku S, Lwamafa DK*, et al*. Bioefficacy of long-lasting insecticidal nets against pyrethroid-resistant populations of *Anopheles gambiae* s.s. from different malaria transmission zones in Uganda. Parasit Vectors. 2013;6:130.

30. Abeku TA, Helinski ME, Kirby MJ, Kefyalew T, Awano T, Batisso E*, et al*. Monitoring changes in malaria epidemiology and effectiveness of interventions in Ethiopia and Uganda: Beyond Garki Project baseline survey. Malar J. 2015;14:337.

31. Helinski M, Nuwa A, Protopopoff N, Feldman M, Ojuka P, Oguttu DW*, et al*. Entomological surveillance following a long-lasting insecticidal net universal coverage campaign in Midwestern Uganda. Parasit Vectors. 2015;8:458.

32. Kristan M, Lines J, Nuwa A, Ntege C, Meek SR, Abeku TA. Exposure to deltamethrin affects development of *Plasmodium falciparum* inside wild pyrethroid resistant *Anopheles gambiae* s.s. mosquitoes in Uganda. Parasit Vectors. 2016;9:100.

33. Okia M, Hoel DF, Kirunda J, Rwakimari JB, Mpeka B, Ambayo D*, et al*. Insecticide resistance status of the malaria mosquitoes: *Anopheles gambiae* and *Anopheles funestus* in eastern and northern Uganda. Malar J. 2018;17:157.

34. World Health Organization: Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. Geneva, Switzerland: World Health Organization; 2013.

35. Lardeux F, Tejerina R, Aliaga C, Ursic-Bedoya R, Lowenberger C, Chavez T. Optimization of a semi-nested multiplex PCR to identify *Plasmodium* parasites in wild-caught

Anopheles in Bolivia, and its application to field epidemiological studies. Trans R Soc Trop Med Hyg. 2008;102:485-492.

36. Bass C, Williamson MS, Field LM. Development of a multiplex real-time PCR assay for identification of members of the *Anopheles gambiae* species complex. Acta Trop. 2008;107:50-53.

37. Bass C, Nikou D, Donnelly MJ, Williamson MS, Ranson H, Ball A*, et al*. Detection of knockdown resistance (*kdr*) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. Malar J. 2007;6:111.

38. Bass C, Nikou D, Vontas J, Williamson MS, Field LM. Development of high-throughput real-time PCR assays for the identification of insensitive acetylcholinesterase (*ace-1*^{R}) in *Anopheles gambiae*. Pestic Biochem Phys. 2010;96:80-85.

39. Raymond M, Rousset F. Genepop (version-1.2) - Population-genetics software for exact tests and ecumenicism. J Hered. 1995;86:248-249.

40. Weir BS, Cockerham CC. Estimating F-Statistics for the Analysis of Population Structure. Evolution. 1984;38:1358-1370.

41. Derua YA, Alifrangis M, Hosea KM, Meyrowitsch DW, Magesa SM, Pedersen EM*, et al*. Change in composition of the *Anopheles gambiae* complex and its possible implications for the transmission of malaria and lymphatic filariasis in north-eastern Tanzania. Malar J. 2012;11:188.

42. Lwetoijera DW, Harris C, Kiware SS, Dongus S, Devine GJ, McCall PJ*, et al*. Increasing role of *Anopheles funestus* and *Anopheles arabiensis* in malaria transmission in the Kilombero Valley, Tanzania. Malar J. 2014;13:331.

43. Bayoh MN, Mathias DK, Odiere MR, Mutuku FM, Kamau L, Gimnig JE*, et al*. *Anopheles gambiae*: historical population decline associated with regional distribution of insecticide-treated bed nets in western Nyanza Province, Kenya. Malar J. 2010;9:62.

44. Mwangangi JM, Mbogo CM, Orindi BO, Muturi EJ, Midega JT, Nzovu J*, et al*. Shifts in malaria vector species composition and transmission dynamics along the Kenyan coast over the past 20 years. Malar J. 2013;12:13.

45. U.S. President's Malaria Initiative: Uganda Malaria Operational Plan FY 2018. 2017: 111.

46. Riehle MM, Guelbeogo WM, Gneme A, Eiglmeier K, Holm I, Bischoff E*, et al*. A cryptic subgroup of *Anopheles gambiae* is highly susceptible to human malaria parasites. Science. 2011;331:596-598.

47. The Anopheles gambiae 1000 Genomes Consortium. Genetic diversity of the African malaria vector *Anopheles gambiae*. Nature. 2017;552:96-100.

48. Kamdem C, Fouet C, Gamez S, White BJ. Pollutants and Insecticides Drive Local Adaptation in African Malaria Mosquitoes. Mol Biol Evol. 2017;34:1261-1275.

49. U.S. President's Malaria Initiative: Uganda Malaria Operational Plan FY 2017. 2016: 98.

50. 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 U S A. 2013;110:9397-9402.

51. Lynd A, Weetman D, Barbosa S, Egyir Yawson A, Mitchell S, Pinto J*, et al*. Field, genetic, and modeling approaches show strong positive selection acting upon an insecticide resistance mutation in *Anopheles gambiae* s.s. Mol Biol Evol. 2010;27:1117-1125.

52. Soderlund DM, Knipple DC. The molecular biology of knockdown resistance to pyrethroid insecticides. Insect Biochem Mol Biol. 2003;33:563-577.

53. Pinto J, Lynd A, Elissa N, Donnelly MJ, Costa C, Gentile G*, et al*. Co-occurrence of East and West African kdr mutations suggests high levels of resistance to pyrethroid insecticides in *Anopheles gambiae* from Libreville, Gabon. Med Vet Entomol. 2006;20:27-32.

54. Mathias DK, Ochomo E, Atieli F, Ombok M, Bayoh MN, Olang G*, et al*. Spatial and temporal variation in the *kdr* allele *L1014S* in *Anopheles gambiae* s.s. and phenotypic variability in susceptibility to insecticides in Western Kenya. Malar J. 2011;10:10.

55. Yewhalaw D, Bortel WV, Denis L, Coosemans M, Duchateau L, Speybroeck N. First evidence of high knockdown resistance frequency in *Anopheles arabiensis* (Diptera: Culicidae) from Ethiopia. Am J Trop Med Hyg. 2010;83:122-125.

56. Alemayehu E, Asale A, Eba K, Getahun K, Tushune K, Bryon A*, et al*. Mapping insecticide resistance and characterization of resistance mechanisms in *Anopheles arabiensis* (Diptera: Culicidae) in Ethiopia. Parasit Vectors. 2017;10:407.

57. Messenger LA, Shililu J, Irish SR, Anshebo GY, Tesfaye AG, Ye-Ebiyo Y*, et al*. Insecticide resistance in *Anopheles arabiensis* from Ethiopia (2012-2016): a nationwide study for insecticide resistance monitoring. Malar J. 2017;16:469.

58. Edi AVC, N'Dri BP, Chouaibou M, Kouadio FB, Pignatelli P, Raso G*, et al*. First detection of *N1575Y* mutation in pyrethroid resistant *Anopheles gambiae* in Southern Cote d'Ivoire. Wellcome Open Res. 2017;2:71.

59. Fossog Tene B, Poupardin R, Costantini C, Awono-Ambene P, Wondji CS, Ranson H*, et al*. Resistance to DDT in an urban setting: common mechanisms implicated in both M and S forms of *Anopheles gambiae* in the city of Yaounde Cameroon. PLoS One. 2013;8:e61408.

60. Abeku TA, Helinski MEH, Kirby MJ, Ssekitooleko J, Bass C, Kyomuhangi I*, et al*. Insecticide resistance patterns in Uganda and the effect of indoor residual spraying with bendiocarb on *kdr L1014S* frequencies in *Anopheles gambiae* s.s. Malar J. 2017;16:156.

61. Hougard JM, Duchon S, Darriet F, Zaim M, Rogier C, Guillet P. Comparative performances, under laboratory conditions, of seven pyrethroid insecticides used for impregnation of mosquito nets. Bull World Health Organ. 2003;81:324-333.

62. Silva AP, Santos JM, Martins AJ. Mutations in the voltage-gated sodium channel gene of anophelines and their association with resistance to pyrethroids - a review. Parasit Vectors. 2014;7:450.

63. Weetman D, Wilding CS, Neafsey DE, Muller P, Ochomo E, Isaacs AT*, et al*. Candidate-gene based GWAS identifies reproducible DNA markers for metabolic pyrethroid resistance from standing genetic variation in East African *Anopheles gambiae*. Sci Rep. 2018;8:2920.

64. Sinka ME, Bangs MJ, Manguin S, Coetzee M, Mbogo CM, Hemingway J*, et al*. The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic precis. Parasit Vectors. 2010;3:117.

Chapter 9. Discussion and conclusions

Widespread use of insecticides has led to an increased frequency and intensity of insecticide resistance in the African malaria vectors, *Anopheles gambiae* s.s., *An. coluzzii*, *An. arabiensis* and *An. funestus*, posing a threat to disease control [1, 2, 3, 4]. However, evidence of the epidemiological impact of resistance on the effectiveness of currently used vector control tools is limited.

The aim of this thesis was to investigate the potential effects of pyrethroids and pyrethroid exposure of *An. gambiae* s.l. vectors on the sporogonic development and transmissibility of *Plasmodium falciparum*, and to understand the effects of both, insecticides and insecticide resistance mechanisms, on parasites, vectors and malaria transmission.

This overall aim was achieved through addressing two objectives. The primary objective was to study the effects of pyrethroids and pyrethroid exposure of *An. gambiae* s.s. on the sporogonic cycle of *P. falciparum*. This objective was fulfilled through studying the effects of sub-lethal doses of deltamethrin on sporogony in wild pyrethroid resistant *An. gambiae* s.s. in Uganda. The results of the study showed that exposure of resistant mosquitoes to sub-lethal doses of pyrethroids significantly reduces both parasite prevalence and intensity of infection [5]. These results imply that although resistant mosquitoes might survive insecticide exposure their vector competence is impaired, suggesting that pyrethroid-based interventions could still have a role in malaria control at least until alternative insecticides are available. Apart from the effect of deltamethrin exposure, the mean ambient temperature during the incubation period, which varied between 25^{o}C and 27^{o}C during the study, was found to have an independent and highly significant effect on risk of infection. The effects of environmental variables and *kdr* insecticide resistance on survival probability and malaria infection rates were then further analysed, showing that apart from the effect of deltamethrin on infection rates, temperature range during the first 24 hours and on day 4 post-infectious feed had a highly significant effect on risk of infection, where an increase in temperature range was associated with lower infection. Significant differences in survival of resistant *Re/Re, Rw/Rw* and *Re/Rw* genotypes in control and high dose groups were detected, with the lowest survival in mosquitoes with *Re/Rw* genotype, which might be due to the fitness costs the two resistant alleles exert when present together. Survival of *kdr* homozygous mosquitoes was still significantly impaired by exposure to deltamethrin, while mean daily temperature and mean daily relative humidity during the seven day incubation

period had an independent and highly significant effect on mosquito mortality. In order to better understand these findings, preliminary investigations into possible mechanisms underlying the observed effects were carried out in the laboratory by determining how much insecticide the parasites might be exposed to, and whether exposure of parasites to insecticide at such concentrations results in direct effects on sporogony. By using High Performance Liquid Chromatography-Photodiode Array (HPLC-PDA) analysis, it was found that each mosquito picks up approximately 10 ng of deltamethrin following exposure to a standard long-lasting insecticidal net (LLIN) (PermaNet 2.0), and that the final dose depends on the length of exposure. Furthermore, rapid colorimetric tests were successfully used to detect deltamethrin on mosquitoes exposed to PermaNet 2.0, showing they could potentially be used by vector control programmes when assessing effectiveness of vector control measures. Finally, using *P. berghei* ookinete cultures we showed that deltamethrin exposure can significantly impair both ookinete conversion and motility at doses that malaria parasites are likely to encounter when mosquitoes are exposed to insecticides in field conditions, which could in turn affect infection levels at oocyst and sporozoite stages and therefore might be one of the mechanisms behind the field findings. These results have important implications as they indicate that pyrethroid-based nets might continue to prevent malaria transmission despite increasing insecticide resistance.

The secondary objective was to investigate whether *kdr* resistance interferes with the development of *P. falciparum* within the vector. This objective was fulfilled through exploring the effects of the *kdr* genotype on *Plasmodium* infection rates in *An. gambiae* s.s. and *An. arabiensis*, and variations in phenotypic and genetic resistance against commonly used insecticides among *An. gambiae* s.l. in mid-western Uganda, where the field study was conducted. Both *kdr* mutations (*Re and Rw)* were found in the two sibling species, but the frequencies of *Re* and *Rw* resistant alleles were significantly higher in *An. gambiae* s.s. than in *An. arabiensis.* Bioassay mortality was only weakly associated with *kdr* genotype in both sibling species, implying that other metabolic resistance mechanisms play a significant role. Oocyst prevalence rates and infection intensity in *An. gambiae* s.s. and *An. arabiensis* were not significantly different between *kdr* genotypes, nor did they vary between the two sibling species. Similarly, sporozoite rates in *An. gambiae* s.s. were also not significantly different between *kdr* genotypes.

9.1 Placing the main findings into context and reflections on challenges encountered

Resistance against all classes of insecticides commonly used in malaria control has been reported in vector populations throughout Africa and elsewhere around the world [6, 7, 8, 9, 10, 11, 12]. Pyrethroid resistance is especially problematic as pyrethroids are the only class of insecticides currently used in all LLINs. These chemicals have also been widely used for indoor residual spraying (IRS) due to their lower cost and longer residual life compared with most alternative chemicals. Although the level of insecticide resistance increased significantly in recent years in many settings [2, 3], it has not always had an epidemiologically significant effect on malaria incidence and has not yet led to widespread malaria control failure, with pyrethroid-based control methods remaining in use even in the presence of resistance in mosquito populations [13, 14, 15, 16, 17].

9.1.1 Field study findings

A number of studies have investigated potential effects of insecticides and insecticide resistance on parasite development, but none involved the use of pyrethroids, nor were they carried out in conditions as closely resembling what actually happens in the field. In one study, exposure to DDT and bendiocarb inhibited development of *P. falciparum* in insecticide-resistant *An. gambiae* s.s. [18], while previous studies found no effect of organochlorines, carbamates and organophosphates on parasite development in mosquitoes [19, 20, 21]. *Plasmodium falciparum*-infected resistant mosquitoes have been found to be more susceptible to DDT than uninfected mosquitoes [22]. Other studies reported that *kdr*-resistant *An. gambiae* exhibited increased susceptibility to the infection with P*. falciparum* [23, 24].

It has been shown that in laboratory conditions pyrethroids reduce the infectivity of *Plasmodium* parasites to mosquitoes and inhibit their development in the vectors [19, 25, 26, 27]. However, these studies were carried out in laboratory conditions, using cultured *P. berghei, P. yoelii* and *P. falciparum* parasites and *An. stephensi* mosquitoes [19, 25, 26, 27]. Human and rodent malaria parasites differ in their interactions with vectors, while vectors do not equally support the development of different parasite species [28, 29, 30, 31, 32, 33]. Wild parasites have co-evolved with and are adapted to their local vectors [32, 34, 35, 36].

These variations must be considered in transmission studies and interpretation of results [28, 37].

The field research presented in this thesis was carried out to study the effect of exposure to sub-lethal doses of deltamethrin on the sporogony of *P. falciparum* in wild-caught, sympatric, pyrethroid resistant *An. gambiae* s.s. The effect of deltamethrin exposure on oocyst prevalence and intensity of infection was examined, using insecticide doses that would mimic those found on used LLINs. We showed that pyrethroids impair parasite development in *kdr* resistant mosquitoes in field conditions, resulting in significant reductions of parasite prevalence and intensity of infection [5]. Our findings might at least partly explain why resistance has not always led to control failure and ITNs seem to remain effective in many situations [9, 38, 39, 40, 41].

Field-based membrane feeding assays, where uninfected mosquitoes are fed on a venous blood sample from gametocytaemic individuals, are recognised as a valuable tool for studying transmission-reducing properties of various interventions [37] and were therefore chosen for this project. One of the inclusion criteria for gametocytaemic volunteers was the presence of microscopically detectable *P. falciparum* gametocytes, which were counted against 200 leucocytes in thick blood smears. Gametocyte density was then calculated assuming a standard leucocyte count of 8,000/µl of blood, which is one of the most commonly used estimates [42]. However, assumption of a constant white blood cell (WBC) count of 8,000/µl of peripheral blood can present a great source of error as large deviations from this assumed value have been recorded, and a large proportion of gametocytes might also be missed during the staining and slide reading procedures [43, 44].

As the study was carried out in a health centre, microscopy was the only means of gametocyte detection available. However, the sensitivity of this method can be unsatisfactory as gametocyte densities are frequently too low to be detected by standard light microscopy [45]. A number of molecular methods have been developed for gametocyte detection, such as reverse transcriptase polymerase chain reaction (RT-PCR) [46], quantitative nucleic acid sequence based analysis (QT-NASBA) [47], and reverse transcriptase loop mediated amplification (RT-LAMP) [48]. Using one of these in conjunction with microscopy would have improved our study and allow us to more accurately measure gametocyte densities.

Moreover, although a number of studies have shown a positive relationship between gametocyte densities and infection outcome in mosquitoes [49, 50, 51, 52, 53, 54, 55, 56, 57], other studies have not [58, 59, 60, 61, 62], and the exact nature of this relationship remains largely unclear [63, 64]. Gametocyte density was included as a fixed-effects explanatory variable in the models described in the thesis but was not found to be significant in any of them. Mosquito infections can result from sub-microscopic gametocytaemias but do not necessarily result from high gametocyte densities [65, 66, 67]. One of the gametocytaemic volunteers recruited in our study had no microscopically detectable gametocytes yet mosquitoes fed on their blood sample became infected.

Furthermore, when mosquitoes feed on blood of naturally infected people, gametocyte densities in the blood meal tend to be low, resulting in lower prevalence and intensity of infection in mosquitoes. A large number of mosquitoes must therefore be used in membrane feeding assays in order to accurately evaluate malaria transmission and any potential transmission blocking effects of a substance being tested [68, 69].

Another of the inclusion criteria for gametocytaemic volunteers was haemoglobin level of > 9.9 g/dl, as measured by HemoCue, to exclude those with moderate and severe anaemia because the clinical officers involved in the study felt that it would not be appropriate to take further blood samples from anaemic patients. Studies show that gametocytaemia and anaemia are positively correlated [70, 71]. However, anaemia may also be correlated with increasing concentrations of transmission-blocking immune factors and can influence transmission [53, 72]. Several properties of blood, including anaemia, can influence mosquito feeding and the size of a blood meal, which can have an impact on vector fitness [73, 74], but feeding on blood of anaemic hosts had no impact on the probability of mosquitoes surviving long enough for malaria parasites to complete their sporogonic development [75]. Haemoglobin level was included as a fixed-effects explanatory variable in the models described in the thesis but was not found to be significant in any of them.

Most previous studies used laboratory-reared mosquitoes that have been adapted to membrane feeding. Such mosquitoes may differ from those in nature due to inbreeding, while it was also shown that local adaptation of parasites to sympatric mosquito vectors probably resulted in better adapted vector–parasite combinations [32]. The use of wild mosquitoes however poses a number of challenges [37]. The main challenge in our study was related to the difficulty of membrane feeding of wild mosquitoes as they are not adapted to experimental membrane feeding. In many instances, refusal of some mosquitoes to feed

resulted in insufficient sample sizes, so experimental data from different insecticide doses had to be pooled for statistical analyses purposes. Yet this is the closest we were able to simulate what would happen under field conditions as controlled trials on the effect of insecticide resistance and malaria infection are not possible. Despite the challenges, we consider the results to reflect natural phenomena at least in the study area.

Mosquito body size can affect both longevity and fecundity of mosquitoes and is thought to be the best predictor of adult fitness [76, 77]. However, the effect of mosquito body size on infection prevalence and intensity is not clear. Although a number of studies found that mosquito body size affects the number of parasites that develop into oocysts, with smaller mosquitoes developing fewer oocysts than larger ones [78, 79, 80], another study found that oocyst prevalence and intensity were not significantly associated with mosquito body size [81]. It has also been shown that mosquito body size can play a role in surviving insecticide exposure, where heavier mosquitoes – especially from a resistant strain – were significantly more likely to survive permethrin exposure [82]. Wing length is often used as a standard indicator of mosquito body size [83]. Wings of each mosquito used in transmission experiments should have been measured to provide additional data. The omission of mosquito body size as a determinant of infection likelihood and insecticide survival therefore represents a drawback of our study.

9.1.2 Potential confounding effects and study limitations

Many endemic malaria settings experience seasonal peaks in rainfall which are followed by peaks in mosquito density and peaks in the number of malaria cases. It has been recognised that both dynamics and distribution of malaria are strongly determined by climatic factors [84], which directly affect mosquitoes and parasites developing in them [85, 86]. However, there is also significant variation in other malaria-related factors that can affect sporogony and malaria transmission.

Season-related patterns in gametocyte prevalence and density were demonstrated in some areas [87, 88, 89, 90]. Although there was some variation in gametocyte densities between the three study rounds in our study, the differences were not significant. Naturally acquired transmission-reducing immune responses, which differ between individuals, were shown to be present in a number of populations in endemic countries, such as the Gambia, Kenya and Cameroon [65]. Whole blood was used in direct membrane feeding assays, without washing or replacing the serum, and these factors, if present, might have affected the outcome of our

transmission experiments. Apart from gametocyte density and transmission-reducing immune responses, other gametocyte-donor related factors such as sex, age, body temperature and use of certain antimalarial drugs, were found not to have a significant effect on the success of experimental infections [57]. Many of these factors were included in our models but were not found to be significant.

Seasonal variation has also been observed in metabolic rate, flight activity, body size and microbiome of vectors, such as *An. coluzzii* in Mali [91, 92]. As mentioned earlier, mosquito body size could affect infection outcome in mosquitoes. Microbiota found in *Anopheles* mosquitoes influence mosquito physiology and fitness, affect their susceptibility to human pathogens and their vectorial capacity [93, 94, 95, 96].

Furthermore, experimental variation can also occur due to the differences in timing of the experiments. Mosquitoes were offered an infectious blood meal at different times during the day, depending on when a suitable volunteer was identified and recruited into the study. This could have had a confounding effect on the outcome of our experiments as studies have shown that daily rhythms in mosquitoes affect metabolic detoxification and insecticide resistance, immunity, and interactions between mosquitoes, hosts and parasites [97, 98, 99]. Although mosquitoes were offered blood within 10 minutes of collection from the volunteer in most of the experiments, blood samples were kept in a water bath for up to 1 ½ hours prior to feeding, which could have an impact on gametocytes and their infectiousness. The length of blood feeding also varied between experiments, as it depended on the willingness of mosquitoes to feed. Although it could affect the experiment outcome it was not significant when included in the models. Although we aimed to expose the mosquitoes to insecticide as soon as possible following the feed, this period varied between 1 – 3 hours, depending on how many mosquitoes fed and therefore had to be sorted into different insecticide exposure groups. Finally, mosquitoes were exposed to insecticides only after the infectious feed, whereas in areas with LLINs, and to some extent IRS, mosquitoes are likely to be exposed to pyrethroids (or insecticides in general) before, during and after blood feeding. Insecticide exposure after the feed was used during field work for practical reasons, as it was not possible to predict on which days and at what time a gametocytaemic volunteer will become available. This decision was also based on the results of the study carried out by Hill (2002) [19] who showed in laboratory experiments using resistant *An. stephensi* mosquitoes and *P. yoelii nigeriensis* parasites that exposure to pyrethroids 24 hours or less before the feed, during the feed, and up to 18-24 hours post-infectious feed had a significant effect on

sporogony. It appears this is mainly determined by parasite development stages with ookinete being the most susceptible stage, and that once oocysts are formed, the parasites are no longer susceptible to insecticides. These experiments will need to be repeated with *P. falciparum* to confirm that the same effects are present when insecticide exposure takes place at different time points in mosquito's gonotrophic cycle. If pyrethroid exposure before, during and after the infectious feed impairs *Plasmodium* sporogony, the results seen in the field should be even more significant.

When data were available (eg. gametocyte density, haemoglobin concentration, whether the volunteer had fever or took antimalarials, length of membrane feed, etc) the factors were included in the models, where relevant. However, some of the data were not available (eg. mosquito body size) yet could have a confounding effect on the outcome of the experiments described and therefore present a limitation of this project.

9.1.3 Effect of environmental variables and *kdr* resistance on survival probability and infection rates in *Anopheles gambiae* s.s.

Environmental factors, especially ambient temperature and relative humidity, affect both mosquitoes and malaria parasites. Temperature affects larval and adult mosquito stages, including mosquito biting rates, blood meal digestion and duration of the gonotrophic cycle, survival, and mosquito immune response [85, 99, 100, 101], which might consequently have an impact on parasite development. Variation in temperature and time of day can alter different aspects of mosquito immunity and physiology [98, 99], and also have an impact on expression of insecticide resistance [102]. Humidity has been shown to be a strong determinant of *kdr* resistance phenotype [103].

Sporogonic development of parasites is also temperature-dependent and it is thought that the effect of temperature is complemented by that of relative humidity [86]. The early part of sporogony, up to the formation of oocysts, is thought to be most sensitive to temperature [86, 99, 101, 104, 105, 106, 107, 108, 109]. Furthermore, high temperatures above 30^{o}C and temperature fluctuation immediately following the infectious blood meal have been shown to be the most damaging for parasites [104, 107]. Additionally, previous studies have shown interactions between pyrethroid exposure and environmental factors such as temperature [110, 111, 112].

Our field study findings indicated that apart from the effect of deltamethrin exposure, the mean ambient temperature during the incubation period had a highly significant effect on the risk of infection [5]. Further analysis of the effects of temperature, relative humidity and the presence of *kdr* mutations on mosquito survival probability and infection rates was carried out, showing that temperature range during the first 24 hours and on day 4 post-infectious feed had a highly significant effect on risk of infection, where an increase in temperature range was associated with lower infection.

Temperature and relative humidity were measured every 30 min throughout the duration of transmission experiments inside the field laboratory using data loggers. Different temperature and humidity variables were then calculated (eg. means during the seven day incubation period, ranges, minimums and maximums, etc), providing environmental data for each individual feed. Forty-two feeds were included in data analyses, providing forty-two different sets of environmental data. As there was significant variability in temperature and humidity between the feeds and also between the study rounds, the environmental variables were included in the analyses, together with other variables such as gametocyte density, haemoglobin concentration, and whether gametocyte donors took antimalarial drugs within seven days prior to the clinic visit. Environmental variables which were included in the models were those recognised to affect sporogony the most, such as average temperature and temperature variations during the first 24 hours post-infectious blood meal and during the entire incubation period (seven days between the infectious blood meal and dissection for oocysts). Studies have shown that different temperature measures impact estimates of extrinsic incubation period (i.e. sporogony) in different ways, with short-term temperature variation potentially having a significantly larger effect than generally thought [113].

Mean daily temperature and mean daily relative humidity during the incubation period also had an independent and highly significant effect on mosquito mortality.

From vector control perspective, it was encouraging to find that deltamethrin significantly impaired survival of *kdr* homozygous mosquitoes. While we were not able to compare survival of all *kdr* genotypes due to low numbers of mosquitoes with a wild type *S* allele in the study area, significant differences in survival of resistant genotypes were detected when they were unexposed or exposed to high doses of deltamethrin. The lowest survival was recorded in mosquitoes with *Re/Rw* genotype, which might be due to fitness costs because of the simultaneous presence of two *kdr* mutations. However, all these mosquitoes were fed

on infected blood, which can impair mosquito fitness, even in the absence of actual mosquito infection [114]. Further studies on the impact of insecticide resistance on malaria infection and vector survival should therefore be carried out, using either blood from uninfected individuals or heat-treated samples which are no longer infectious as controls.

9.1.4 How much insecticide do mosquitoes pick up after contact with treated surfaces

In order to understand potential transmission-blocking effects of pyrethroids, it is important to identify the likely sites of action where parasites could come into contact with doses of insecticides high enough to harm them. These doses would inevitably depend on how much insecticide mosquitoes come into contact with as a result of vector control interventions.

Insecticide doses used in vector control are high enough to kill all susceptible mosquitoes and are based on laboratory tests which determine intrinsic insecticidal activity, diagnostic doses and discriminating concentrations [115, 116, 117]. However, only a few studies have previously attempted to measure the amount of insecticides mosquitoes pick up after contact with treated surfaces [118, 119]. Such information would be useful for the purposes of optimising vector control tools, even more so to take advantage of any potential transmission-blocking characteristics of pyrethroids.

Mosquito – LLIN interactions have been characterized using infrared video tracking [120], showing that the most intense mosquito activity around the net happens during the initial 10 minutes. However, this does not entirely reflect what happens in households occupied in the night by people and domestic animals, or where cooking fire would be used indoors, producing smoke, potentially affecting mosquito behaviour in addition to nets and repellents being used.

Pyrethroids are contact insecticides and must cross the cuticle to reach their target sites [121]. Following tarsal application, insecticide was detected in mosquito body within 15 minutes [122], while previous studies using permethrin show that some insecticide reaches the midgut and can be detected in the blood meal, where it could potentially affect developing parasites, within one hour after feeding [19].

Using HPLC-PDA analysis we showed that mosquitoes pick up approximately 10 ng of deltamethrin following exposure to a standard LLIN (PermaNet 2.0, treated with 55 mg/m^2 deltamethrin), and that the final dose depends on the length of exposure. Furthermore,

colorimetric tests which are used for detection of deltamethrin on ITNs [123] and sprayed walls [124], were successfully used for the first time to detect deltamethrin on mosquitoes following exposure to PermaNet 2.0 net. As new interventions with insecticide mixtures are introduced, detection of active ingredients that cause the greatest mortality may potentially be used to assess the effectiveness of interventions.

The lack of replicates was the main limitation of this study. Using a larger number of samples might have helped us to determine why the disparity between the colorimetric method and HPLC-PDA occurred, and it might have reduced the variation in the amount of deltamethrin detected for each exposure time.

9.1.5 Direct effects of pyrethroids on *Plasmodium* parasites

Apart from the observed effects of pyrethroids, little is known regarding the specific time and developmental stage at which the parasite's transmissibility is affected [19, 25, 26, 27]. The observed effects of sub-lethal doses of pyrethroids on the sporogonic cycle could be caused by one or more of the following mechanisms:

- Direct effect of insecticide on the parasite inside the mosquito via as yet unidentified target site.
- Indirect effect of insecticide on the parasite caused by the physiological and other changes of the environment inside the mosquito as a consequence of insecticide exposure.
- Differential insecticidal killing of infected mosquitoes if infection restores phenotypic susceptibility in resistant mosquitoes.

Apart from affecting the *para* voltage-gated sodium channels (VGSC) on the insect's neurons [125, 126], pyrethroids also target a number of other channels, enzymes and receptors, contributing to their overall toxicity [127, 128, 129, 130]. Some of these can be found on vector-borne parasites as well as vectors, potentially allowing insecticides to also function as antiparasitic substances.

Plasmodium parasites have no known 'receptors' or target sites for pyrethroids. However, deltamethrin could potentially directly affect the parasites in a number of ways. For example, pyrethroids can set off generation of oxygen reactive species (ROS), causing oxidative stress [110], which can act as a trigger for apoptosis of ookinetes [131]. Inhibitory effects of pyrethroids on mitochondrial Complex I have also been observed [128], which

could potentially lead to disruption of sporogony [132]. Furthermore, deltamethrin was shown to be a potent calcium (Ca^{2+}) channel agonist [127] and calcineurin inhibitor [129], both of which have an important role in key transition points of *Plasmodium* life cycle [133, 134, 135]. Furthermore, a limited time period during which pyrethroids were shown to affect parasite development suggests that once parasites are established as oocysts, insecticides no longer have an impact on them [19]. This was why part of the present study also focused on ookinete development and motility.

Plasmodium berghei ookinete cultures were used to investigate whether pyrethroids directly affect the parasites or if the interaction with a mosquito is necessary to produce the effects previously reported in laboratory and field conditions [5, 19, 25, 26, 27]. The results showed that deltamethrin exposure significantly impairs both ookinete conversion and motility at doses that are close to what parasites would be exposed to through mosquitoes in the field. Further studies will be required to unravel the underlying mechanisms and explore how the effects observed at ookinete stage manifest at oocyst and sporozoite stage. Because deltamethrin was shown to affect ookinete conversion rates and motility in a concentration-dependent manner, more precise measurements of insecticide quantities on mosquito abdomens should be carried out in the future, especially using mosquitoes exposed to LLINs in the field.

While production of *P. falciparum* ookinetes in culture has been described [136], it is not as successful and as routinely used as *P. berghei* ookinete cultures [137]. Because of the differences between human and rodent malaria parasites – including different external temperature requirements during sporogony of both *Plasmodium* species - and possible effects of unnatural vector-parasite combinations [28], similar experiments should be repeated with *P. falciparum*, both *in vitro* and *in vivo.*

9.1.6 Effect of *kdr* resistance genotype on malaria infection rates in *Anopheles gambiae* s.s.

Plasmodium parasites must pass through tissues, such as the midgut wall and the salivary gland wall in the mosquito, to complete their sporogonic cycle. Insecticide resistance mechanisms may affect vector competence through modifications of the environment the pathogens are exposed to within vectors and might impair fitness of resistant vectors [138, 139, 140]. It is therefore possible that parasite survival and transmission may be directly affected by the insecticide resistance status of insects [141]. For example, it has been

reported that esterase-based insecticide resistance in mosquitoes can interfere with development of the filarial worm *Wuchereria bancrofti* [142].

Laboratory studies using *An. gambiae* s.s. mosquitoes with a *L1014F-kdr* mutation showed that they may be more susceptible to *P. falciparum* infection [23, 24, 143], although results from different studies were conflicting in terms of the effect on infection intensity. While field observations from Bioko Island showed that sporozoite rates were lower in mosquitoes homozygous for *kdr* than in heterozygous and non-*kdr* mosquitoes [144], a study from Tanzania showed that significantly higher sporozoite rates were detected in *kdr*-homozygous mosquitoes compared to mosquitoes without *kdr* mutation, with heterozygotes showing intermediate sporozoite rates [145].

The study on the association of *kdr* and infection rates was intended to explore the effect of insecticide resistance on *P. falciparum* sporogony, to better understand the impact of resistance in the field. Oocyst prevalence rates and infection intensity in *An. gambiae* s.s. and *An. arabiensis* were not significantly different between *kdr* genotypes, while sporozoite rates in *An. gambiae* s.s. were also not significantly different between *kdr* genotypes. As mentioned previously, due to low numbers of mosquitoes with a wild type *S* allele in the study area we were not able to compare the effect of all *kdr* genotypes on malaria infection or on survival in mosquitoes.

Different resistance mechanisms can be present in wild mosquitoes simultaneously, and some of the resistance alleles can become fixed, making the ultimate determination of the impact of resistance on *Plasmodium* infection and malaria transmission difficult. As DNA markers for metabolic resistance become available [103] it will be easier to have a complete picture of resistance mechanisms present in individual mosquitoes, together with their phenotype, and to interpret results of studies such as this one.

9.1.7 Variations in phenotypic and genetic resistance against commonly used insecticides among *An. gambiae* s.s. and *An. arabiensis* in mid-western Uganda

The impact of insecticide resistance on malaria transmission does not appear to be uniform in all affected areas and cannot be generalized due to varying conditions such as the presence of different vector and parasite species and strains, local climate conditions, ecological interactions between vectors, parasites and the environment, the presence of

different types of insecticide resistance mechanisms, local population characteristics, and the use of vector control measures [146].

Insecticide resistance is caused by a number of mechanisms [6, 147]. Whether a local vector population is resistant is usually assessed by WHO susceptibility tests, which discriminate between susceptible and resistant phenotypes in a given mosquito population [117]. Molecular tools which detect mutations such as the genotype at the *kdr* locus [148] do not automatically indicate the presence of resistance, and are most commonly used in addition to the WHO susceptibility tests [149]. Moreover, the presence of *kdr* genotype might explain only a portion of variation in resistance due to the role additional resistance mechanisms may play [150].

Insecticide resistance is widespread in Uganda [15, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160] and was previously detected in Kyankwanzi [151, 153]. This part of the present study examined *kdr*-associated insecticide resistance in *An. gambiae* s.s. and *An. arabiensis* in Kyankwanzi, mid-western Uganda, and explored the relationship between bioassay survival and genotype at the *kdr* locus.

Both *kdr* mutations, *L1014S (Re)* and *L1014F (Rw)* were found in the two sibling species, but the frequencies of *Re* and *Rw* resistant alleles were significantly higher in *An. gambiae* s.s. than in *An. arabiensis.* The latter tends to be less endophilic and endophagic than *An. gambiae* s.s. and is less likely to be exposed to insecticides in ITNs. In *An. gambiae* s.s. *Re* mutation was present at high frequencies, approaching fixation, with the *Re/Re* homozygotes increasing from 80% to 90%. A double resistant heterozygote genotype (*Re/Rw*) was also detected in *An. gambiae* s.s. in all three rounds, reaching 9% frequency in round 3.

Observed survival rates in *An. gambiae* s.l. following bioassay exposure to pyrethroids and DDT indicate resistance to all of these insecticides. All *An. gambiae* s.s. that survived the bioassays had at least one copy of either *Re* or *Rw* alleles, yet a significant association was detected only between the *Re* mutation and DDT resistance. Frequencies of the *Rw* allele were higher in resistant than susceptible mosquitoes and were associated with resistance to deltamethrin.

On the contrary, while in *An. arabiensis* the wild type *S* allele remained dominant (frequencies between 83% and 94%), high levels of bioassay survival were observed with both permethrin (mortality 83%) and lambda-cyhalothrin (mortality 62%).

Bioassay mortality was only weakly associated with *kdr* genotype in both sibling species. It is therefore likely that other metabolic resistance mechanisms play a significant role.

The findings of this study add some further information to the wider picture on insecticide resistance in Uganda. Continued surveillance of vector populations for resistance monitoring purposes will be needed to detect any additional changes and modify the response of the malaria vector control programme accordingly.

9.2 Future work and recommendations

The main finding of this project is that exposure to the pyrethroid insecticide deltamethrin affects development of *Plasmodium falciparum* inside wild pyrethroid resistant *Anopheles gambiae* s.s. mosquitoes in field conditions, reducing both infection prevalence and intensity [5], while the results of laboratory experiments indicate that ookinetes might be the stage which is affected by insecticides.

However, a number of questions regarding the effect of exposure to pyrethroids remain unanswered. Pyrethroids are also increasingly being used in combination with synergists (e.g. piperonyl-butoxide, PBO) or non-pyrethroid insecticides such as chlorfenapyr on new-generation LLINs. The effect of these additional compounds on *Plasmodium* sporogony is unknown.

Previous studies [19] show that pyrethroids significantly affect sporogony only if exposure takes place 24 hours or less prior to the infective feed, during the feed, or up to 18 hours after the feed. Once oocysts are formed, at around 24 hrs post infective blood meal, the insecticides seem to no longer have an impact on the parasites. Further experiments should be carried out to determine if exposure to insecticides at a later stage in sporogonic cycle affects mature oocysts and even sporozoites, and whether additional insecticide exposure post infection has a further protective effect.

Although the present study found that exposure to sub-lethal doses of deltamethrin resulted in reduced prevalence and intensity of infection at the oocyst stage, it is important to understand whether surviving oocysts produce viable sporozoites and whether the number of sporozoites produced per oocyst is also affected.

The results obtained so far show that the effect of pyrethroids is dose-dependent. Further work should be carried out in the field with repeated exposure to a wider range of sub-lethal doses.

The laboratory experiments to study whether pyrethroids affect the developing parasites directly were carried out using cultures of the rodent parasite *P. berghei.* While the results indicate that ookinetes might be affected by insecticides, further experiments using *P. falciparum* malaria parasites will be required to confirm the findings.

Further work should also be carried out using the synergist PBO and non-pyrethroid insecticides such as indoxacarb, chlorfenapyr, pyriproxyfen, clothianidin, to study their potential effects on sporogony.

Finally, the effect of insecticide resistance on sporogony should be addressed. Metabolic resistance mechanisms were shown to affect the development of filarial worms in their mosquito vectors [140] and are due to their nature also most likely to change the internal environment in mosquitoes, which parasites are exposed to.

These additional studies will help us to increase further our understanding of the impact of resistance on malaria transmission and vector control measures.

9.3 Conclusions

If pyrethroids or other insecticides impair the parasite's development within the mosquito, then insecticide resistance would pose less of a threat and interventions such as pyrethroid-based LLINs could continue to be used at least until alternative cost-effective tools or compounds are available. Where vector populations are resistant, pyrethroid-based interventions might no longer reduce vector density and survival substantially, but they might still have an impact on transmission through the effect of insecticides on the parasite.

The issue of pyrethroids and pyrethroid resistance is currently a global priority, owing to the importance of these chemicals in malaria prevention and control. While efforts in the search for alternative chemicals are continuing, sound decisions are needed in the immediate future regarding the continued use of proven interventions. Increased understanding of the additional effects of pyrethroids and insecticide resistance on malaria transmission will have obvious practical significance in making such decisions.

9.4 References

1. 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.

2. Hemingway J, Ranson H, Magill A, Kolaczinski J, Fornadel C, Gimnig J*, et al*. Averting a malaria disaster: will insecticide resistance derail malaria control? Lancet. 2016;387:1785-1788.

3. Ranson H, Lissenden N. Insecticide resistance in African *Anopheles* mosquitoes: a worsening situation that needs urgent action to maintain malaria control. Trends Parasitol. 2016;32:187-196.

4. World Health Organization. Global Plan for Insecticide Resistance Management in Malaria Vectors. Geneva: World Health Organization; 2012.

5. Kristan M, Lines J, Nuwa A, Ntege C, Meek SR, Abeku TA. Exposure to deltamethrin affects development of *Plasmodium falciparum* inside wild pyrethroid resistant *Anopheles gambiae* s.s. mosquitoes in Uganda. Parasit Vectors. 2016;9:100.

6. Hemingway J, Hawkes NJ, McCarroll L, Ranson H. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem Mol Biol. 2004;34:653-665.

7. Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Berge JB, Devonshire AL*, et al*. Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae* s.s. Insect Mol Biol. 1998;7:179-184.

8. Ranson H, Jensen B, Vulule JM, Wang X, Hemingway J, Collins FH. Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. Insect Mol Biol. 2000;9:491-497.

9. World Health Organization. World Malaria Report 2017. Geneva: World Health Organization; 2017.

10. 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 Vectors. 2014;7:76.

11. VectorBase. https://www.vectorbase.org/popbio.

12. IR Mapper. http://www.irmapper.com/.

13. Corbel V, Chandre F, Brengues C, Akogbeto M, Lardeux F, Hougard JM*, et al*. Dosage-dependent effects of permethrin-treated nets on the behaviour of *Anopheles gambiae* and the selection of pyrethroid resistance. Malar J. 2004;3:22.

14. Kleinschmidt I, Mnzava AP, Kafy HT, Mbogo C, Bashir AI, Bigoga J*, et al*. Design of a study to determine the impact of insecticide resistance on malaria vector control: a multi-country investigation. Malar J. 2015;14:282.

15. Okia M, Ndyomugyenyi R, Kirunda J, Byaruhanga A, Adibaku S, Lwamafa DK*, et al*. Bioefficacy of long-lasting insecticidal nets against pyrethroid-resistant populations of *Anopheles gambiae* s.s. from different malaria transmission zones in Uganda. Parasit Vectors. 2013;6:130.

16. Tokponnon FT, Ogouyemi AH, Sissinto Y, Sovi A, Gnanguenon V, Cornelie S*, et al*. Impact of long-lasting, insecticidal nets on anaemia and prevalence of *Plasmodium falciparum* among children under five years in areas with highly resistant malaria vectors. Malar J. 2014;13:76.

17. World Health Organization: Implications of insecticide resistance for malaria vector control. Geneva: World Health Organization; 2016: 4.

18. Alout H, Djegbe I, Chandre F, Djogbenou LS, Dabire RK, Corbel V*, et al*. Insecticide exposure impacts vector-parasite interactions in insecticide-resistant malaria vectors. Proc Biol Sci. 2014;281.

19. Hill N. Effects of sublethal doses of pyrethroids on malaria vectors. *PhD Thesis.* London: London School of Hygiene & Tropical Medicine, University of London; 2002.

20. Prasittisuk C, Curtis CF. Absence of effects of insecticides on susceptibility of anophelines to *Plasmodium yoelii nigeriensis*. Southeast Asian J Trop Med Public Health. 1982;13:127-132.

21. Rifaat MA, Khalil HM, Gad AM, Sadek S. Effect of sublethal concentrations of the insecticides DDT, Abate and Sevin applied to 3rd stage larvae of *Anopheles pharoensis* on malaria cycle in the adult mosquito. J Egypt Public Health Assoc. 1974;49:329-340.

22. Alout H, Yameogo B, Djogbenou LS, Chandre F, Dabire RK, Corbel V*, et al*. Interplay between *Plasmodium* infection and resistance to insecticides in vector mosquitoes. J Infect Dis. 2014;210:1464-1470.

23. Alout H, Ndam NT, Sandeu MM, Djegbe I, Chandre F, Dabire RK*, et al*. Insecticide resistance alleles affect vector competence of *Anopheles gambiae* s.s. for *Plasmodium falciparum* field isolates. PLoS One. 2013;8:e63849.

24. Ndiath MO, Cailleau A, Diedhiou SM, Gaye A, Boudin C, Richard V*, et al*. Effects of the *kdr* resistance mutation on the susceptibility of wild *Anopheles gambiae* populations to *Plasmodium falciparum*: a hindrance for vector control. Malar J. 2014;13:340.

25. Carle PR, Coz J, Elissa N, Gasquet M, Sannier C, Richard A*, et al*. [Intravector anti-*Plasmodium* activity of a pyrethrinoid: deltamethrin]. C R Acad Sci III. 1986;303:565-568.

26. Elissa N, Sannier C, Coz J. [Étude de la réceptivité à *Plasmodium yoelii yoelii* d'*Anopheles stephensi* soumis au stade adulte à des doses toxiques de différents pyréthrinoïdes]. Cah ORSTOM, ser Ent méd et Parasitol. 1987;25:187-192.

27. Elissa N, Sannier C, Richard A: [La Receptivite a *Plasmodium yoelii yoelii* d'*Anopheles* Soumis a des Subletales de Deltamethrine]. In: *IVe Congres Sur La Protection De La Sante Humaine Et Des Cultures En Milieu Tropical*. Marseille, France; 1986: 599-604.

28. Aguilar R, Dong Y, Warr E, Dimopoulos G. *Anopheles* infection responses; laboratory models versus field malaria transmission systems. Acta Trop. 2005;95:285-291.

29. Vaughan JA, Hensley L, Beier JC. Sporogonic development of *Plasmodium yoelii* in five anopheline species. J Parasitol. 1994;80:674-681.

30. Vaughan JA, Noden BH, Beier JC. Sporogonic development of cultured *Plasmodium falciparum* in six species of laboratory-reared *Anopheles* mosquitoes. Am J Trop Med Hyg. 1994;51:233-243.

31. Ramsdale CD, Coluzzi M. Studies on the infectivity of tropical African strains of *Plasmodium falciparum* to some southern European vectors of malaria. Parassitologia. 1975;17:39-48.

32. Harris C, Morlais I, Churcher TS, Awono-Ambene P, Gouagna LC, Dabire RK*, et al*. *Plasmodium falciparum* produce lower infection intensities in local versus foreign *Anopheles gambiae* populations. PLoS One. 2012;7:e30849.

33. Beier JC, Beier MS, Vaughan JA, Pumpuni CB, Davis JR, Noden BH. Sporozoite transmission by *Anopheles freeborni* and *Anopheles gambiae* experimentally infected with *Plasmodium falciparum*. J Am Mosq Control Assoc. 1992;8:404-408.

34. Beier JC, Copeland RS, Mtalib R, Vaughan JA. Ookinete rates in Afrotropical anopheline mosquitoes as a measure of human malaria infectiousness. Am J Trop Med Hyg. 1992;47:41-46.

35. Harris C, Lambrechts L, Rousset F, Abate L, Nsango SE, Fontenille D*, et al*. Polymorphisms in *Anopheles gambiae* immune genes associated with natural resistance to *Plasmodium falciparum*. PLoS Pathog. 2010;6:e1001112.

36. Lambrechts L, Halbert J, Durand P, Gouagna LC, Koella JC. Host genotype by parasite genotype interactions underlying the resistance of anopheline mosquitoes to *Plasmodium falciparum*. Malar J. 2005;4:3.

37. Bousema T, Churcher TS, Morlais I, Dinglasan RR. Can field-based mosquito feeding assays be used for evaluating transmission-blocking interventions? Trends Parasitol. 2013;29:53-59.

38. Henry MC, Assi SB, Rogier C, Dossou-Yovo J, Chandre F, Guillet P*, et al*. Protective efficacy of lambda-cyhalothrin treated nets in *Anopheles gambiae* pyrethroid resistance areas of Cote d'Ivoire. Am J Trop Med Hyg. 2005;73:859-864.

39. Lindblade KA, Mwandama D, Mzilahowa T, Steinhardt L, Gimnig J, Shah M*, et al*. A cohort study of the effectiveness of insecticide-treated bed nets to prevent malaria in an area of moderate pyrethroid resistance, Malawi. Malar J. 2015;14:31.

40. Tokponnon FT, Ogouyemi AH, Sissinto Y, Sovi A, Gnanguenon V, Cornelie S*, et al*. Impact of long-lasting, insecticidal nets on anaemia and prevalence of *Plasmodium falciparum* among children under five years in areas with highly resistant malaria vectors. Malar J. 2014;13:76.

41. 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 U S A. 2012;109:19063-19070.

42. World Health Organization: Malaria parasite counting. Malaria microscopy standard operating procedure - MM-SOP-09. Geneva: World Health Organization; 2016: 5.

43. Greenwood BM, Armstrong JR. Comparison of two simple methods for determining malaria parasite density. Trans R Soc Trop Med Hyg. 1991;85:186-188.

44. McKenzie FE, Prudhomme WA, Magill AJ, Forney JR, Permpanich B, Lucas C*, et al*. White blood cell counts and malaria. J Infect Dis. 2005;192:323-330.

45. Okell LC, Ghani AC, Lyons E, Drakeley CJ. Submicroscopic infection in *Plasmodium falciparum*-endemic populations: a systematic review and meta-analysis. J Infect Dis. 2009;200:1509-1517.

46. Babiker HA, Abdel-Wahab A, Ahmed S, Suleiman S, Ranford-Cartwright L, Carter R*, et al*. Detection of low level *Plasmodium falciparum* gametocytes using reverse transcriptase polymerase chain reaction. Mol Biochem Parasitol. 1999;99:143-148.

47. Schneider P, Schoone G, Schallig H, Verhage D, Telgt D, Eling W*, et al*. Quantification of *Plasmodium falciparum* gametocytes in differential stages of development by quantitative nucleic acid sequence-based amplification. Mol Biochem Parasitol. 2004;137:35-41.

48. Buates S, Bantuchai S, Sattabongkot J, Han ET, Tsuboi T, Udomsangpetch R*, et al*. Development of a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) for clinical detection of *Plasmodium falciparum* gametocytes. Parasitol Int. 2010;59:414-420.

49. Bonnet S, Gouagna C, Safeukui I, Meunier JY, Boudin C. Comparison of artificial membrane feeding with direct skin feeding to estimate infectiousness of *Plasmodium falciparum* gametocyte carriers to mosquitoes. Trans R Soc Trop Med Hyg. 2000;94:103-106.

50. Boudin C, Van Der Kolk M, Tchuinkam T, Gouagna C, Bonnet S, Safeukui I*, et al*. *Plasmodium falciparum* transmission blocking immunity under conditions of low and high endemicity in Cameroon. Parasite Immunol. 2004;26:105-110.

51. Carter R, Graves PM. Gametocytes. Malaria: Principles and Practice of Malariology. Edinburgh: Churchill Livingstone; 1988. p. 253-305.

52. Collins WE, Jeffery GM. A retrospective examination of mosquito infection on humans infected with *Plasmodium falciparum*. Am J Trop Med Hyg. 2003;68:366-371.

53. Drakeley CJ, Secka I, Correa S, Greenwood BM, Targett GA. Host haematological factors influencing the transmission of *Plasmodium falciparum* gametocytes to *Anopheles gambiae* s.s. mosquitoes. Trop Med Int Health. 1999;4:131-138.

54. Gouagna LC, Bonnet S, Gounoue R, Tchuinkam T, Safeukui I, Verhave JP*, et al*. The use of anti-Pfs 25 monoclonal antibody for early determination of *Plasmodium falciparum* oocyst infections in *Anopheles gambiae*: comparison with the current technique of direct microscopic diagnosis. Exp Parasitol. 1999;92:209-214.

55. Gouagna LC, Mulder B, Noubissi E, Tchuinkam T, Verhave JP, Boudin C. The early sporogonic cycle of *Plasmodium falciparum* in laboratory-infected *Anopheles gambiae*: an estimation of parasite efficacy. Trop Med Int Health. 1998;3:21-28.

56. Mulder B, Tchuinkam T, Dechering K, Verhave JP, Carnevale P, Meuwissen JH*, et al*. Malaria transmission-blocking activity in experimental infections of *Anopheles gambiae* from naturally infected *Plasmodium falciparum* gametocyte carriers. Trans R Soc Trop Med Hyg. 1994;88:121-125.

57. Tchuinkam T, Mulder B, Dechering K, Stoffels H, Verhave JP, Cot M*, et al*. Experimental infections of *Anopheles gambiae* with *Plasmodium falciparum* of naturally infected gametocyte carriers in Cameroon: factors influencing the infectivity to mosquitoes. Trop Med Parasitol. 1993;44:271-276.

58. Boudin C, Olivier M, Molez JF, Chiron JP, Ambroise-Thomas P. High human malarial infectivity to laboratory-bred *Anopheles gambiae* in a village in Burkina Faso. Am J Trop Med Hyg. 1993;48:700-706.

59. Graves PM. Studies on the use of a membrane feeding technique for infecting *Anopheles gambiae* with *Plasmodium falciparum*. Trans R Soc Trop Med Hyg. 1980;74:738-742.

60. Graves PM, Burkot TR, Carter R, Cattani JA, Lagog M, Parker J*, et al*. Measurement of malarial infectivity of human populations to mosquitoes in the Madang area, Papua, New Guinea. Parasitology. 1988;96 (Pt 2):251-263.

61. Shute PG, Maryon M. A study of gametocytes in a West African strain of *Plasmodium falciparum*. Trans R Soc Trop Med Hyg. 1951;44:421-438.

62. Muirhead-Thomson RC. Factors determining the true reservoir of infection of *Plasmodium falciparum* and *Wuchereria bancrofti* in a West African village. Trans R Soc Trop Med Hyg. 1954;48:208-225.

63. Churcher TS, Bousema T, Walker M, Drakeley C, Schneider P, Ouedraogo AL*, et al*. Predicting mosquito infection from *Plasmodium falciparum* gametocyte density and estimating the reservoir of infection. eLife. 2013;2:e00626.

64. Da DF, Churcher TS, Yerbanga RS, Yameogo B, Sangare I, Ouedraogo JB*, et al*. Experimental study of the relationship between *Plasmodium* gametocyte density and infection success in mosquitoes; implications for the evaluation of malaria transmission-reducing interventions. Exp Parasitol. 2015;149:74-83.

65. Bousema T, Drakeley C. Epidemiology and infectivity of *Plasmodium falciparum* and *Plasmodium vivax* gametocytes in relation to malaria control and elimination. Clin Microbiol Rev. 2011;24:377-410.

66. Paul RE, Bonnet S, Boudin C, Tchuinkam T, Robert V. Aggregation in malaria parasites places limits on mosquito infection rates. Infect Genet Evol. 2007;7:577-586.

67. Rosenberg R. Malaria: some considerations regarding parasite productivity. Trends Parasitol. 2008;24:487-491.

68. Goncalves BP, Kapulu MC, Sawa P, Guelbeogo WM, Tiono AB, Grignard L*, et al*. Examining the human infectious reservoir for *Plasmodium falciparum* malaria in areas of differing transmission intensity. Nat Commun. 2017;8:1133.

69. Okell LC, Bousema T, Griffin JT, Ouedraogo AL, Ghani AC, Drakeley CJ. Factors determining the occurrence of submicroscopic malaria infections and their relevance for control. Nat Commun. 2012;3:1237.

70. Price R, Nosten F, Simpson JA, Luxemburger C, Phaipun L, ter Kuile F*, et al*. Risk factors for gametocyte carriage in uncomplicated *falciparum* malaria. Am J Trop Med Hyg. 1999;60:1019-1023.

71. von Seidlein L, Drakeley C, Greenwood B, Walraven G, Targett G. Risk factors for gametocyte carriage in Gambian children. Am J Trop Med Hyg. 2001;65:523-527.

72. Cameron A, Reece SE, Drew DR, Haydon DT, Yates AJ. Plasticity in transmission strategies of the malaria parasite, *Plasmodium chabaudi*: environmental and genetic effects. Evol Appl. 2013;6:365-376.

73. Ferguson HM, Rivero A, Read AF. The influence of malaria parasite genetic diversity and anaemia on mosquito feeding and fecundity. Parasitology. 2003;127:9-19.

74. Taylor PJ, Hurd H. The influence of host haematocrit on the blood feeding success of *Anopheles stephensi*: implications for enhanced malaria transmission. Parasitology. 2001;122:491-496.

75. Emami SN, Ranford-Cartwright LC, Ferguson HM. The impact of low erythrocyte density in human blood on the fitness and energetic reserves of the African malaria vector *Anopheles gambiae*. Malar J. 2013;12:45.

76. Russell TL, Lwetoijera DW, Knols BG, Takken W, Killeen GF, Ferguson HM. Linking individual phenotype to density-dependent population growth: the influence of body size on the population dynamics of malaria vectors. Proc Biol Sci. 2011;278:3142-3151.

77. Sangare I, Michalakis Y, Yameogo B, Dabire R, Morlais I, Cohuet A. Studying fitness cost of *Plasmodium falciparum* infection in malaria vectors: validation of an appropriate negative control. Malar J. 2013;12:2.

78. Lyimo EO, Koella JC. Relationship between body size of adult *Anopheles gambiae* s.l. and infection with the malaria parasite *Plasmodium falciparum*. Parasitology. 1992;104 (Pt 2):233-237.

79. Okech BA, Gouagna LC, Yan G, Githure JI, Beier JC. Larval habitats of *Anopheles gambiae* s.s. (Diptera: Culicidae) influences vector competence to *Plasmodium falciparum* parasites. Malar J. 2007;6:50.

80. Takken W, Smallegange RC, Vigneau AJ, Johnston V, Brown M, Mordue-Luntz AJ*, et al*. Larval nutrition differentially affects adult fitness and *Plasmodium* development in the malaria vectors *Anopheles gambiae* and *Anopheles stephensi*. Parasit Vectors. 2013;6:345.

81. Emami SN, Ranford-Cartwright LC, Ferguson HM. The transmission potential of malaria-infected mosquitoes (*An.gambiae*-Keele, *An.arabiensis*-Ifakara) is altered by the vertebrate blood type they consume during parasite development. Sci Rep. 2017;7:40520.

82. Owusu HF, Chitnis N, Muller P. Insecticide susceptibility of *Anopheles* mosquitoes changes in response to variations in the larval environment. Sci Rep. 2017;7:3667.

83. Takken W, Klowden MJ, Chambers GM. Effect of body size on host seeking and blood meal utilization in *Anopheles gambiae* sensu stricto (Diptera: Culicidae): the disadvantage of being small. J Med Entomol. 1998;35:639-645.

84. Rogers DJ, Randolph SE. Climate change and vector-borne diseases. Adv Parasitol. 2006;62:345-381.

85. Afrane YA, Githeko AK, Yan G. The ecology of *Anopheles* mosquitoes under climate change: case studies from the effects of deforestation in East African highlands. Ann N Y Acad Sci. 2012;1249:204-210.

86. Boyd MF. Malariology: A Comprehensive Survey of All Aspects of this Group of Diseases from a Global Standpoint. Philadelphia and London: W. B. Saunders & Co.; 1949.

87. Nacher M, Carrara VI, Ashley E, McGready R, Hutagalung R, Nguen JV*, et al*. Seasonal variation in hyperparasitaemia and gametocyte carriage in patients with *Plasmodium falciparum* malaria on the Thai-Burmese border. Trans R Soc Trop Med Hyg. 2004;98:322-328.

88. Ouedraogo AL, de Vlas SJ, Nebie I, Ilboudo-Sanogo E, Bousema JT, Ouattara AS*, et al*. Seasonal patterns of *Plasmodium falciparum* gametocyte prevalence and density in a rural population of Burkina Faso. Acta Trop. 2008;105:28-34.

89. van der Kolk M, Tebo AE, Nimpaye H, Ndombol DN, Sauerwein RW, Eling WM. Transmission of *Plasmodium falciparum* in urban Yaounde, Cameroon, is seasonal and age-dependent. Trans R Soc Trop Med Hyg. 2003;97:375-379.

90. Gadalla AA, Schneider P, Churcher TS, Nassir E, Abdel-Muhsin AA, Ranford-Cartwright LC*, et al*. Associations between season and gametocyte dynamics in chronic *Plasmodium falciparum* infections. PLoS One. 2016;11:e0166699.

91. Huestis DL, Yaro AS, Traore AI, Dieter KL, Nwagbara JI, Bowie AC*, et al*. Seasonal variation in metabolic rate, flight activity and body size of *Anopheles gambiae* in the Sahel. J Exp Biol. 2012;215:2013-2021.

92. Krajacich BJ, Huestis DL, Dao A, Yaro AS, Diallo M, Krishna A*, et al*. Investigation of the seasonal microbiome of *Anopheles coluzzii* mosquitoes in Mali. PLoS One. 2018;13:e0194899.

93. Linenberg I, Christophides GK, Gendrin M. Larval diet affects mosquito development and permissiveness to *Plasmodium infection*. Sci Rep. 2016;6:38230.

94. Romoli O, Gendrin M. The tripartite interactions between the mosquito, its microbiota and *Plasmodium*. Parasit Vectors. 2018;11:200.

95. Dennison NJ, Jupatanakul N, Dimopoulos G. The mosquito microbiota influences vector competence for human pathogens. Curr Opin Insect Sci. 2014;3:6-13.

96. Dennison NJ, BenMarzouk-Hidalgo OJ, Dimopoulos G. MicroRNA-regulation of *Anopheles gambiae* immunity to *Plasmodium falciparum* infection and midgut microbiota. Dev Comp Immunol. 2015;49:170-178.

97. Rund SS, O'Donnell AJ, Gentile JE, Reece SE. Daily rhythms in mosquitoes and their consequences for malaria transmission. Insects. 2016;7.

98. Balmert NJ, Rund SS, Ghazi JP, Zhou P, Duffield GE. Time-of-day specific changes in metabolic detoxification and insecticide resistance in the malaria mosquito *Anopheles gambiae*. J Insect Physiol. 2014;64:30-39.

99. Murdock CC, Paaijmans KP, Bell AS, King JG, Hillyer JF, Read AF*, et al*. Complex effects of temperature on mosquito immune function. Proc Biol Sci. 2012;279:3357-3366.

100. Murdock CC, Moller-Jacobs LL, Thomas MB. Complex environmental drivers of immunity and resistance in malaria mosquitoes. Proc Biol Sci. 2013;280:20132030.

101. Murdock CC, Paaijmans KP, Cox-Foster D, Read AF, Thomas MB. Rethinking vector immunology: the role of environmental temperature in shaping resistance. Nat Rev Microbiol. 2012;10:869-876.

102. Glunt KD, Paaijmans KP, Read AF, Thomas MB. Environmental temperatures significantly change the impact of insecticides measured using WHOPES protocols. Malar J. 2014;13:350.

103. Weetman D, Wilding CS, Neafsey DE, Muller P, Ochomo E, Isaacs AT*, et al*. Candidate-gene based GWAS identifies reproducible DNA markers for metabolic pyrethroid resistance from standing genetic variation in East African *Anopheles gambiae*. Sci Rep. 2018;8:2920.

104. Eling W, Hooghof J, van de Vegte-Bolmer M, Sauerwein R, van Gemert G-J. Tropical temperatures can inhibit development of the human malaria parasite *Plasmodium falciparum* in the mosquito. Proc Exper Appl Entomol. 2001;12:151-156.

105. Noden BH, Kent MD, Beier JC. The impact of variations in temperature on early *Plasmodium falciparum* development in *Anopheles stephensi*. Parasitology. 1995;111 (Pt 5):539-545.

106. Okech BA, Gouagna LC, Kabiru EW, Walczak E, Beier JC, Yan G*, et al*. Resistance of early midgut stages of natural *Plasmodium falciparum* parasites to high temperatures in experimentally infected *Anopheles gambiae* (Diptera: Culicidae). J Parasitol. 2004;90:764-768.

107. Paaijmans KP, Blanford S, Bell AS, Blanford JI, Read AF, Thomas MB. Influence of climate on malaria transmission depends on daily temperature variation. Proc Natl Acad Sci U S A. 2010;107:15135-15139.

108. Rastogi M, Pal NL, Sen AB. Effect of variation in temperature on development of *Plasmodium berghei* (NK 65 strain) in *Anopheles stephensi*. Folia Parasitol (Praha). 1987;34:289-297.

109. Vanderberg JP, Yoeli M. Effects of temperature on sporogonic development of *Plasmodium berghei*. J Parasitol. 1966;52:559-564.

110. Tu HT, Silvestre F, Meulder BD, Thome JP, Phuong NT, Kestemont P. Combined effects of deltamethrin, temperature and salinity on oxidative stress biomarkers and acetylcholinesterase activity in the black tiger shrimp (*Penaeus monodon*). Chemosphere. 2012;86:83-91.

111. Cutkomp LK, Subramanyam B. Toxicity of pyrethroids to *Aedes aegypti* larvae in relation to temperature. J Am Mosq Control Assoc. 1986;2:347-349.

112. Hodjati MH, Curtis CF. Effects of permethrin at different temperatures on pyrethroid-resistant and susceptible strains of *Anopheles*. Med Vet Entomol. 1999;13:415-422.

113. Blanford JI, Blanford S, Crane RG, Mann ME, Paaijmans KP, Schreiber KV*, et al*. Implications of temperature variation for malaria parasite development across Africa. Sci Rep. 2013;3:1300.

114. Ferguson HM, Mackinnon MJ, Chan BH, Read AF. Mosquito mortality and the evolution of malaria virulence. Evolution. 2003;57:2792-2804.

115. World Health Organization: Guidelines for laboratory and field testing of long-lasting insecticidal nets. 2013: vii, 89.

116. World Health Organization: Guidelines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets. 2006: viii, 60.

117. World Health Organization: Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. 2nd edn. Geneva, Switzerland: World Health Organization; 2016.

118. Pennell JT, Miskus R, Craig R. The use of gas chromatography for the quantitative determination of micro-amounts of insecticide picked up by mosquitos. Bull World Health Organ. 1964;30:91-95.

119. Smith A, Webley DJ. A verandah-trap hut for studying the house-frequenting habits of mosquitoes and for assessing insecticides. 3. The effect of DDT on behavior and mortality. Bull Entomol Res. 1968;59:33-46.

120. Parker JE, Angarita-Jaimes N, Abe M, Towers CE, Towers D, McCall PJ. Infrared video tracking of *Anopheles gambiae* at insecticide-treated bed nets reveals rapid decisive impact after brief localised net contact. Sci Rep. 2015;5:13392.

121. Gerolt P. Insecticides: their route of entry, mechanism of transport and mode of action. Biol Rev Camb Philos Soc. 1983;58:233-274.

122. Yahouedo GA, Chandre F, Rossignol M, Ginibre C, Balabanidou V, Mendez NGA*, et al*. Contributions of cuticle permeability and enzyme detoxification to pyrethroid resistance in the major malaria vector *Anopheles gambiae*. Sci Rep. 2017;7:11091.

123. Green MD, Atieli F, Akogbeto M. Rapid colorimetric field test to determine levels of deltamethrin on PermaNet surfaces: association with mosquito bioactivity. Trop Med Int Health. 2009;14:381-388.

124. Russell TL, Morgan JC, Ismail H, Kaur H, Eggelte T, Oladepo F*, et al*. Evaluating the feasibility of using insecticide quantification kits (IQK) for estimating cyanopyrethroid levels for indoor residual spraying in Vanuatu. Malar J. 2014;13:178.

125. Davies TG, Field LM, Usherwood PN, Williamson MS. DDT, pyrethrins, pyrethroids and insect sodium channels. IUBMB Life. 2007;59:151-162.

126. Soderlund DM, Bloomquist JR. Neurotoxic actions of pyrethroid insecticides. Annu Rev Entomol. 1989;34:77-96.

127. Symington SB, Zhang A, Karstens W, Van Houten J, Clark JM. Characterization of pyrethroid action on ciliary calcium channels in *Paramecium tetraurelia*. Pestic Biochem Physiol. 1999;65:181-193.

128. Gassner B, Wuthrich A, Scholtysik G, Solioz M. The pyrethroids permethrin and cyhalothrin are potent inhibitors of the mitochondrial complex I. J Pharmacol Exp Ther. 1997;281:855-860.

129. Enan E, Matsumura F. Specific inhibition of calcineurin by type II synthetic pyrethroid insecticides. Biochem Pharmacol. 1992;43:1777-1784.

130. Schleier III JJ, Peterson RKD. Pyrethrins and Pyrethroid Insecticides. In: Lopez O, Fernandez-Bolanos JG, editors. Green Trends in Insect Control: Royal Society of Chemistry; 2011. p. 94-131.

131. Matthews H, Ali M, Carter V, Underhill A, Hunt J, Szor H*, et al*. Variation in apoptosis mechanisms employed by malaria parasites: the roles of inducers, dose dependence and parasite stages. Malar J. 2012;11:297.

132. Sturm A, Mollard V, Cozijnsen A, Goodman CD, McFadden GI. Mitochondrial ATP synthase is dispensable in blood-stage *Plasmodium berghei* rodent malaria but essential in the mosquito phase. Proc Natl Acad Sci U S A. 2015.

133. Ishino T, Orito Y, Chinzei Y, Yuda M. A calcium-dependent protein kinase regulates *Plasmodium* ookinete access to the midgut epithelial cell. Mol Microbiol. 2006;59:1175-1184.

134. Moon RW, Taylor CJ, Bex C, Schepers R, Goulding D, Janse CJ*, et al*. A cyclic GMP signalling module that regulates gliding motility in a malaria parasite. PLoS Pathog. 2009;5:e1000599.

135. Philip N, Waters AP. Conditional degradation of *Plasmodium* calcineurin reveals functions in parasite colonization of both host and vector. Cell Host Microbe. 2015;18:122-131.

136. Warburg A, Schneider I. In vitro culture of the mosquito stages of *Plasmodium falciparum*. Exp Parasitol. 1993;76:121-126.

137. Arambage SC, Grant KM, Pardo I, Ranford-Cartwright L, Hurd H. Malaria ookinetes exhibit multiple markers for apoptosis-like programmed cell death *in vitro*. Parasit Vectors. 2009;2:32.

138. Agnew P, Berticat C, Bedhomme S, Sidobre C, Michalakis Y. Parasitism increases and decreases the costs of insecticide resistance in mosquitoes. Evolution. 2004;58:579-586.

139. Berticat C, Rousset F, Raymond M, Berthomieu A, Weill M. High *Wolbachia* density in insecticide-resistant mosquitoes. Proc Biol Sci. 2002;269:1413-1416.

140. McCarroll L, Hemingway J. Can insecticide resistance status affect parasite transmission in mosquitoes? Insect Biochem Mol Biol. 2002;32:1345-1351.

141. Rivero A, Vezilier J, Weill M, Read AF, Gandon S. Insecticide control of vector-borne diseases: when is insecticide resistance a problem? PLoS Pathog. 2010;6.

142. McCarroll L, Paton MG, Karunaratne SH, Jayasuryia HT, Kalpage KS, Hemingway J. Insecticides and mosquito-borne disease. Nature. 2000;407:961-962.

143. Mitri C, Markianos K, Guelbeogo WM, Bischoff E, Gneme A, Eiglmeier K*, et al*. The *kdr*-bearing haplotype and susceptibility to *Plasmodium falciparum* in *Anopheles gambiae*: genetic correlation and functional testing. Malar J. 2015;14:391.

144. 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 U S A. 2013;110:9397-9402.

145. Kabula B, Tungu P, Rippon EJ, Steen K, Kisinza W, Magesa S*, et al*. A significant association between deltamethrin resistance, *Plasmodium falciparum* infection and the *Vgsc-1014S* resistance mutation in *Anopheles gambiae* highlights the epidemiological importance of resistance markers. Malar J. 2016;15:1-5.

146. Alout H, Labbe P, Chandre F, Cohuet A. Malaria Vector Control Still Matters despite Insecticide Resistance. Trends Parasitol. 2017;33:610-618.

147. 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;27:91-98.

148. Bass C, Nikou D, Donnelly MJ, Williamson MS, Ranson H, Ball A*, et al*. Detection of knockdown resistance (*kdr*) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. Malar J. 2007;6:111.

149. Weetman D, Donnelly MJ. Evolution of insecticide resistance diagnostics in malaria vectors. Trans R Soc Trop Med Hyg. 2015;109.

150. Donnelly MJ, Corbel V, Weetman D, Wilding CS, Williamson MS, Black WCt. Does *kdr* genotype predict insecticide-resistance phenotype in mosquitoes? Trends Parasitol. 2009;25:213-219.

151. Abeku TA, Helinski ME, Kirby MJ, Kefyalew T, Awano T, Batisso E*, et al*. Monitoring changes in malaria epidemiology and effectiveness of interventions in Ethiopia and Uganda: Beyond Garki Project baseline survey. Malar J. 2015;14:337.

152. Abeku TA, Helinski MEH, Kirby MJ, Ssekitooleko J, Bass C, Kyomuhangi I*, et al*. Insecticide resistance patterns in Uganda and the effect of indoor residual spraying with bendiocarb on *kdr L1014S* frequencies in *Anopheles gambiae* s.s. Malar J. 2017;16:156.

153. Helinski M, Nuwa A, Protopopoff N, Feldman M, Ojuka P, Oguttu DW*, et al*. Entomological surveillance following a long-lasting insecticidal net universal coverage campaign in Midwestern Uganda. Parasit Vectors. 2015;8:458.

154. Verhaeghen K, Bortel WV, Roelants P, Okello PE, Talisuna A, Coosemans M. Spatio-temporal patterns in *kdr* frequency in permethrin and DDT resistant *Anopheles gambiae* s.s. from Uganda. Am J Trop Med Hyg. 2010;82:566-573.

155. Verhaeghen K, Van Bortel W, Roelants P, Backeljau T, Coosemans M. Detection of the East and West African *kdr* mutation in *Anopheles gambiae* and *Anopheles arabiensis* from Uganda using a new assay based on FRET/Melt Curve analysis. Malar J. 2006;5:16.

156. Mawejje HD, Wilding CS, Rippon EJ, Hughes A, Weetman D, Donnelly MJ. Insecticide resistance monitoring of field-collected *Anopheles gambiae* s.l. populations from Jinja, eastern Uganda, identifies high levels of pyrethroid resistance. Med Vet Entomol. 2013;27:276-283.

157. Ojuka P, Boum Y, 2nd, Denoeud-Ndam L, Nabasumba C, Muller Y, Okia M*, et al*. Early biting and insecticide resistance in the malaria vector *Anopheles* might compromise the effectiveness of vector control intervention in Southwestern Uganda. Malar J. 2015;14:148.

158. Ramphul U, Boase T, Bass C, Okedi LM, Donnelly MJ, Muller P. Insecticide resistance and its association with target-site mutations in natural populations of *Anopheles gambiae* from eastern Uganda. Trans R Soc Trop Med Hyg. 2009;103:1121-1126.

159. Rubaihayo J, Ephraim T, Andrew A. Reduced susceptibility to pyrethroid insecticide treated nets by the malaria vector *Anopheles gambiae* s.l. in western Uganda. Malar J. 2008;7:92.

160. U.S. President's Malaria Initiative: Uganda Malaria Operational Plan FY 2018. 2017: 111.