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Impact of insecticide resistance on the efficacy of malaria vector control

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ABSTRACT

The entomological work required to characterize the effectiveness of vector control tools against insecticide-resistant mosquitoes is one of the highest priorities of contemporary malaria research. Yet, current methods for resistance monitoring and evaluation of vector control tools leave out the most important parameters for malaria transmission. Since longevity, blood feeding success and biting rate over the lifetime affect the ability of mosquitoes to transmit malaria, they should be an integral part of the evaluation of long-lasting insecticide-treated nets (LLINs) efficacy.

In this thesis, the direct and delayed effects of the most widely distributed LLINs in Africa have been evaluated with resistant mosquitoes in Côte d'Ivoire. First, we quantified the impact of LLIN exposures on the capacity to survive several gonotrophic cycles and the benefices of taking one or more blood meals during the lifetime. Second, we studied several parameters influencing mosquito behavior in interaction with LLINs by comparing the condition of the mosquito net (damaged or not), access to the host (direct or indirect), choice between a protected or non-protected host, the distance from the host, different larval diets, and the ability to detect odors.

In our laboratory studies, mosquitoes (pre-) exposed to a LLIN had reduced host searching and feeding activity. One of the mechanisms involved is probably the absence of odor detection by mosquitoes for a short period of time after an exposure to a LLIN. Nonetheless, semi-field experiments mitigated these results as the LLIN direct efficacy seemed to be compromised by resistance as it offered only slight personal protection. That being said, blood fed mosquitoes surviving a first sublethal dose of insecticide in the laboratory did not necessarily go through additional gonotrophic cycles, either because they died before that, or because of a lower ability to take further blood meals later in life. Besides, when mosquitoes missed a chance to feed, being blocked by an intact LLIN, their chance to take bloodmeals later in life was lower compared to mosquitoes having the opportunity to feed directly through a net upon their first attempt. However, given the nuances in the direct effect of insecticide on feeding success in laboratory and semi-field settings, caution is needed when translating the results of laboratory outputs into normative guidelines for testing the efficacy of LLINs. This is reinforced by the influence of the juvenile environment on the variability of adult mosquito behavior.

Overall, this work demonstrates LLINs efficacy despite insecticide resistance and underlines the urgency of finding new standardized methods to monitor the sub-lethal effects on mosquitoes for a better understanding of the consequence of resistance on malaria transmission. It also stresses the need for a better consideration of the effect of insecticide on odor detection, host recognition and memory in the malaria vector.

Keywords

Anopheles gambiae, pyrethroid resistance, sublethal effect, vector control, host searching, blood feeding behavior

RESUME

Le travail entomologique nécessaire à la caractérisation de l'efficacité des outils de lutte antivectorielle contre les moustiques résistants aux insecticides est l'une des plus grandes priorités de la recherche contemporaine sur le paludisme. Pourtant, les méthodes actuelles de surveillance de la résistance et d'évaluation des outils de lutte ne tiennent pas compte des paramètres les plus importants pour la transmission du paludisme. En effet, la longévité, la réussite à se gorger et la fréquence des piqûres au cours d'une vie affectent la capacité des moustiques à transmettre le paludisme, et ils devraient faire partie intégrante de l'évaluation de l'efficacité des moustiquaires imprégnées d'insecticide de longue durée (MILD).

Dans cette thèse, les effets directs et retardés des MILDs couramment distribuées en Afrique ont été évalués avec des moustiques résistants en Côte d'Ivoire. Tout d'abord, nous avons quantifié l'impact d'une ou plusieurs expositions aux MILDs sur la capacité à survivre plusieurs cycles gonotrophiques et aussi les avantages pour la survie de prendre un ou plusieurs repas sanguins. Ensuite, nous avons étudié les conditions influençant le comportement des moustiques en interaction avec les MILDs, en comparant la qualité de la moustiquaire (abimée ou non), l'accès à l'hôte (direct ou indirect), le choix entre un hôte protégé ou non protégé, la distance jusqu'à l'hôte, l'effet de différents régimes larvaires et le potentiel de détection des odeurs.

Dans nos études en laboratoire, les moustiques (pré-) exposés à une MILD étaient moins motivés à trouver un hôte pour se gorger qu'en l'absence d'insecticide. L'un des mécanismes impliqués est probablement l'absence de détection d'odeur pendant une courte période de temps juste après une exposition à une MILD. Néanmoins, les expériences en conditions semi-naturelles ont nuancé ces résultats et l'efficacité directe des MILDs semble être compromise par la résistance en n'offrant qu'une légère protection personnelle. Cela étant dit, les moustiques nourris de sang et survivant à une première dose sublétale d'insecticide au laboratoire n'ont pas nécessairement pris plus de repas sanguins après la première fois, soit parce qu'ils sont morts avant la fin du cycle, soit en raison d'une capacité réduite à prendre des repas sanguins plus tard dans la vie. De plus, lorsque les moustiques n'ont pu se nourrir une première fois en raison d'une MILD intacte, leur chance de se gorger de sang plus tard dans la vie fut plus faible que celle des moustiques ayant eu la possibilité de se nourrir directement à travers le filet. Mais compte tenu de la divergence des résultats concernant l'effet direct de l'insecticide sur le succès du repas sanguin entre le laboratoire et les conditions semi-naturelles, il convient d'être prudent lors de la traduction des résultats de laboratoire en normes d'évaluation de

l’efficacité des MILDs. Cela est d’autant plus vrai étant donné l’impact qu’à l’environnement larvaire sur le comportement du moustique adulte.

Dans l'ensemble, ce travail démontre l'efficacité des MILDs malgré la résistance aux insecticides et souligne la nécessité de trouver de nouvelles méthodes de suivi des effets sublétaux sur les moustiques afin de mieux comprendre les conséquences de la résistance sur la transmission du paludisme. Cette thèse souligne également l’intérêt de considérer l'effet de l'insecticide sur la détection des odeurs, la reconnaissance de l'hôte et la mémoire chez le vecteur du paludisme.

Mots clés

Anopheles gambiae, résistance aux pyréthroïdes, effet sublétal, lutte antivectorielle, recherche d'hôte, repas sanguin

LIST OF ABBREVIATIONS

Abbreviation	Description
ACE-1 (G119 S)	Acetylcholinesterase target site mutation
ANOVA	Analyze of Variance
ATSB	Attractive Toxic Sugar Bait
CDC	Centers for Disease Control and Prevention
CI	Confidence Interval
DDT	Dichloro-Diphenyl-Trichloroethane
DEET	N, N-Diethyl-meta-toluamide
DNA	Deoxyribonucleic Acid
EAG	Electroantennogram
EIP	Extrinsic Incubation Period of the parasite
F0 & F1	First generation (F0) of mosquitoes and the offspring (F1)
GLM, GLMM and LMER	(Generalized) Linear Model and (Generalized) Linear Mixed-Effects Model
GTS	Global Technical Strategy for Malaria 2016-2030
HSD	Honestly Significant Difference in the Tukey test
IRs & ORs	Ionotrophic and Odorant Receptors
IRS	Indoor Residual Spraying
KDR	Knockdown Resistance
LD (of 50)	Lethal dose killing (50 % of) a tested group
LLIN	Long-Lasting Insecticidal Net
M- and S- forms	Mopti and Savannah, molecular subdivision of *Anopheles gambiae*
P-value, p (significance p > 0.05)	less than 5 % probability the null hypothesis is correct
P450 (cytochrome)	Superfamily of hemoproteins involved in insecticide resistance
PBO	synergist Piperonyl Butoxide
PCR	Polymerase Chain Reaction
RS, RR & SS forms	heterozygotes, resistant and susceptible homozygotes
RH	Relative Humidity
SE, se	Standard Error
SEM	piece-wise Structural Equation Model
s.l.	sensu lato
s.p.p.	several species
UTN	Untreated Net
VC	Vectorial Capacity
WHO	World Health Organization

CONTENTS

CHAPTER ONE

General introduction

1.1. Background

1.1.1. Mosquito biology

1.1.1.a. Life cycle of the malaria vector

The mosquito (Diptera, Culicidae) has been characterized as the deadliest animal in the world, and malaria the vector-borne disease with the most destructive impact[1]. With millions of malaria cases worldwide and over half of the world's population at risk of an infection, this disease is responsible for the death of a child every two minutes[2].

From the 3,548 known species of mosquitoes, less than 40 species belonging to the genus *Anopheles* are important vectors of human malaria[3,4]. While transmission of malaria parasites is intimately linked to blood feeding, the life cycle of the adult mosquito involves much more than taking and digesting a blood meal. A young adult mosquito emerges from the aquatic larval habitat with a small reserve of energy[5]. Both male and female mosquitoes then consume sugars, mainly obtained from floral and extra-floral nectar, and honeydew[6]. Mating does not occur for a couple of days after the adults emerge. Males form mating swarms and virgin females enter these swarms, locate a male and then exit as a couple to mate[7]. To complete egg production, a female mosquito must next seek a blood meal. The host could be a human or, depending on the feeding behavior, an alternative vertebrate such as a cow[8]. Feeding can take place indoors or outdoors depending on the species and their populations[9]. To digest a blood meal safely and before the onset of searching for an oviposition site, a female will rest for 2-3 days. Resting can take place indoors or outdoors, again depending on the species[10]. After blood digestion, a female has to find a suitable oviposition site, which could be distant and take several days to locate, during which there is likely more demand for sugars[11]. Those mosquito species that are the most important malaria vectors tend to be relatively long-lived, highly anthropophilic, endophilic, and blood feed late at night[12,13]. Because the malaria parasite usually takes 8 to 15 days to complete the sporozoite cycle within the mosquito under optimum temperatures (and this can be considerably longer under suboptimal conditions)[14–16], female mosquitoes will engage in three to six gonotrophic cycles before being able to transmit malaria[17] (Figure 1). Infected mosquitoes can normally live up to a month (or more in captivity), but the average longevity in nature seems shorter[18]. In fact, most infected mosquitoes die before the end of the development of infectious sporozoites in the salivary glands, when they are ready to be injected in the next person bitten[19].

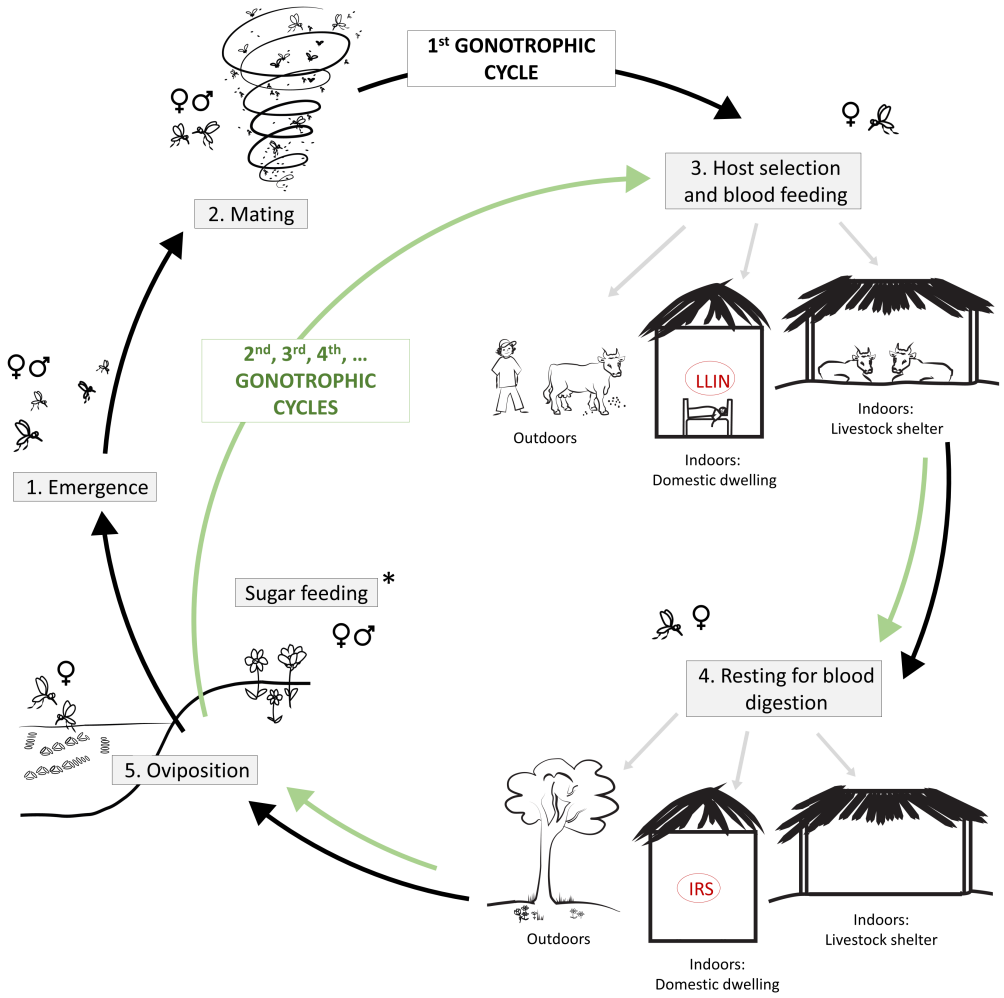

Figure 1: Behaviors and activities of adult malaria mosquitoes as they progress from emergence through to egg laying over one or more gonotrophic cycles. Adult mosquitoes emerge from aquatic habitats (1) and mate within a few days (2), potentially taking a sugar meal for energy (*). Male mosquitoes then tend to die quite quickly, while females go in search of a blood meal (3). Blood feeding could be on a diversity of hosts, either indoors or outdoors. After blood feeding the mosquitoes will tend to rest for 2–4 days while they digest the blood to produce eggs (4). Resting can occur in a range of indoor or outdoor environments. Once the eggs are fully developed the mosquitoes then search for a suitable oviposition site (5), potentially taking another sugar meal (*) to boost energy reserves for flight. Once a suitable aquatic habitat is located and the eggs are laid, female mosquitoes can repeat the blood feeding and egg production process over subsequent days to complete multiple gonotrophic cycles.

1.1.1.b. Mosquito olfactory system

Olfaction is essential to mosquito survival, as it plays a key role in finding food, and in case of females, finding suitable oviposition sites for their offspring[20]. Mosquito sense the chemical and physical stimuli thanks to three sexually dimorphic organs : a pair of antennae, a pair of maxillary palps and a pair of labella at the tip of the proboscis[21]. Antennae and maxillary palps are involved in the long-distance

odor detection while the proboscis is used for close-range odor and taste detection[22].

The three pairs of "noses" in mosquitoes are covered in hundreds of hair-like sensilla, especially the antennae[23,24]. Found also on other chemosensory tissues as the head and legs, these morphologically distinct structures are involved in the perception of olfactory cues and each typically houses the sensory dendrites of two to four bipolar olfactory receptor neurons[21,25]. Those neurons encode odor quality, as for example the molecular structure and odor concentration while expressing one or more olfactory receptor (ORs) proteins and variant ionotropic receptors (IRs), that are responsible for the specific detection of odor molecules[23,26]. In *An. gambiae* s.l, the ORs family of 79 members responds to various volatile odorants but is more and more specialized[27]. The more conservative family of 46 variant IRs is not functionally characterized for *An. gambiae* s.l. yet but it has already been shown that they respond to acids and amines and probably others cues, such as carboxylic acids[26,28]. Note that IRs are not only meditators of sensory transduction, but they also are gustative sensors complementary to the gustatory receptors and thermal and humidity sensors[29].

When volatile odor cues enter through pores in the cuticle into sensilla, odorant-binding proteins bind and solubilize the odorant molecules to finally transfer them to chemoreceptors (ORs and IRs) where they are peripherally recognized. At this point, the nerve cell fires and emits a signal directly to the brain about the compound's presence[23,30].

1.1.1.c. Host seeking and feeding behavior

Female mosquitoes are the only ones taking blood meals and have, therefore, up to four times more olfactory sensilla than males[21]. Thanks to this dimorphism, they can locate a potential blood meal at long range by following the olfactory cues emanating from a host. With no odor stimuli mosquitoes either stay still or fly upwind in order to maximize the chance to encounter an odorant air flow[31]. When activated during a flight, the trajectory changes to orient into the right direction and if an odor plume is lost, mosquitoes zigzag crosswind until it is found again[32].

Human odors, made of numerous volatiles and aromatic compounds like carbon dioxide, octen-3-ol and other molecules found in sweat, play a consistent role in guiding the mosquitoes from a long distance[33–36]. At a closer range, heat, moisture and visual cues are also helpful to target a host[32]. Even though it is still unclear how hundreds of human odorant molecules interact together to attract mosquitoes, it has been shown that *An. gambiae* s.l. female use both host-specific odors and ubiquitous/general odors in host detection[37,38]. For example, lactic acid is quantitatively specific to the

human odor, with a higher concentration in the human skin emanation compare to others animal emanations[39]. Adding lactic acid to several animal odors increases their attractiveness to anthropophilic mosquito to a level similar to that of human odour[40–42]. But sulcatone makes human scent distinctly host specific and is recognize as a signal for a source of blood[43,44].

The process of obtaining a blood meal consists of probing, feeding, and at the end of the meal in diuresis. During probing, the proboscis is introduced into the host body and moves until it contacts a blood vessel. Mosquitoes then feed on the host and blood is ingested[45]. Salivary proteins are injected in the blood during blood feeding that allows vasodilatation, anti-coagulation and an immune response from its host[46]. At the end of the meal, the osmosis and ionic regulations start, and the metabolic waste is expelled. This last mechanism helps to control the temperature and quantity of sodium chloride and water as the human blood content disturbs the homeostasis in the hemolymph. The diuresis lasts 30h and it is only when both the hematin is digested and excreted and the blood meal is degraded that oviposition is possible[47].

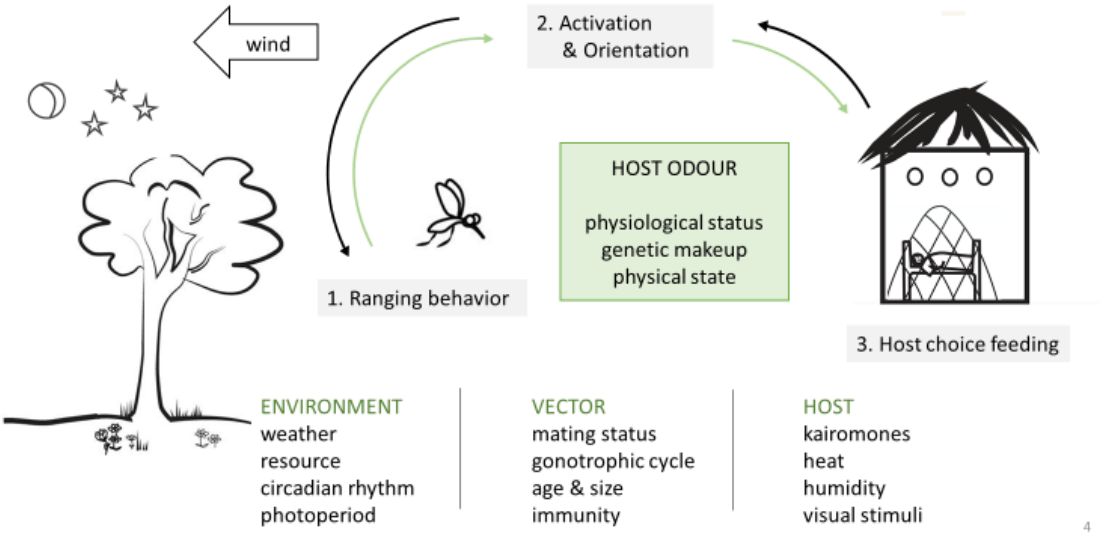

Figure 2: Endo- and exogenous factors from the environment, the vector and the host influencing different mosquito feeding behaviors such as ranging behavior, activation and orientation, host choice and feeding.

Regulated by endo- and exogenous factors, the localization of a host is not a simple "stimulus-response" behavior[48] (Figure 2). Both the physical and physiological states of the mosquitoes, determined by parameters such as mating status, age, size, immunity, and infection status and play a role in their decision to fly toward an odor plume, and find a host. For example, the evolutionary pressure to increase the parasite's transmission success favors changes in host behavior for an

enhanced pathogen spread[49,50]. The changes in mosquito's biting rate may be a potential by-product of malaria infection due to tissue damage from invasion[51,52]. Also, the malaria parasite has been described as a vector "manipulator" that limits the host-seeking behavior and decreases the risky biting behavior at the un-mature stage of the parasite, and then decreases the blood meal size which increases the biting rate during the night at the mature stage[53–55]. Recent studies also show that mosquitoes seem to be preferentially attracted to infected individuals during transmissible stages of malaria and that the infectious stage on the parasite influences their host preference[56–59]. All these changes in human feeding rates could have substantial impacts on transmission potential[60]. Moreover, mosquitoes learn to avoid hosts based on scent and swatting[61] and seeking and feeding behaviors are strongly determined by the color, temperature, moisture and mass of the body. These characteristics might in turn be affected by genetic make-up, age, diet, gender and physical state[62]. The circadian rhythm, the photoperiod, the weather, the wind direction and speed as well as the resources available in the environment are other parameters that influence the mosquito physiological and physical state, resulting in changes in mosquito behavior, such as flight activity[63–68].

1.1.1.d. Influence of environmental factors on feeding behavior

Feeding behavior at both the larval and adult stages fluctuates during life depending on mosquito needs and nutritional reserves[69]. For example, blood-seeking is naturally stopped after a blood meal as well as during oogenesis[70–73]. Sugar feeding can also regulate biting behavior and feeding decision for a short period of time (less than a week)[74,75]. In some mosquito species, autogenous immature females are able to lay eggs without a previous blood meal and their food reserves are higher than anautogenous mosquitoes[76,77]. Change in larval environmental factors (such as temperature, density and food resources) play a great role in host seeking and feeding behaviors as they influence mosquitoes nutritional reserves at the juvenile and adult stages[63,78–80]. Yet, the complex effects of larval environment on adult mosquito feeding behavior are not well understood, especially in *Anopheles* mosquitoes. Healthier adults generally come out of larval environments with higher resources[81], with female mosquitoes containing more protein, lipid and glycogen upon emergence, which could increase the frequency of bites and the blood meal duration[82,83]. Nonetheless, limitations in larval food and competition could decrease survival[68,84,85] and motivate females with low somatic reserves, or that are sugar deprived, to take supplementary blood meals to restock energy for future activities (such as reproduction)[86–88]. In some cases, however, a stressful environment may also constrain juvenile development with carry over effects on adult longevity. Thus being smaller may become favorable in terms of longevity, and supplementary blood meals may not be necessary[89].

Beyond potential effects on behavior, nutritional condition can also affect traits such as parasite development and vector competence. Undernourishment during the larval stage and high temperature results in smaller *An. stephensi* adults[78] and a delay in malaria parasite development duration (EIP) in case of infection[90]. On the other hand, larval competition has been shown to shorten the EIP of dengue virus in *Aedes albopictus*[91]. On a side note, while environmental variations extend or reduce the length of the gonotrophic cycle, the biting activity of mosquitoes and the malaria parasite EIP could be rhythmically modulated to avoid the situation where the parasite is ready to be transmitted in the middle of the gonotrophic cycle[92]. When in this situation, parasites in the salivary glands need to wait for the next delayed bloodmeal and this asynchrony with the vector could negatively impact transmission. With no fitness gains to asynchrony between EIP and gonotrophic cycles, parasites may have evolved strategies to optimize EIP (by making it shorter or longer) in response to physiologically constrained vectors that bite less frequently when mounting an immune response[93,94]. For example, co-infection with other pathogens could result in fitness costs[95–98] and in some case in a longer EIP[99].

1.1.2. The current and principal vector control tools

Long-Lasting Insecticide-treated Nets (LLINs) and Indoor Residual Spraying (IRS) with insecticides have substantially contributed to a decline in the burden of malaria over the last 15 years[100,101]. These control tools work by lowering contact rate between humans and vectors, either because the insecticide changes the normal feeding or host-searching behavior (repellency or deterrence)[102], and/or the insecticide causes mosquito death, affecting the age structure of the mosquito population and potentially adult mosquito density[103]. The WHO recently published its 'Global Technical Strategy for Malaria 2016-2030' (GTS), which sets out a vision and strategic framework to reduce malaria transmission by at least 90 % over the next 15 years and prevent its re-establishment in countries that are currently free of malaria. Similar ambitious targets are set out in the 'Aspiration to Action' document prepared by the Bill and Melinda Gates Foundation[104], which calls for a halving in transmission every 10 years, leading to ultimate eradication by 2040. Inter-country alliances, such as the Asian Pacific Malaria Elimination Network, aim for regional elimination by 2030[105]. This plan is informed by a modeling analysis, which explores a range of future intervention scenarios that vary in terms of access to vector control (LLINS and IRS) and drug treatments (both seasonal malaria chemoprevention and first line treatments with artemisinin combination therapy). The modeling analysis reveals several key insights (Figure 3). First, if vector control and drug use remain at current levels, malaria mortality is expected to increase in the next 10-15 years due to changing immunity profile in the human population, wherein people born after the current interventions were scaled up

are exposed more slowly and acquire their first and subsequent cases at an older age. Second, if effectiveness of existing tools falls (e.g. through evolution of resistance) the rebound in malaria burden will likely be more pronounced. Third, further intensification of existing core tools to 80 or 90 % coverage can lead to reductions in malaria burden and even elimination in some settings but fails to reach the anticipated targets in areas of intense transmission. As such, new tools are required if the WHO targets are to be achieved. Nonetheless, increasing the coverage and overall effectiveness of vector control is key to achieving the targets of the GTS for malaria, and the broader goals of elimination and eradication. LLIN and IRS provide the foundation and intensifying their use is a priority.

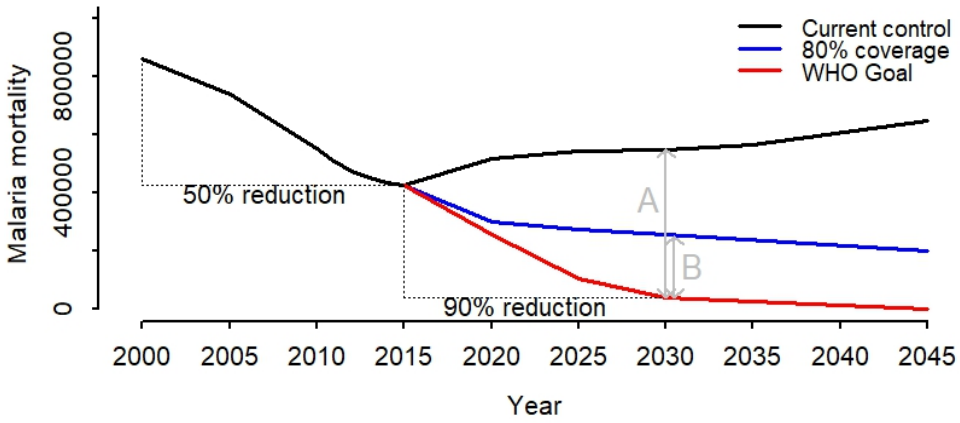

Figure 3: Estimate of historic and projected global deaths due to malaria based on different control scenarios The figure (modified from Figure 1B of Griffin and al.[106]) shows estimates of global malaria deaths from 2000-2045. The 50 % decline in malaria related mortality recorded from 2000-2015 is largely attributable to the wide scale implementation of vector control tools (Long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS))[107,108]. The future projections are based on a model analysis that considers different scenarios of access to vector control, together with malaria drug treatments. The back line indicates resurgence in malaria deaths if control efforts remain at current levels. The blue line is the predicted decline in deaths assuming coverage of current control tools can be increased to reach 80 % of the population at risk. The red line represents the target set out in the WHO Global Technical Strategy[109], which aims for a 90 % decline in malaria deaths by 2030 and then ultimate elimination thereafter. The arrows A and B illustrate the differences between the WHO target and the two control scenarios. Business as usual clearly represents a massive failure (A). Perhaps more notably, even substantial intensification of existing tools still yields a substantial shortfall (B). These gaps in control demonstrate the need for new interventions.

1.1.3. Insecticide resistance

1.1.3.a. Insecticides and mode of action

There are only few insecticides used for vector control. Insecticides recommendations are based on their characteristics like mechanism of action, level of toxicity to the target and safety for humans.

The selected pesticides can be organized in five categories of neurotoxic insecticides: pyrethroids, organochlorines, carbamates, organophosphates and neonicotinoids; a category of juvenile hormone agonist widely used in pest control, pyridines; and an unique category of respiratory disruptor, pyrroles[110,111].

Pyrethroids

Pyrethroids were until recently the only class of insecticide authorized by WHO for LLINs[112]. They are efficient at a low dose, safe for humans and mammals but fatal and/or irritating for mosquitoes[112]. The toxic effects of pyrethroids slow down the closure of the voltage-gate sodium channels that normally would open during depolarization[113]. While the axonic excitoxin keeps the channel in the open state, the propagation of an action potential persists after repolarization of the membrane[114], thereby paralyzing mosquitoes. This tetany is also commonly called « knockdown » effect. Pyrethroids are synthetic derivatives of pyrethrins. Based on toxicological and physical properties they are divided in 2 sub-groups. Pyrethroids of the type I are non-α-cyano, such as permethrin, produce a tremor syndrome, with symptoms that include convulsive twitching, coma, and death. Pyrethroids of the type II α-cyano, such as ß-cyfluthrin, cypermethrin and deltamethrin, cause the choreoathetosis with salivation syndrome, followed by death[115]. To increase their effectiveness, pyrethroids can be combined with piperonyl butoxide (PBO), a man-made pesticide synergist. The efficacy of this combination result in the inhibition of some of the enzymes that break down insecticides like pyrethroids, allowing less chance to recover and enhancing the susceptibility of mosquitoes[116].

Organochlorines

Organochlorines were first used for IRS with Dichlorodiphenyltrichloroethane (DDT)[117]. Despite their efficacy in terms of toxicity against vectors and persistence[118], these pesticides were largely disused due to their noxious effect on the environment[119,120]. DDT works like pyrethroid by altering the nervous transmission and disturbing the way sodium channels work[121,122]. Other organochlorines like fipronil disrupt the insect central nervous system by blocking gamma-Aminobutyric acid gated chloride channels and glutamate-gated chloride channels[123].

Carbamates and organophosphates

Carbamates, such as bendiocarb and propoxur, and organophosphates, such as malathion, fenitrothion and pirimiphos-methyl, are used for IRS purposes and organophosphates are also used in larval source management[124,125]. They have the same mode of action: they target and inhibit the enzyme acetylcholinesterase with the neurotransmitter acetylcholine, normally released by neurons

to send signals to other cells, or by phosphorylation. This disturbance of the nerve impulse between two neurons, leads to an accumulation of acetylcholine in the synaptic cleft, a paralysis and finally the death of insects[126,127].

Neonicotinoids

Neonicotinoids (or "new nicotine-like insecticides") are a new class of insecticide formulations prequalified by WHO for IRS[128]. Mosquito control is made through tarsal contact, blocking the nicotinic acetylcholine receptors on the postsynaptic membrane and therefore interrupting the transmission of nerve impulses in the mosquito central nervous system[129,130]. Clothianidin, one of the six insecticides in this group, has low mammalian toxicity its use for IRS products demonstrated promising results to target multiple resistance vector species and showed efficient reduction in local vector house entry[131,132].

Pyridine and pyrrole

Pyridine and pyrrole are two aromatic heterocycles that do not target mosquito nervous system but use different modes of action and they also are the only non-pyrethroid insecticides authorized by the WHO to be used on LLIN[133]. Pyriproxyfen, a pyridine-based pesticide is an insect growth regulator preventing the metamorphosis from the larvae into the adult mosquito[134,135]. Its properties may also reduce fecundity and longevity and even sterilize adult mosquito[136–139]. It is efficient at extremely low doses against mosquitoes and effective for 5 to 9 months[136,140]. The chlorfenapyr, a pyrrole, works upon contact, spreads throughout the mosquito body, then the P450 enzyme metabolizes the insecticide, as a result a deadly active agent is formed, the production of adenosine triphosphate molecules in the mitochondria is disrupted and this leads to loss of energy, cell dysfunction and death[141,142].

1.1.3.b. Emergence of insecticide resistance mechanisms

Since the large-scale use of insecticides in agriculture during the 1950s and 60s and implementation of vector control tools[143], physiological (and to a lesser extent behavioral) resistance is now widespread across mosquito species and populations, threatening the effectiveness of the frontline insecticide-based interventions[109,144]. In two thirds of countries with malaria today, all major vector species are insecticide resistant and often "multi-resistant" to all known classes of insecticide[145,146]. Resistance is found against each class of insecticide[147,148] and when in a class, it often also confers cross-resistance to other insecticides in the same class (with a similar mode of action).

There are four forms of known insecticide resistance to pyrethroids: (1) target site insensitivity, (2)

metabolic resistance, (3) behavioral resistance and (4) cuticular resistance[149,150]. Target-site insensitivity is due to a single nucleotide polymorphism (mutation) resulting in an overall change in the protein being produced and a reduction in the neurotoxic efficacy of the insecticide[151]. Metabolic resistance is due to an over-expression of enzymes such as α-esterases, ß-esterases, P450 oxidases, or glutathione-S-transferases that detoxify or sequester the insecticide through an increase in gene copy number and/or an increase gene expression[151,152]. Behavioral resistance helps avoid the lethal effects of insecticide. In response to an extensive indoor insecticide use, mosquitoes may adapt to a stressful environment by altering their behavior so that they bite and rest outdoors, take a blood meal earlier in the evening before people have gone to sleep, or increase zoophagy[153,154]. Changes in phenotypic expression of resistance (or non-genetic factor influencing the response and expression of insecticide) to facilitate avoidance of insecticides may result in changes in genetic background[155]. Note that unlike physiological resistance, behavioral resistance is difficult to investigate in simple exposure assays in the lab or directly in the field as it is uneasy to differentiate behavioral resistance from the insecticide repellency effect on mosquito behavior[156,157]. The fourth mechanism reduces penetration of the insecticide though the mosquito cuticle. This is made possible with the modification in the structure (cuticular sclerotization) or composition of the cuticle (increase in the lipid and protein content of the cuticle)[158]. Penetration resistance is often working in combination with the two others forms of resistance[159]. Recently, it has been shown that resistant mosquitoes remodel their legs via a higher amount of both cuticular hydrocarbons that seal legs and cuticular proteins and chitin that thicken the leg cuticles. These alterations are associated with a reduced effect of insecticides[160].

1.1.3.c. Effect of insecticide exposure and resistance-associated cost

Sub-lethal exposure to permethrin and deltamethrin of 3 different strains of mosquitoes (*Culex quinquefasciatus*, *Anopheles albimanus* and *Aedes aegypti*) has been shown to alter the host seeking behavior (loss of host-seeking flight and orientation abilities) up to 24h post exposure[161]. The presence of deltamethrin decreases the blood feeding success of both kdr homozygous and kdr heterozygous mosquitoes but permethrin increases the blood-feeding success of kdr homozygous mosquitoes. Nets containing one or the other of these insecticides alters the behavior (feeding duration, pre-diuresis duration and blood meal size) of these laboratory strains[162]. Also, the effect of a sub-lethal exposure to permethrin during blood feeding of kdr resistant lab strain decreases the insecticide irritancy at the following exposure[163]. Sub-lethal exposure to permethrin may reduce blood seeking behavior of both susceptible mosquitoes for 48h[164] and different insecticide-resistant mosquitoes strains for 6h[165]. However, rodent malaria infection may reduce that inhibition period and the repellency effect of permethrin in insecticide-susceptible mosquitoes[164,166]. Several exposures to DEET also negatively

influences the proportion of blood engorgement, the number of eggs laid and the proportion of offspring that becomes adult in kdr resistant mosquitoes. Yet, mosquito blood-seeking behavior of DEET-pre-exposed females is increased 3-4 days later[167]. The delayed efficacy of LLINs affects the longevity of insecticide resistant female and several exposures cut the malaria transmission potential by two thirds[168]. In an experimental study too, human malaria-infection is found to increase the lethal effect of DEET by killing more kdr-resistant and infected mosquitoes than un-infected mosquitoes[169]. Interestingly, kdr homozygous mosquitoes are found to prefer feeding on a host protected by a LLIN over a host protected by a untreated net, suggesting that LLINs are detectable by insecticide resistant mosquitoes and that a behavioral cost is associated with insecticide resistance mutation[170]. Moreover, despite their choice preference the kdr homozygous mosquitoes are the least efficient at going through a damaged net (with holes) treated with deltamethrin or permethrin and this may be due to the reduction in blood-seeking in comparison to homozygous susceptible and especially kdr heterozygous mosquitoes that search for blood more often and are more efficient at finding holes in order to penetrate pyrethroid-treated nets. This shows that heterozygotes are not affected by the cost observed for kdr homozygous females and have a better fitness than both homozygotes[171].

The physiological state of mosquitoes can also influence the behavior and the sensitivity to insecticides in genetically resistant mosquitoes. For example, fungi or microsporidian parasitism[172], larval diet[173,174], ingestion of xenobiotics at the larval stage[158,175] and age[176–180] increase sensitivity or restore susceptibility to insecticides; whereas a blood-meal[177,181,182] and an increasing temperature[183] can increase resistance. Other parameters from the physiological state of mosquitoes such as the gonotrophic cycles, the satiety status[184], the circadian rhythm[63] and the insemination status[185] are fluctuating parameters and could potentially affect insecticide susceptibility. In return, insecticide resistance does affect life-history traits of mosquitoes[186,187]. Studies have shown that organophosphate resistant *Culex pipiens* mosquitoes die more rapidly due to food stress or high larval densities, and are also killed more often due to predation or parasitism in comparison to susceptible mosquitoes[186–188]. But an infection by the microsporidian parasite *Vavraia culicis* has been shown to reduce the difference in relative fitness costs between resistant and susceptible *Cu. pipiens*[186]. Moreover, the exposure to the insecticide temephos increases the fitness cost of permethrin resistance in that same mosquito species[189] and those mosquitoes are also more susceptible to the fungal pathogens *Beauveria bassiana* and *Metarhizium anisopliae*[190]. Another study on *An. gambiae* has shown that resistant mosquitoes die more at the pupal stage[191]. Also, when carrying the kdr mutation in comparison to not being kdr resistant, the malaria vectors also have higher initial malaria infection rates but smaller malaria parasite loads[192]. However, other studies have shown that

resistance can result in an increase in *Wolbachia* parasite load due to lower energy reserves which in turn affect survival[193,194]. Bearing in mind that the evolution of one life-history trait often comes at the cost of another[195], any costs associated with resistance (as for example longevity) may impact the vectorial capacity of mosquitoes, and the malaria transmission potential may increase with the diminishing resistance costs over time due to gene replacement[196].

1.1.3.d. Insecticide resistance management plan

To maintain the effectiveness of current vector control tools, there is a need for novel chemical actives that circumvent insecticide resistance. One of the challenges is that many of the candidate insecticides are pyrethroid-based[197], and resistance is already wide spread[198,199]. Therefore, the development of next generation LLIN and IRS products is an important ongoing activity[189]. PermaNet® 2.0 (Vestergaard Frandsen SA, Aarhus, DK) is a LLIN coated with the pyrethroid deltamethrin to a target dose of 55 mg/m^2 ± 25 %. It is not efficient to directly kill extremely resistant mosquitoes anymore[200–202]. PermaNet® 3.0 polyester side panel is coated with 85 mg/m^2 ± 25 % deltamethrin and the monofilament polyethylene roof panel is coated with a mixture of 121 mg/m^2 ± 25 % deltamethrin and 759 mg/m^2 ± 25 % piperonyl butoxide (PBO)[203]. Similarly, Olyset Net (Sumitomo Chemicals, Osaka, Japan) is a multifilament polyester net dipped in an aqueous solution of 25 mg/m^2 % deltamethrin and Olyset Plus is a version with added PBO. Pyrethroid-LLINs with the addition of PBO can restore mortality of vectors with moderate-intensity mono-oxygenase resistance[204]. However a loss of efficacy of PBO-based LLIN has already been observed in Mozambique[205]. Olyset Duo is a LLIN containing 2 % weight for weight permethrin and 1 % pyriproxyfen incorporated into polyethylene fibres[206]. Interceptor® LN (BASF Corporation, Germany) is a polyester net coated with 200 mg/m^2 ± 25 % alpha-cypermethrin[207] and Interceptor® G2 (BASF Corporation, Germany) combines an alternative to pyrethoids with 200 mg/m^2 chlorfenapyr[207]. Olyset Duo and G2 LLINs are now available to target the most resistant mosquito populations and are the first ones in more than 30 years that are not singly pyrethroid-based. The competition in the LLIN market is strong, but next generation nets are usually more expensive than current LLINs. The goal of resistance management is not to mass-produce innovative LLIN treated with only a single insecticide class nor a single product but to target a given vector populations with the right tools based on appropriate entomological indicators.

For IRS, two new types of formulations with neonicotinoid have been developed: SumiShield^{TM} 50 water dispersible granules of 300 mg clothianidin per square meter and Fluodora Fusion^{TM} wettable powder in a water-soluble bag of a mixture with 200 mg clothianidin and 25 mg deltamethrin per square meter. Their best implementation strategy, like LLINs, relies on rotation with other IRS

products to mitigate resistance selection pressure[208,209].

Exploration of new products that supplement existing vector control is also a rich area of research[133,210]. New vector control tools are required to target behavioral as well as physiological resistance, address the challenges of residual transmission, and in general target mosquitoes more broadly. Moreover, in order to avert an anticipated rebound in malaria due to waning natural immunity and potential impacts of insecticide resistance, it is essential that new tools enter into operational use within the next years to work in combination with other intervenations[211]. For example, the wide choice of candidate stomach toxins available for ATSB creates options for control of mosquitoes resistant to the currently used contact insecticides[212]. For the development of products that do not target mosquitoes near humans like swarm sprays and livestock endectocides, it might well be possible to use different chemical products than those approved for use inside domestic dwellings, providing opportunities for resistance management[213]. Beyond diversifying the active ingredients available for vector control, the flexibility and potential for rapid turnover could provide a real opportunity to implement insecticide resistance management strategies that use insecticide rotations, mosaics, or mixtures[208]. However, the development of non-insecticide vector control tools working as complementary tools to LLINs and IRS products should also be considered.

There is a considerable interest in the potential of new gene editing technologies for developing transgenic mosquitoes for use in population replacement or population suppression strategies[214–219]. Approaches to reduce vector competence by manipulating elements of the mosquito microbiome[98,220,221], or via trans-infection with endosymbionts such as *Wolbachia*[222,223], are also being examined. However, given the current exploratory nature of this research (in most cases the research has yet to progress beyond lab-based proof of principle studies), together with the challenges and timelines of regulatory approval, it is questionable whether such technologies will achieve wide scale operational use for malaria control within the next 5-10 years. This argument does not mean that these technologies cannot make valuable contributions somewhere down the line. Nonetheless, it is very difficult to see how they can play a substantial role in averting the present-day insecticide resistance crisis, or in driving down malaria transmission in the next decade (Figure 3).

1.2. Thesis introduction

Although there is evidence for widespread resistance and major concerns that this will lead to control failure and inability to achieve targets like those set out in the GTS, the evidence that resistance has

impacted the efficacy of LLINs is limited to date. Even though standard WHO assays point to the impact of pyrethroids in sub-Saharan Africa and India being reduced[224,225], and mathematical models predict that this could ultimately result in an increase of malaria cases[226], until now there is limited evidence for apparent control failure of pyrethroid-based LLINs. For example, a recent WHO-coordinated cohort study in 5 countries (Benin, Cameroon, India, Kenya and Sudan) found that irrespective of resistance, LLINs were still effective at reducing the risk of malaria infection[227]. The question is why? One billion LLINs distributed in Africa have contributed substantially to reductions in the burden of malaria in the last 15 years[146], but it is still unclear how this current tool influences highly insecticide-resistant mosquito's behavior and especially how it contributes to reduce ultimate transmission. Therefore, there is a pressing need to better understand the issue of insecticide resistance and assess how it impacts host searching, feeding behavior and longevity of field mosquitoes.

One of the key issues is that the way resistance is typically characterized using either simple genetic markers or highly standardized WHO assays, does not capture how mosquitoes experience insecticide exposure in the field. WHO tests use single controlled exposures against young (3-5 days old) females and monitor mortality up to 24h. Yet in the field, mosquitoes vary in condition (one or more blood feeds, presence or absence of the parasite, different larval conditions), age (only mosquitoes that are at least 12-14 days old mosquitoes can transmit malaria and it has been shown previously that resistance declines with age), exposure history (potentially multiple exposures over time as mosquitoes encounter nets at each gonotrophic cycle) and patterns of exposure (how a mosquito searches around a net could give different exposure time and deposition of insecticide relative to 1 hour forced exposure against a diagnostic dose in a WHO tube test, or a 3 minute exposure on an LLIN using cone test). All these factors will combine to determine the overall number and capacity of mosquitoes in a cohort potentially able to transmit malaria. While there are numerous approaches underway to improve malaria control, there is also a pressing need to develop appropriate test protocols to better understand the impact of resistance on current tools.

1.3. Aim of this thesis

The aim of this thesis is to better determine how LLINs might impact the potential of a highly resistant mosquito strain to transmit malaria and assess whether existing nets currently provide any function beyond a simple physical barrier. To address this aim we considered four key questions:

(1) Do LLINs cause delayed mortality?

To answer this first question, we characterized the effects of resistance on mosquito longevity. We aimed to understand how realistic exposure(s) to an LLIN (once or multiple times) affects the number of mosquitoes that ultimately live long enough to be able to transmit the malaria parasite. We also studied the time spent in contact with a treated surface, the time spent blood feeding, and the feeding success in the presence of insecticide, in relation to the longevity of insecticide resistant mosquitoes.

(2) Do LLINs change the normal host-searching behavior or feeding of mosquitoes?

Our work extended beyond impacts on survival/longevity to examine host-feeding behaviors, host choice, and behavioral insecticide effects such as excito-repellency, deterrence, and inhibition. Mosquitoes can only acquire and transmit the malaria parasite if they feed at least twice on appropriate human hosts (once to acquire the parasite from an infectious carrier and once to pass infection onto a susceptible host after the parasite has completed its development within the mosquito). If contact with insecticide alters host searching and feeding success, LLINs could impact transmission even if mosquitoes survive exposure. Accordingly, we examined how sub- or pre-lethal exposure to an LLIN affects egg laying and the ability of mosquitoes to locate hosts and successfully feed (considering elements of appetite, attraction, activation, and orientation).

(3) Does variation in environmental factors affect mosquito feeding rate over a lifespan?

Variations in the larval environment influences insecticide susceptibility[158,173], but it is still unknown how it impacts lifelong mosquito behavioral responses to a human host protected by pyrethroid-treated LLINs in malaria endemic regions. Moreover, the quality of the net (intact or damaged) and position of the host behind the net (against the net or not) force mosquitoes to interact differently with a LLIN in order to take a blood meal. We investigated how the sub-lethal effects of insecticide on mosquito lifelong host seeking, blood feeding, egg laying, and longevity may be influenced by these different factors.

(4) Do LLINs physiologically impair mosquito odor detection mechanism?

There is evidence that exposure to insecticide temporarily impairs mosquito behavior[228,229]. To investigate whether this could be due to changes in a physiological mechanism or not, we tested the effect of insecticide exposure on neural responses of mosquitoes to human and plant odors.

A secondary aim underlying this research was to explore possible novel assay methods which might provide better insights into the likely functional significance of insecticide resistance. The current resistance monitoring and behavioral assays in use are primarily designed as either surveillance tools

(e.g. WHO tube tests) or comparative bio-efficacy tests (e.g. WHO cone tests) and do not represent the full impact of the active ingredient or product tested on the lifetime transmission potential of a mosquito. There is a scope for the development of novel behavioral assays that include sub-lethal effects, a natural suite of lifelong host seeking and feeding behavior, and insecticide exposures history, to fill this knowledge gap.

1.4. Thesis outline

Our study system was based around a commonly used LLIN (PermaNet© 2.0 impregnated with the pyrethroid deltamethrin) and a highly resistant strain of *An. gambiae* s.l. from Bouaké, Côte d'Ivoire, which is known to be intensely pyrethroid resistant (> 1700-fold resistance to deltamethrin[165]), with both metabolic and target site resistance mechanisms[230].

Ideally, the efficacy of LLINs should be investigated in the presence of a human host to elicit host-seeking behaviors. In chapter 2, we performed modified WHO assays and individual 'cup assays' that were designed to measure the direct effect of insecticide on mosquito host searching and feeding behavior, and sub-lethal effects of insecticide on mosquito's subsequent blood meals and longevity. We focused on expression of insecticide resistance considering the effect of mosquito age, feeding status and insecticide exposure patterns considering one or multiple insecticide exposures.

To ensure a steady supply of mosquitoes for the various experiments we established and successfully maintained a mosquito colony from the field. Chapter 3 describes how we generated this line of resistant mosquitoes collected from rice fields around Bouake. The methods used to both characterize the resistance status of the colony and maintain a high level of resistance in the colony are also reported in this chapter. The following chapter build in complexity to fully explore the effects of LLIN exposure on this resistant colony.

In Chapter 4, we next explored more realistic searching behavior using modified baited tunnels allowing the investigation of the repellent and/or irritant effects of LLINs on insecticide resistant mosquitoes. The assay system allowed mosquitoes the choice to take a blood meal on a human foot enveloped in either an untreated net, or an LLIN, every day or every four days. We tested the effect of insecticide exposure on the transmission potential of mosquitoes considering parameters such as host searching, feeding success, feeding rate, egg laying, number of gonotrophic cycles and longevity. Those parameters were expected to correlate with age, vector control tool usage (treated net vs a

non-treated net) and difficulty to reach the host due to net quality (intact net vs a damaged net). To complete the story, the chapter 5 also describes how larval environmental variations, namely larval density and diet, influence phenotypic expression of host searching and feeding behavior in adult female mosquitoes over the entire life.

In chapter 6, we evaluated behavioral consequences of LLIN exposures and complemented this work with mechanistic studies examining effects of insecticide exposure on mosquito olfaction. As the chapter 4 showed a difference in feeding success depending on the access to the host and net quality, the goal here was to better understand if detection of odor and host searching behavior in mosquitoes is altered following an exposure to insecticide. Even though extremely resistant mosquitoes do not necessarily die from an exposure and are not knocked down, they might suffer a behavioral "hangover" from the exposure. The direct and life-long effect of insecticide on subsequent blood meals was investigated in short-range assays in the laboratory. Additionally, electrophysiological sensory responses to human and plant odors in wild-type insecticide resistant mosquitoes were measured to provide information on whether insecticide exposure impacted detection of odors and hence, searching behavior.

Finally, in chapter 7, we assessed whether mosquitoes are actively and successfully host seeking and feeding in the night following an exposure to insecticide in a semi-field experiment with two enclosed housed working as a large distance dual-choice assay. We quantified the host searching and feeding rate to better understand the spatial and excito-repellency properties of deltamethrin against field mosquitoes, to better understand if repelled mosquitoes are diverted to an unprotected host, and if being pre-exposed to insecticide induced changes in mosquito's host searching strategy.

CHAPTER TWO

Importance of host searching, blood feeding and history of insecticide exposure for the evaluation of insecticide resistance in malaria mosquitoes

2.1. Abstract

Long-Lasting Insecticidal Nets (LLINs) appear to continue to protect against malaria despite the increase of insecticide resistance. A possible reason is that resistance is defined by the mortality of mosquitoes 24 hours after a standard exposure to an insecticide, which ignores that, for example, time of and age at exposure vary in nature and which fails to capture possible impacts of insecticides on other parameters relevant for malaria transmission, such as the feeding success and longevity of the mosquitoes that are exposed but not rapidly killed.

We measured the behavior of highly insecticide resistant mosquitoes faced with a LLIN (PermaNet® 2.0) or an untreated net in three experiments. First, we exposed mosquitoes to a PermaNet® 2.0 for 1, 3, or 5 minutes, gave them the opportunity or not to take a blood-meal during this time, and assessed whether the duration of exposure and feeding success would affect the longevity after exposure. Second, we gave the mosquitoes the possibility to bite through a net (PermaNet® 2.0, PermaNet® 3.0 and untreated net) for five minutes. In contrast to the first experiment the mosquitoes contacted the net only if they tried to take a bloodmeal. We measured the time spent on the net, the feeding success, the time spent blood feeding, and the longevity after exposure. Third, we assessed whether multiple exposure to a sublethal dose of insecticide shortens the mosquito's lifespan.

In the first experiment, unfed mosquitoes lived on average 16 days, independent of exposure. Blood-feeding increased the longevity to 18 days after an exposure of 1 minute and 20 days after an exposure of 5 minutes. In the second experiment, the blood-fed mosquitoes exposed to a PermaNet® 2.0 lived 25 % longer than unfed mosquitoes, and the longer they spent on the LLIN, the longer they lived. However, the insecticide shortened the time mosquitoes contacted the net, almost halved the likelihood that they obtained blood and shortened the duration of the blood meal. In the third experiment, during which mosquitoes were exposed to the insecticide in WHO cones, a delayed mortality effect decreased the mosquitoes' lifespan by about a quarter and multiple exposures further shortened the lifespan.

Thus, although extremely resistant mosquitoes do not die within 24 hours of an LLIN exposure, the insecticide affected other parameters that are relevant for transmission. The standard WHO test procedures may therefore be only weak indicators of malaria transmission potential. We need alternative assay methods to better characterize the functional significance of insecticide resistance.

2.2. Introduction

Thanks in large part to the introduction of over 1 billion long-lasting insecticidal nets (LLINs) in the tropics, malaria cases have been dramatically reduced in the past 15 years[108]. LLINs provide a physical barrier that protects us from mosquitoes that attempt to feed at night. The insecticidal compound adds further to personal protection, it kills mosquitoes, protecting people who do not use a net from being bitten and thus contributes to a community-wide benefit[231,232]. However, with the increase of insecticide resistance genes, particularly the target-site mutation of the voltage-gate sodium channel gene (kdr mutation)[233–236] throughout malarious regions, LLINs have become less able to kill mosquitoes[201,205,225,230,237].

Nevertheless, LLINs treated with pyrethroid remain more effective than untreated nets[201]. One of the reasons is that the effectiveness of malaria control depends not only on killing the mosquito shortly after its exposure to the insecticide but can also depend on sub-lethal effects of the insecticide on host-seeking, feeding or longevity. In particular, insecticides will block the transmission of malaria providing they kill infected mosquitoes before the parasite has completed its extrinsic incubation period (EIP)[168,238]. A recent study, for example, showed that LLINs can kill highly resistant mosquitoes several days after exposure, reducing the potential of transmission of malaria by two thirds[168]. This effect may well be enhanced by repeated exposure to the insecticide[168,238]. Furthermore, we do not know whether resistance changes how mosquitoes respond in the face of the repellent and irritant pyrethroids properties of pyrethroids, and thus how resistance affects the personal protection offered by LLINs.

We therefore studied how insecticides affect the blood-feeding success and the longevity of *Anopheles gambiae* with more than 1700-fold insecticide resistance. Our main aim was to evaluate the overall effect of insecticides on the transmission potential of malaria vectors. A second aim was to understand the extent to which standard forced exposure assays (like WHO tube or cone tests), provide insights about malaria transmission.

2.3. Material and general methods

2.3.1. Mosquito populations

These experiments were done with *Anopheles gambiae* s.l. collected from natural breeding habitats. 90 % of the tested mosquitoes were collected in Yao Koffikro (-5.09000W longitude and 7.68000 N

latitude) with mostly S-form *An. gambiae* s.s (unpublished data) and 10 % of the tested mosquitoes were collected in M'be (5.209963 W longitude and 7. 970241 N latitude) with 99 % M-form *An. coluzzi*, in central Côte d'Ivoire[239–241]. Those suburban villages of Bouaké are dominated by highly insecticide resistant *An. gambiae* s.l that exhibit both kdr and metabolic resistance and breed in vegetable and rice fields[165,230,242]. CDC bottle assays indicate more than 1700-fold resistance to deltamethrin relative to a standard susceptible stain[165]. The collected larvae were reared to adults with Tetramin™ baby fish food at 27 ± 2 °C and at a density of 300 larvae in metallic bowls containing 1 liter of deionized water. Before being tested, adult mosquitoes were kept in 32.5 cm^3 mosquito cages and maintained on 10 % sugar solution at 27 ± 2 °C, 60 ± 20 % RH and ambient light. They were tested when they were 4 or 5 days old, and then kept in plastic cups covered with netting. They were provided a cotton ball soaked in 10 % sugar solution that was renewed daily.

2.3.2. Human host preparation

An experimenter, who was monitored daily for malaria infection as not to infect mosquitoes, was involved in all three experiments. Her arm was washed with unscented soap and rinsed with water the day before a test. She avoided the use of fragrance, repellent products, tobacco, and alcohol for 12 hours before and during testing.

2.3.3. LLINs

We used three types of netting: unwashed PermaNet® 2.0 and PermaNet® 3.0 manufactured by Vestergaard Frandsen SA (DK) and an untreated polyester net distributed by Coghlan's used as the control. The PermaNet® 2.0 is made of polyester and coated with 55 mg/m^2 ± 25 % deltamethrin. The PermaNet® 3.0 has polyester side panels coated with 85 mg/m^2 ± 25 % deltamethrin and a polyethylene roof panel coated with 121 mg/m^2 ± 25 % deltamethrin and 759 mg/m^2 ± 25 % Piperonyl Butoxide (PBO)[203]. PBO increases the insecticide penetration rate into the insect cuticle and prevents enzymatic insecticide breakdown[149], thus increasing the efficacy of the insecticide. We confirmed the efficacy of the nets by exposing sensitive mosquitoes (Kisumu strain) to pieces of each net; all the mosquitoes died within 24 hours when exposed to insecticide, while the untreated net (UTN) killed none. For the rest of the article, we refer to the PermaNet® 2.0 and the side panels of PermaNet® 3.0 as LLIN (despite the different concentrations of insecticides) because we found no appreciable difference between both nets in our experiments; and refer to the roof panel of PermaNet® 3.0 as LLIN+PBO. Ultimately, we were interested in "delta-only" nets compared to "delta+PBO" nets.

2.4. Behavioral assays and insecticide susceptibility tests

2.4.1. Effect of the duration of exposure on blood meal success and longevity

In two replicates, we exposed a total of 341 field-collected, 4-5 days-old unfed female mosquitoes to the insecticide for 1, 3 or 5 minutes in WHO tubes lined with a piece of LLIN (PermaNet® 2.0). In two additional replicates, we exposed 140 mosquitoes for 5 minutes in WHO tubes lined with a piece of LLIN. During the exposure, half of the mosquitoes had the opportunity to take a blood meal on one of the experimenter's arms; the other half could not feed, but the experimenter held one of her arms 1 cm from the tube to provide host cues. Mosquitoes were chosen haphazardly to give similar sample sizes for each duration of exposure and feeding opportunity (Table 1). Mosquito feeding status was recorded, and mosquitoes were then kept individually in plastic cups covered with netting and a 10 % sugar solution cotton that was renewed daily. Fed females could lay eggs in their cups on a wet cotton pad. Survival was monitored daily until all mosquitoes had died.

2.4.2. Effect of LLIN type on the time spent blood-seeking, blood-feeding, and longevity

This experiment relaxed the constraint of 'forced contact' imposed by WHO tubes by keeping instead mosquitoes in plastic cups that were not lined with the a bednet, but that were covered with a LLIN, a LLIN+PBO or an UTN at the top. Thus, mosquitoes only contacted the net if they were blood-feeding or if they chose to rest on it. In three replicates, a total of 246 field-collected, 4-5-days old unfed female mosquitoes were placed individually for 5 min into a cup. In replicate 4 there are no data for the LLIN+PBO. One of the experimenter's arms was placed onto the net at the top of the cup to attract mosquitoes and enable blood feeding. The time mosquitoes were in contact with the net and the duration of their blood-meal were recorded. After 5 min, mosquitoes were placed individually into their rearing plastic cups. Mosquito survival was monitored daily until all of them had died.

2.4.3. Effect of repeated exposure to the insecticide on longevity

This experiment investigated whether repeated exposure to a non-lethal dose could kill mosquitoes. 342 field collected mosquitoes were haphazardly assigned and maintained in groups of ten in plastic cups up to their death. Each group was assayed using WHO cones containing an LLIN (PermaNet® 2.0) or an untreated net (UTN) either once, every four days, or every day. The mosquitoes were 4-5 days old at their first exposure. They were then given access to a 10 % sugar solution cotton that was renewed daily. Survival was monitored daily until all mosquitoes in the insecticide treatments had died.

Exp.	Replicate	LLIN exposure(s)	Access to blood	Bednet	Exposure time (min)	Sample size	Sample size per replicate
1	1	1	Yes	LLIN	1	34	219
		1	Yes	LLIN	3	32	
		1	Yes	LLIN	5	30	
		1	No	LLIN	1	40	
		1	No	LLIN	3	40	
		1	No	LLIN	5	43	
	2	1	Yes	LLIN	1	24	122
		1	Yes	LLIN	3	21	
		1	Yes	LLIN	5	16	
		1	No	LLIN	1	16	
		1	No	LLIN	3	21	
		1	No	LLIN	5	24	
	3	1	Yes	LLIN	5	46	84
		1	No	LLIN	5	38	
	4	1	Yes	LLIN	5	24	56
		1	No	LLIN	5	32	
2	1	1	Yes	UTN	5	21	61
		1	Yes	LLIN	5	20	
		1	Yes	LLIN+PBO	5	20	
	2	1	Yes	UTN	5	18	58
		1	Yes	LLIN	5	20	
		1	Yes	LLIN+PBO	5	20	
	3	1	Yes	UTN	5	47	127
		1	Yes	LLIN	5	55	
		1	Yes	LLIN+PBO	5	25	
	4	1	Yes	UTN	5	61	123
		1	Yes	LLIN	5	62	
3	1	1	No	UTN	3	48	342
		1	No	LLIN	3	50	
		Every 4 days	No	UTN	3	74	
		Every 4 days	No	LLIN	3	70	
		Daily	No	UTN	3	50	
		Daily	No	LLIN	3	50	

Table 1: Sample size used for each replicate in experiment 1.: Effect of Long-Lasting Insecticidal Net (LLIN) exposure time on blood meal success and insecticide susceptibility, 2.: Effect of LLIN type on time spent host seeking, blood feeding and longevity and 3.: Effects of multiple insecticide exposures on insecticide susceptibility. This table also informs about the species, number of exposures, access to blood, insecticide exposure treatments and exposure time for each treatment given to the tested mosquitoes. LLIN: Long-Lasting Insecticidal Net. LLIN+PBO: Long-Lasting Insecticidal Net with the addition of piperonyl butoxide. UTN: Untreated Net.

2.5. Statistical analysis

In all analyses, contrasts among treatments were assessed with the *multcomp* package version 1.4-10 and the function *glht* with Tukey's honestly significant difference test (Tukey HSD). All statistical analyses and graphs were done in the software R© version 3.6.1.

2.5.1. Effect of LLIN exposure time on blood meal success and insecticide susceptibility

Using a Generalized Linear Model (GLM) with a binomial distribution (Bernoulli trial involving outcomes classified into two events: probability of success and failure), the blood feeding success of mosquitoes given access to a blood source was analyzed to investigate whether the proportion that was fed depended on the duration of insecticide exposure and the replicates.

We analyzed the longevity after exposure with a weighted Cox regression (using the R package *coxphw* due to the violation of the proportional hazards assumption in a Cox regression model) regarding the blood feeding categories (no access to a blood source; access to a blood source but unfed; access to a blood source and fed), the duration of insecticide exposure, their interaction, and the replicates as co-variate.

Moreover, mosquito longevity in two additional replicates was analyzed together with the other replicates considering mosquitoes exposed in WHO tubes for 5 min only. A weighted cox model was used to investigate the effect of the blood feeding categories, the replicates, and their interaction.

2.5.2. Effect of LLIN type on the time spent host seeking, blood feeding, and longevity

We analyzed the time spent on the net with a gaussian GLM and an identity link function (we assumed that the random variable is normal) including the type of bed net (UTN, LLIN, LLIN+PBO) and replicate as nominal factors.

We analyzed the proportion of mosquitoes that fed with a binomial GLM and the time spent feeding with a gaussian GLM, both including the type of bed nets, the time spent on the net, their interaction and replicate as factors.

We analyzed the longevity of the mosquitoes with a weighted cox proportional hazards model with the type of bed net, the feeding status, their interactions, and the replicate as factors. Considering fed mosquitoes alone, the same analysis was done adding the time spent on the net and the time spent feeding. Then considering unfed mosquitoes alone, the analysis was repeated with the time spent on the net.

2.5.3. Effect of multiple insecticide exposures on insecticide susceptibility

A binomial GLM analyzed the 24h mortality considering the type of bed net (untreated net or LLIN). We analyzed the longevity with a weighted cox proportional-hazards analysis including the type of bed net (untreated net or LLIN), the number of exposures, their interaction, and the cup as factors.

2.6. Results

2.6.1. Effect of LLIN exposure time on blood meal success and insecticide susceptibility

The LLIN did not prevent biting and almost all the mosquitoes with access to a blood meal fed, 84.7 (95 % Confidence interval: 78.1 to 89.9) %, irrespective of the duration of the exposure (χ^2 = 0.58, df = 1, p = 0.44). The replicates did not influence the blood feeding success (p > 0.05).

The same LLIN that was 100 % efficient at killing susceptible mosquitoes (Kisumu strain) failed to effectively kill insecticide-resistant mosquitoes 24h post-exposure. In this first experiment, mosquitoes lived an average of (mean ± se) 16.2 ± 0.34 days. A blood meal added 2.5 days to the average lifespan of fed mosquitoes (18.7 ± 0.59 days), which is 4 more days in comparison to unfed mosquitoes (14.6 ± 0.37 days) (χ^2 = 33.22, df = 2, p < 0.001) (Figure 1). If mosquitoes were not blood-fed there was no influence of exposure time on longevity [1min: 15.9 ± 0.76; 3 min: 14.4 ± 0.55 days; 5 min: 13.8 ± 0.58 days] (p > 0.05).

If mosquitoes were blood-fed, longer exposure to insecticide (LLIN) led to a longer life (χ^2 = 11.42, df = 1, p = 0.003). When exposed for 1 or 3 minutes, fed mosquitoes lived an average of 2.8 more days than unfed mosquitoes [1+3 min, fed: 17.9 ± 0.59 days; 1+3 min, unfed: 15.1 ± 0.47 days] (χ^2 = 29.64, df = 1, p < 0.001) and when exposed for 5 minutes, fed mosquitoes lived another 6.6 more days [1min:

18.2 ± 0.80; 3 min: 17.5 ± 0.89 days; 5 min: 20.4 ± 1.36 days]. Thus, while the interaction between exposure time and blood-feeding was significant on longevity, there was no main effect of exposure duration itself (χ^2 = 0.11, df = 1, p = 0.73).

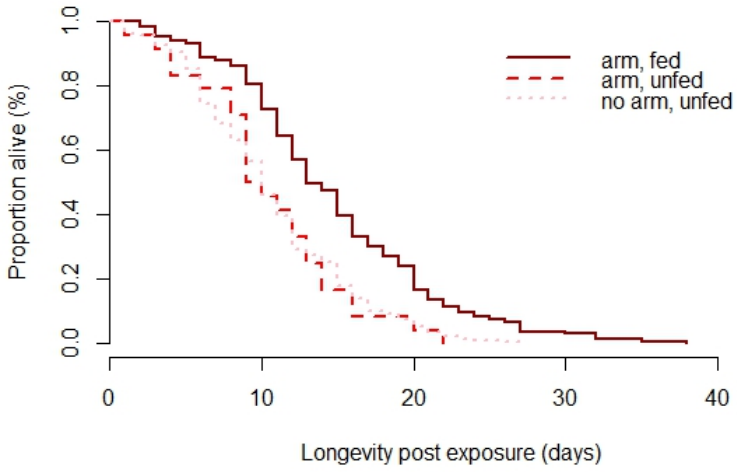

Figure 1: Survival curves for mosquitoes exposed in World Health Organization bioassays tubes against a PermaNet® 2.0 for 1, 3 or 5 min and with or without access to a human host (arm). The 3 lines represent the survival curves of mosquitoes that took a blood meal when they had access to an human arm (solid dark red line), mosquitoes that did not take a blood meal while having access to an human arm (dotted red line) or mosquitoes with no access to a blood source (smaller dotted pink line).

Two additional replicates, in which the mosquitoes were all only exposed for 5 minutes corroborated these results. When combining these two replicates with mosquitoes exposed to insecticide for 5 min in the first two replicates, similar results were found. A blood meal extended the lifespan of fed mosquitoes with 3.5 days more than the average lifespan [fed: 20.6 ± 0.87 days and unfed: 13.5 ± 0.37 days] (χ^2 = 65.17, df = 2, p < 0.001). The replicate 2 had an overall higher mean longevity compared to the overall mean of the others replicates, 17.6 ± 1.20 days against 15.4 ± 0.45 days respectively (χ^2 = 15.79, df = 1, p < 0.001). In this analysis as well, no interaction was found between the 3 feeding categories (taking a blood meal when access to an arm, not taking a bloodmeal when access to an arm, and no access to an arm) and the replicates (χ^2 = 0.52, df = 2, p = 0.77).

2.6.2. Effect of LLIN type on time spent host seeking, blood feeding and longevity

When mosquitoes could choose to contact the LLIN or not, the average contact time was lower than the maximum potential contact time of 5min (300 seconds) (Figure 2.1) (F = 14.67, df = 3, p < 0.001). Mosquitoes exposed to insecticide with PBO spent on average around one minute in contact with the net, which is half the average time spent on a LLIN without PBO, and 2.8 times less than the average time spent on an UTN [respectively 59.1 ± 9.25 sec, 121.4 ± 9.50 sec and 167.7 ± 13.06 sec].

Similarly, fed mosquitoes exposed to a LLIN with PBO spent a 1.3-times shorter period feeding on the net than mosquitoes exposed to a LLIN without PBO (Figure 2.2) and those mosquitoes exposed to a LLIN spent 1.6 less time feeding than mosquitoes exposed to an UTN [respectively, 105.0 ± 15.85 sec, 135.4 ± 9.80 sec and 219.3 ± 8.83 sec] ($F = 72.63$, $df = 3$, $p < 0.001$).

The presence of insecticide led to a reduction in the proportion of mosquitoes that fed successfully (Figure 2.3) ($\chi^2 = 48.06$, $df = 3$, $p < 0.001$). Mosquitoes exposed to a LLIN without PBO took 1.6 times fewer blood meals than those exposed to an UTN, respectively 38.9 (95 % Confidence Interval: 29.1 to 49.5) % and 60.5 (95 % CI: 49.3 to 70.8) %. Only 9.2 (95 % CI: 3.46 to 19.0) % of mosquitoes exposed to a LLIN with PBO took a blood meal. In addition, the longer the time spent in contact with the net, the more successful the blood meal attempt ($\chi^2 = 111.78$, $df = 1$, $p < 0.001$). Blood fed females spent 3.6 more time in contact with nets than unfed ones [respectively 216.8 ± 7.38 sec and 59.80 ± 6.22 sec].

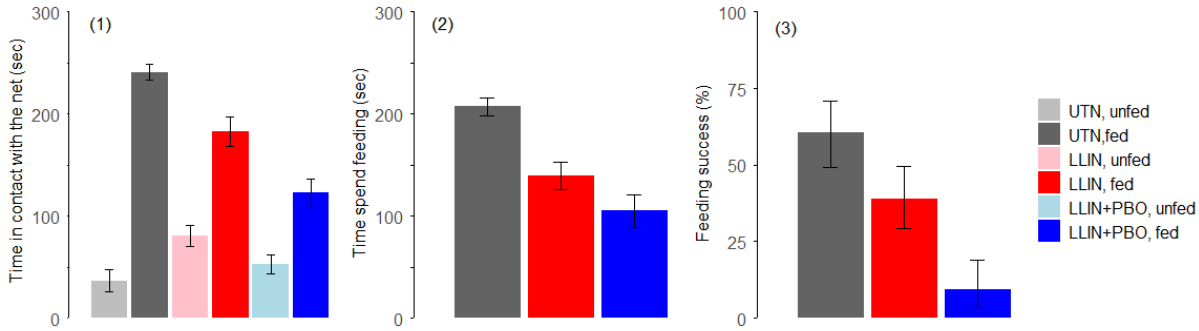

Figure 2: Panel representing the (1) mean time (in seconds) spent on the net, (2) mean time (in sec) spent taking a blood meal and (3) percentage of feeding success (in %) for mosquitoes exposed in a plastic cup against a LLIN (PermaNet® 2.0 or a PermaNet® 3.0 side), a LLIN with PBO (PermaNet® 3.0 roof), or an untreated net (UTN) for 5 min and with access to an human host. The black and grey bars represent respectively mosquitoes exposed to an UTN that did or did not take a blood meal. The red and pink colors represent respectively mosquitoes exposed to an LLIN that did or did not take a blood meal. The blue and light blue and blue colors represent respectively mosquitoes exposed to an LLIN+PBO that did or did not take a blood meal. The replicate 3 is not represented in this figure as there is no data for LLIN+PBO. ± standard error bars are shown in the figure (1) Time in contact with the net and (2) Time spent feeding and 95 % confidence interval is shown in the figure (3) Feeding success.

As observed in the first experiment, a delayed mortality due to insecticide was associated with the initial exposure ($\chi^2 = 142.12$, $df = 2$, $p < 0.001$). Mosquitoes exposed to an UTN survived 1.7 times longer than those exposed to insecticide (both LLIN and LLIN+PBO combined), with an average lifespan of 20.9 ± 0.74 days and 12.1 ± 0.54 days, respectively (Figure 3). The effect of the LLINs on longevity

was the same, [mean longevity of PermaNet® 2.0: 16.1 ± 0.78 days and the PermaNet® 3.0 side: 15.3 ± 1.03 days], but mosquitoes exposed to a LLIN with PBO had a shorter lifespan compared to all treatments, with a mean of 6.7 ± 0.45 days.

However, if the mosquitoes had blood fed, they survived 1.7 times longer, with fed females having an average survival time of 20.1 ± 0.83 days and the unfed females 12.1 ± 0.51 days (χ^2 = 24.13, df = 1, p < 0.001). While this observation is true for fed mosquitoes exposed to a LLIN or an UTN [respectively for the fed ones the average lifespan was 18.3 ± 1.10 days and 23.1 ± 0.99 days; and for the unfed ones was 14.2 ± 0.67 days and 17.4 ± 0.81 days], but in presence of PBO fed mosquitoes did not have a longer life than the unfed ones [with average survival time of 5.3 ± 0.21 days and 6.9 ± 0.49 days, respectively].

Despite the blood meal benefit on longevity, the fed females died more quickly when exposed to insecticide than those exposed to a UTN (χ^2 = 7.93, df = 2, p = 0.019). The time fed mosquitoes spent on the net did not influence longevity (χ^2 = 1.27, df = 1, p = 0.26) but the time taken for a blood meal did influence longevity (increase longevity χ^2 = 4.31, df = 1, p = 0.04). An interaction was found between the net type and the time taken to blood feed (χ^2 = 7.81, df = 2, p = 0.02).

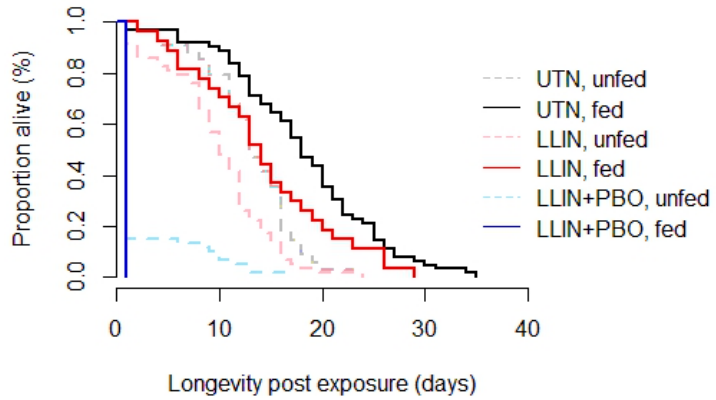

Figure 3: Survival curves for mosquitoes exposed in a plastic cup against a PermaNet® 2.0 or a PermaNet® 3.0 side (LLIN), the roof of a PermaNet® 3.0 (LLIN+PBO), or an untreated net (UTN) for 5 min and with access to a human host. The dotted lines and light colors represent the survival of mosquitoes that did not take any blood meal even though they had access to a human arm, the solid lines and darker colors represent mosquitoes that successfully fed. The blue lines represent mosquitoes exposed to a LLIN+PBO, the red lines mosquitoes exposed to a LLIN, the black lines mosquitoes exposed to an UTN. The replicate 3 is not represented in this figure as there is no data for LLIN+PBO.

One additional replicate was added to an analysis comparing the effects of one LLIN (PermaNet 2.0) and UTN exposure only. The presence of insecticide reduced the mean time spent on the net 1.5 times [LLIN: 118.5 ± 7.64 sec and UTN: 173.3 ± 10.11 sec] ($F = 9.51$, $df = 2$, $p < 0.001$). No difference was found in between replicates influencing the time spent in contact with the net ($F = 0.01$, $df = 1$, $p = 0.93$).

The insecticide exposure treatment influenced blood feeding success ($\chi^2 = 18.77$, $df = 2$, $p < 0.001$). 1.6- times fewer mosquitoes exposed to a LLIN took blood meals in comparison to those exposed to an UTN [38.2 (95 % CI: 30.6 to 46.3) % against 61.9 (95 % CI: 53.6 to 69.8) %]. Also, mosquitoes that spent a longer period on the net were proportionally more successful in taking a blood meal ($\chi^2 = 206.16$, $df = 1$, $p < 0.001$). When mosquitoes spent less than 1 minute on the net, the feeding success was not different for both the insecticide treatment and the control. However, over 1 min, the more time mosquitoes spent on an UTN, the greater the blood meal success, but this pattern did not hold for mosquitoes exposed to a LLIN ($\chi^2 = 13.58$, $df = 2$, $p = 0.001$). The blood feeding in the different replicates was not significantly different ($\chi^2 = 0.90$, $df = 1$, $p = 0.34$).

There was an overall effect of the insecticide exposure on longevity ($\chi^2 = 29.30$, $df = 1$, $p < 0.001$). The mean survival time of fed mosquitoes was 1.3 times higher than for unfed mosquitoes, respectively 21.2 ± 0.59 days and 15.9 ± 0.47 days ($\chi^2 = 40.06$, $df = 1$, $p < 0.001$). For fed mosquitoes, there was also an interaction between insecticide exposure treatment and time spent feeding ($\chi^2 = 5.12$, $df = 1$, $p = 0.024$) and for unfed mosquitoes an interaction was found between the time spent in contact with the net and net type ($\chi^2 = 6.00$, $df = 1$, $p = 0.014$).

2.6.3. Effect of multiple insecticide exposures on insecticide susceptibility

An initial exposure to insecticide led to a negligible 3.8 (95 % CI: 2.0 to 6.4) % mortality difference at 24h, as 1.8 (95 % CI: 0.4 to 5.1) % of mosquitoes exposed to an UTN died at 24h post exposure against 5.8 (95 % CI: 2.8 to 10.4) % of mosquitoes exposed to a LLIN ($\chi^2 = 4.97$, $df = 1$, $p = 0.032$) (Figure 4). In line with the results above, there was also an apparent delayed mortality due to insecticide and overall longevity was reduced by 31.3 % relative to controls; LLIN pre-exposed mosquitoes had average survival times of 12.4 ± 0.35 days against 17.0 ± 0.43 days for the control group ($\chi^2 = 84.37$, $df = 1$, $p < 0.001$). The effect of one exposure on mean longevity was already important, as mosquitoes exposed to insecticide had reduced average survival (14.1 ± 10.64 days) compared with those exposed to an untreated net (18.1 ± 0.76 days). But the effect of several LLIN exposures had an even greater effect ($\chi^2 = 9.34$, $df = 2$, $p = 0.009$). In fact, the mortality due to insecticide exposures every four days

resulted in a reduction in average survival time of 5.2 days in comparison to the control group (11.7 ± 0.48 days and 16.9 ± 0.65 days, respectively). Interestingly, the impact of daily exposure appeared to have no additional impact above and beyond exposure every four days, both treatments having a mean lifespan of respectively 11.8 ± 0.69 days and 16.2 ± 0.89 days. We did not find an effect of the cup mosquitoes were kept in (χ^2 = 0.06, df = 1, p = 0.81) nor an effect of the interaction between the insecticide exposure treatment and the number of exposures (χ^2 = 0.45, df = 2, p = 0.80).

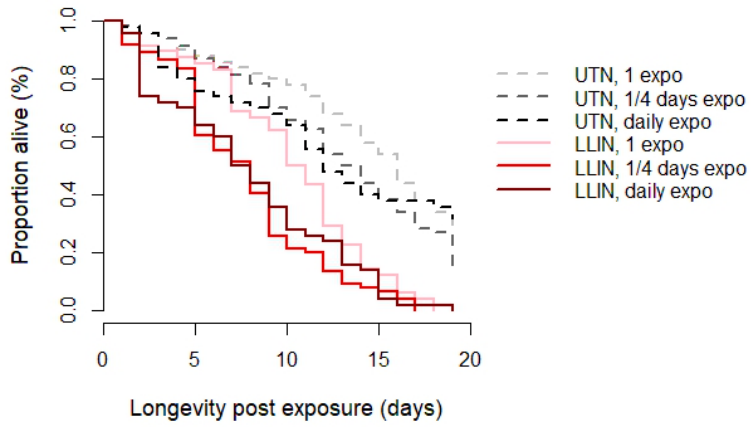

Figure 4: Survival curves following 3-min insecticide exposure in WHO cones: one exposure, an exposure every 4 days, or an exposure every day to a deltamethrin treated net (PermaNet® 2.0, LLIN) or an untreated net (UTN). The data were censored 23 days after the first exposure. The grey dotted lines represent the long-term survival of mosquitoes exposed to an UTN and the red dotted lines represent the long-term survival of mosquitoes exposed to a LLIN. The light grey and pink lines represent one exposure treatment. The grey and red lines represent an exposure every four days and the black and dark red lines represent an exposure every day. The survival curves begin 24h after the initial insecticide exposure with 5 days old female.

2.7. Discussion

The initial experiment confirms that field *Anopheles gambiae* collected around Bouaké, Cote d'Ivoire are extremely resistant to pyrethroids[230] such that even forced exposure to an LLIN fails to instantly kill mosquitoes, or prevent probing and blood-feeding[165,182,201]. However, the second experiment adds some realism to the assays by letting mosquitoes feed more "naturally" and hence have more realistic contact with the LLIN than a forced exposure. Our data also show that the 3-minute exposure time recommended by the WHO for the evaluation of LLINs does not necessarily correspond to the time mosquitoes spend in contact with the tested net during host searching and blood feeding in nature[243]. According to data acquired by Diop et al.[171], mosquitoes exposed to insecticide do most likely bounce on the LLIN until they decide to probe and take a blood meal. How long they choose to stay on the net depends most likely on the level of toxicity of the net as well as on the presence of a host[244]. In our

study, the effects of deltamethrin described here [245,246], and especially in combination with the synergist PBO, reduces the time spent on the treated net but does not completely prevent mosquitoes from biting through it. Interestingly, the fact that LLINs do not repel highly resistant mosquitoes, may help maximize the potential sublethal effects of insecticide against them, which would increase LLIN efficacy[168,238].

In the first experiment, a high percentage of mosquitoes were able to blood feed and a forced contact for up to 5 minutes was not obviously more likely to kill mosquitoes than 1 minute. Moreover, those mosquitoes that did blood feed, and these are the ones we care about as they could potentially pick up malaria, lived longer. When given the possibility to choose to contact the net (rather than forced exposure), the presence of insecticide led to a reduction in the proportion of mosquitoes that fed successfully. Thus, even though mosquitoes do not die instantly, some personal protection is expected against these highly resistant mosquitoes. Contact irritancy could influence feeding success by dissuading and reducing persistence to find a host and/or a hole on the net[162,247].

Insecticide exposure not only reduced the time spent in contact with the net, it also reduced the time spent on the net feeding. Spending less time on a net during blood feeding does not necessarily mean that the blood meal size is smaller and/or insufficient for malaria transmission[248]. The presence of insecticide may reduce the capacity to engorge blood[182], or on the contrary, it may motivate mosquitoes to take more blood on a shorter period in order to minimize the contact time with the treated net. A shorter time in contact with the treated net could also lower the insecticide dosage received by blood feeders and this could be a behavioral adaptation of mosquitoes living in areas with high use of LLINs.

Interestingly, unfed mosquitoes exposed freely to insecticide were found to spend more time in contact with the net than unfed mosquitoes exposed to an untreated net and, in the end, less mosquitoes exposed by their own choice to insecticide fed, suggesting that a significant proportion of mosquitoes failed to take a blood meal through a LLIN, not because they avoided the net or were repelled by it, but more likely because contact irritancy seemed to reduce their feeding capacities while they were still sitting on the LLIN.

Whether mosquitoes were forcefully exposed to insecticide or free to take a blood meal on host protected by a LLIN, the 24h survival post-exposure corroborates our first results and previous findings, that LLIN exposure, whether against a PermaNet® 2.0 or the side of a PermaNet® 3.0, fails to

immediately kill 4-5 day old, unfed, resistant mosquitoes[201,225] and the higher concentration in deltamethrin on the PermaNet® 3.0 did not increase mortality. However, this standard 24h monitoring[168] misses the long-term effect of an exposure to a LLIN on the proportion of mosquitoes surviving long enough to potentially transmit malaria. Our data show that exposure to an LLIN results in delayed mortality for highly insecticide resistant mosquitoes and corroborated the results from Viana et al.[168]. This observation motivated the third set of experiments with mosquitoes exposed multiple times across their life in order to test for a cumulative effect of insecticide exposures on longevity. Indeed, only mosquitoes strong enough to take two blood meals in their life (one to acquire the parasite and one to pass it on) and survive the extrinsic incubation period of the parasite matter for malaria transmission[238,249].

In the third experiment, exposure every four days simulates likely contact rate as mosquitoes attempt to take a blood meal every new gonotrophic cycle (3-4 days)[250]. The daily exposure treatment simulates what might happen if mosquitoes repeatedly failed to take a blood meal and so potentially contact a net every night as they continue to host search. This high frequency of exposure serves as an extreme scenario to contrast with the usual single exposure assays.

Repeat exposure to LLINs reduced overall survival, although there was no difference between daily exposure and exposure every 4 days. In both cases, < 10 % of mosquitoes survived the 12-14 days parasite incubation period compared with 40-60 % in treatments exposed to an untreated net. This result suggests that in areas of high LLIN coverage where the chances of repeat exposure are greatest, LLINs can still reduce the transmission potential of even highly resistant mosquitoes.

The delayed mortality effects of insecticide exposure appear reduced if mosquitoes take a blood meal. These data highlight the importance of blood feeding in the evaluation of insecticide resistance. An additional interesting insight is that the effect of resistance depends on whether you consider resistance to be the difference between an LLIN and an untreated net (which is like asking how bad has a LLIN become), or between a LLIN and a resistance-breaking net (which is like asking how good could a LLIN be). Our data (together with others[251,252]) indicate that PBO can restore susceptibility, suggesting improved control potential of resistance breaking nets in areas of high insecticide resistance. The high level of PBO synergism may indicate that the involvement of cytochrome P450 monooxygenase is the dominant resistance mechanism in these mosquitoes[253]. However, this does not mean that conventional LLINs have no impact at all.

Our data suggest that insecticide exposure could help reduce transmission potential of highly resistant mosquitoes. The effects derive partly from reduced feeding rate and partly from delayed mortality following exposure. Blood feeding tended to increase survival, whereas increased frequency of exposure reduced survival. Whether these patterns are also apparent in mosquitoes infected with malaria parasites is unclear, yet it is these mosquitoes that we ultimately care most about. Malaria infection has been shown to alter feeding rates through impacts on apyrase activity, an enzyme that helps locate blood vessels and take longer blood meals that is less active in case of malaria infection[254] and/or because an immune challenge induces changes in insulin signaling in the mosquito gut resulting in alteration in feeding behavior[255]. Whether these behavioral changes affect blood feeding and exposure rates on LLINs is not known. Also, insecticide exposure could impact vector competence[192,256–258] and the presence of malaria parasites could affect expression of insecticide resistance[163,164]. More research is needed to fully understand the full consequences of malaria infection for mosquito behavior and the functional significance of resistance.

2.8. Conclusion

Taken together the data suggest that forced contact and single exposure assays miss important effects of LLINs that reduce feeding and longevity of resistant mosquitoes under more realistic exposure scenarios. These effects do not mean that LLINs are as effective against resistant mosquitoes as they are against susceptible ones (the PBO net induced much greater overall mortality and feeding inhibition, for example). However, they provide evidence that LLINs retain at least some function even against mosquitoes that are more than 1700-fold resistant. The experiments also highlight the fact that the standard WHO test procedures for evaluating resistance and the bio-efficacy of LLINs are weak indicators of malaria transmission risks, supporting the need for alternative assay methods to better characterize the functional significance of insecticide resistance.

CHAPTER THREE

Colonization, maintenance, and characterization of wild insecticide-resistant *Anopheles gambiae* s.l.

3.1. Abstract

One of the biggest challenges of working with mosquitoes collected from the field is that supply depends on the variability in quantity and quality of mosquitoes collected in variable natural larval habitats. I established a colony of field-derived insecticide resistant *Anopheles gambiae* mosquitoes as an alternative to field-collected mosquitoes to first allow the continuity of the work during the dry season, and second assure more controlled ecological conditions to standardize experiments. The challenge was to maintain a genetic diversity and level of resistance close to the field.

Anopheles gambiae s.l. mosquitoes were collected in rice and vegetable fields from Bouaké in central Côte d'Ivoire. To ensure resistant offspring, the colony mosquitoes fed on a human foot through a PermaNet® 2.0 and was regularly infused with new larval collections of field mosquitoes. One-way tunnel bioassays were performed to screen for change in the feeding success and fecundity of the colony. The WHO cone bioassays were used several times to monitor resistance prevalence and one CDC bottle test helped characterize the level of resistance after 16 months of colonization. Species identification and Taqman™ assays were used to screen for a shift from one species to another (sub-groups of *Anopheles gambiae* s.l.) and target site mutation resistance alleles (kdr and ace-1), in order to check for potential contamination with others laboratory strains and/or loss of resistance.

The high level of pyrethroid resistance did not drop and the diversity was maintained from July 2017 to December 2018. The WHO cone bioassays showed no changes in resistance and the level of pyrethroid resistance stayed high with on average only 1.5 % mortality at 24 hours. A high level of pyrethroid resistance was found with 56.5 % homozygotes and 43.5 % heterozygotes at the beginning of the colonization, and 17 months of colonization later, the frequency of kdr mutations was still at 94.4 %, with non-significant change. The ace-1 mutation was detected at a low frequency (1.6 %) at the start but was lost after 1.5 years. The CDC bottle test confirmed that the resistance intensity to the pyrethroid deltamethrin stayed extremely high as mosquitoes were found to be 1250-fold resistant. The acclimation to the lab helped the colony improve its feeding success over time.

The colonization of wild insecticide resistant mosquitoes was a success. The method used was a great alternative to working with field collected larvae. Whether this colony could have been maintained without regular additions of wild mosquitoes is not known. However, we hypothesized that our method helped maintain genetic diversity inside the mosquito population and this needs confirmation.

3.2. Introduction

Given the rapid spread of pyrethroid resistance there is considerable interest in understanding how resistance intensity affects mosquito life history traits and malaria transmission potential[211]. Conducting extensive exposure assays and life history studies on field-caught adult mosquitoes is challenging, as it is difficult to control for factors such as mosquito age, condition, size, infection status (malaria or other pathogens) and insecticide exposure history. Collection of mosquito larvae or pupae can control for some of these factors but there is still potential for variation due to quality of larval habitat. Availability is also likely to vary substantially between wet and dry seasons.

An alternative to continuous field collection is to establish a colony[259]. In principle, a colony should provide more predictable numbers and permit control of rearing conditions leading to less variation in life history traits. However, one challenge in a establishing a resistant colony is to maintain the diversity and level of resistance found in the field[260]. Artificial conditions (e.g. temperature, humidity, and photoperiod) can lead to unwanted selective pressures, with lab adaptation influencing the feeding success and feeding duration[261], mosquito size, mating and oviposition behaviors[11,262]. This adaptation can lead to inbreeding, genetic drift, and bottlenecks[263,264].

Here we present a hybrid model in which we established a colony that we maintained under periodic insecticide selection, and also included regular infusion of field material (in case it is needed to maintain genetic diversity). We report on the methods of establishing the colony and various quality assurance measures to confirm species identity and resistance status of mosquitoes used in other experimental chapters.

3.3. Material and methods

3.3.1. Mosquito collections

The majority of field mosquitoes larval collections were conducted in M'be in central Côte d'Ivoire (5.209963 W longitude and 7. 970241 N latitude), where previous research suggests the populations are dominated (99 %) by the Mopti (M)-form (now *An. coluzzi*)[239–241]. *An. gambiae* s.l. from M'be and other villages surrounding Bouaké are known to be highly insecticide resistant, exhibiting both kdr and metabolic resistance mechanisms[165,230,242]. Mosquito larvae were collected by a team of 2 or 3 technicians from the Institut Pierre Richet (Bouaké, Côte d'Ivoire) with typically 400-1000 larvae collected per prospection. Before sampling, a 1-2 min waiting period was observed letting larvae rise

to the surface. Then, a 350-ml dipper was used to capture larvae following the standard dipping method[265]. Following collection, larvae were transported to a laboratory in the vector control products evaluation center in Bouaké. Mosquitoes larvae were morphologically identified, and only immature *Anopheles* spp. were kept for mosquito colonization.

3.3.2. Mosquito colonization

Field-collected larvae were reared to adults at 27 ± 2 °C, 60 ± 20 % RH and the light in that room was on during the day from 8.00 am to 8.00 pm. 300 larvae per plastic boxes (capacity 2 liters) identified as being *Anopheles gambiae* s.l. mosquitoes were reared in 1 liter of either deionized water, or bottled spring water (the latter for a 5-months period when water supply was limited by a city-wide water shortage). The larvae were fed following the high larval food regime (our colony standard here) as described in Kulma and al., 2013[266] for 1 larvae: day of hatching: 0.04 mg of Tetramin^{TM} fish food per larva; day 1 after hatching: 0.06 mg; day 2: 0.08 mg; day 3: 0.16 mg; day 4: 0.32 mg; day 5 and more: 0.6 mg. Three hundred pupae were then placed in standard plastic rearing cages (l = 32.5 cm^3). Upon emergence inside the cages, adult mosquitoes had access to a cotton ball soaked in a 10 % sugar solution placed on top of a plastic cup. At all times, there were at least four cages of adult mosquitoes.

3.3.3. Colony maintenance

From July 2017 to December 2018, each generation of 4-5 days old females were given the possibility to take a blood meal on the same host's foot during 30 min (up to 1h in the beginning when feeding rates were lower). The volunteer was being monitored every day for malaria parasite to avoid any contamination of the colony with malaria-infected blood. The foot was placed directly inside a rearing cage to allow blood feeding and maximized the feeding success. During the first weeks, the feeding success after one opportunity was not enough to build a next generation of mosquitoes, therefore mosquitoes were fed two days in a row in the absence of insecticide. After 1-2 months, one blood meal per week was enough to maintain colony numbers (enough eggs were laid to ensure offspring). After blood meal digestion, engorged females could lay eggs on a water-soaked cotton in a petri dish sealed by a filter paper. The eggs obtained were then placed in a plastic box for larval rearing and a new generation was obtained.

Each one to two months, depending on larval prospection opportunities, around 50-100, 4-5-days old wild male and female adult mosquitoes were added to the colony in order to maintain the level of genetic diversity and resistance intensity. Those mosquitoes were added in the rearing cages

containing the youngest mosquitoes from the colony. During the dry season, especially from November to January, the larval collections were sparser and therefore less than 50 wild mosquitoes were regularly added.

3.3.4. Characterization of the colony

3.3.4.a. Evolution of feeding success and eggs laying

Every two generations, mosquitoes were exposed to insecticide by requiring them to access a blood meal through an untreated net (UTN) made of polyester (Coghlan's) or an un-washed Long-lasting Insecticidal Net (LLIN, PermaNet® 2.0) manufactured by Vestergaard Frandsen SA (DK) and made of polyester coated with 55 mg/m^2 ± 25 % deltamethrin. To allow blood feeding, the Bugdorm© rearing cage (Figure 1, b) was linked to a Bugdorm© experimental cage (a) via a transparent Plexiglas® tube (L= 30 cm, d= 14,6 cm). In the experimental cage, a foot was used as blood source and was enveloped in a "sock", a piece of UTN or LLIN (25 cm^2).

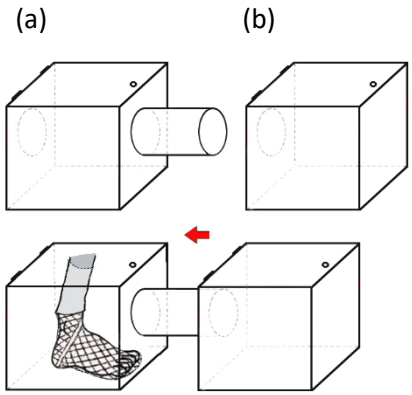

Figure 1: The one-way tunnel with one rearing cage (b), one Plexiglas® tube and an adjacent experimental cage (a) with a direct access to an experimenter's foot enveloped in an un-treated net (UTN) or a Long-Lasting Insecticidal Net (LLIN, PermaNet®2.0). When needed, the 32.5 cm^3 rearing cage containing the mosquitoes from the colony on the right was linked to another a 32.5 cm^3 experimental cage (the left one on the picture) containing the food source, a foot enveloped in a piece of an UTN or LLIN (l= 25 cm^2) thanks to a transparent Plexiglas® tube (L= 30 cm, d= 14,6 cm).

Female mosquitoes (800 in total; 50 mosquitoes x 8 replicates x 2 treatments) at 3-5 days old were tested in a one-way tunnel following the method explained in the preceding section (Figure 1). Once released inside the tunnel, mosquitoes had 30 min to go to the other side and take a blood meal on a human foot enveloped in a bed net. Half of them had access to a foot enveloped inside a piece of UTN and the other half to a foot enveloped inside a piece of LLIN. After 30 min, the number of blood fed

mosquitoes was counted, and mosquitoes were placed back in their rearing cage. The feeding success was here the number of blood fed mosquitoes divided by the number of mosquitoes tested. 24h later, mosquitoes were given the possibility to lay eggs on a filter paper recovering a water-soaked cotton in a petri dish. The number of eggs laid was then counted and divided by the number of females alive in the cage.

3.3.4.b. Insecticide susceptibility assays

To assess the prevalence of resistance, bioassays on 3-5 days old females were conducted multiple times using World Health Organization (WHO) cone (see Table 1 for more information). Those tests were carried out in day light and with non-blood fed females using either a LLIN or an UTN. Mosquitoes were exposed ten by ten in WHO plastic cones for 3 min, the cones were angled at 40° to favor the contact between the mosquitoes and the net, and a ball of cotton closed each cone's entry[267]. Following the WHO guideline, the knock-downs at 5 min and 1 hour after the exposure were recorded[268]. Mosquitoes were then kept in plastic cups covered with netting and supplied with cotton balls soaked in a 10 % sugar solution, for 24 hours when mortality was then checked.

3.3.4.c. Resistance intensity assays: “CDC bottle assays”

After maintaining the colony for 17 months, resistance intensity to deltamethrin (the insecticide used on the widely distributed PermaNet® 2.0) was measured using Centers for Disease Control and Prevention (CDC) bottle assays. Bottles were prepped and coated following the CDC guidelines[269]. Those assays were adapted based on the chosen concentrations that are frequently tested for insecticide resistance monitoring in the laboratory in Bouaké, Côte d'Ivoire[270]. The deltamethrin concentrations ranged from 0.001 µg/ml to 1 µg /ml for the Kisumu strain (SS), the susceptible strain used as reference, and from 1.25 µg/ml to 250 µg /ml for our colony. Four replicates of 25, 3-5-days old females were tested per deltamethrin concentration for one hour. For each strain, Kisumu and the colony, four replicates of 25 mosquitoes were also evaluated against a control bottle coated only with acetone. The number of dead mosquitoes was monitored at different time intervals (every 5 min from 5 to 40 min).

3.3.4.d. Mosquito species and target site resistance mechanisms identifications

Genomic DNA was extracted from randomly chosen portion of the F0 generation and 4 others following generations (see Table 3 for further information) and polymerase chain reaction (PCR) assays were performed 5 times to screen mosquito samples for molecular forms identification of the species in the *An. gambiae* complex (sibling species: the savannah (S) or mopti (M) molecular forms[271]) and

L1014F kdr mutations in the voltage gated sodium channel and ace-1 mutation in acetylcholinesterase. Following the protocol described by Zoh and al.[230] The genomic DNA of each tested mosquito body was grinded and boiled in 200 µl of Cethyl Trimethyl Ammonium Bromide 2 % at 65 °C for 5 min. After adding 200 µl of chloroform, the preparation was centrifuged for 5 min at 12000 rpm. The superior phase was selected and mixed with 200 µl of Isopropanol. After 15 min of centrifugation at 12000 rpm, the isopropanol was removed and 200 µl of ethanol at 70 % was added. The mix was centrifugated again at 12000 rpm, the ethanol removed, and the DNA suspended in 20 µl of sterile H_20 overnight.

Once the ribosomal DNA was extracted, it was used for polymerase chain reaction (PCR) analysis for the identification of the species following the procedure described by Favia et al[272]. The M-form took the name *Anopheles coluzzi* and the S-form retains the name *Anopheles gambiae*. The initial denaturation was set for 3 min at 94 °C and was followed by 28 cycles (one cycle: 94 °C for 30 s, 63°C for 30 s and 72°C for 45 s). The last elongation cycle lasted 5 min at 72°C. Primers used in the PCR were: R5 (5'-GCC AAT CCG AGC TGA TAG CGC-3'), R3 (5'-CGA ATT CTA GGG AGC TCC AG-3'), Mop int (5'-GCC CCT TCC TCG ATG GCA T-3') and B/S int (5'-ACC AAG ATG GTT CGT TGC-3')[230].

The PCR method described by Martinez-Torres et al[273] allowed the screening of 6 mosquito samples for mutation in the voltage gated sodium channel (L1014F kdr alleles). The PCR amplified the resistant sequence of 195 base pairs (bp) and the susceptible sequence of 137 bp with these two specific primers: Agd3 (5'-AAT TTG CAT TAC TTA CGA CA-3') and Agd4 (5'-CTG TAG TGA TAG GAA ATT TA-3'). The two primers used to amplify the common sequence of 293 base pairs (bp) in *Anopheles gambiae* s.l. were Agd1 (5'-ATA GAT TCC CCG ACC ATG-3') and Agd2 (5'-AGA CAA GGA TGA TGA ACC-3'). Amplified fragments were analyzed on a 1 % agarose gel.

The PCR method used by Weill et al., 2004[274] was used for the detection of (ace-1^R G119S) mutation in the acetylcholinesterase for all mosquito samples. The initial denaturation was set for 3 min at 94 °C and was followed by 35 cycles (94 °C for 30 s, 62 °C for 30 s and 72 °C for 20 s). The last elongation cycle lasted 5 min at 72 °C. Amplified fragments were digested with *Alu*I restriction enzyme and were analyzed on a 2 % agarose gel. The two primers used were: Ex3Agdir (5'-GAT CGT GGA CAC CGT GTT CG-3') and Ex3Agrev (5'-AGG ATG GCC CG CTG GAA CAG-3'). They amplified for a 403 bp fragment. The restriction enzyme *Alu*I digested this fragment in susceptible homozygous mosquitoes (SS) or cut the fragment in two (253 bp and 150 bp) for homozygous resistant (RR). Heterozygous individuals (RS) showed a combined pattern of (SS) and (RR).

3.3.4.e. Statistical analysis and data interpretation

Feeding performance (proportion of mosquitoes that successfully took a blood meal while tested) of the colony was analyzed using an Analyze of Variance (ANOVA) regarding the effect of the exposure treatment (LLIN or UTN) and colony establishment time. The proportion of eggs laid per female was analyzed using also an ANOVA regarding the exposure treatment and colony establishment time. The Shapiro-Wilk normality tests of model residuals confirmed that the data were normally distributed.

For the cone tests, WHO criteria[268] were adopted to define resistance status. 24h mortality rate between 98 %-100 % indicated full susceptibility, 24h mortality rate between 90 %-97 %, meant that the population was considered tolerant or suspected of resistance to the tested insecticide and 24h mortality rates < 90 % did equal resistance to the tested insecticides. We analyzed mosquito knock down at 1h and mosquito survival at 24h using quasibinomial Generalized Linear Models regarding the effects of the exposure treatment (exposure to either a LLIN or an UTN), the time (in months) after the colony establishment F(0), and their interaction. We used the *multicomp* package and the function *ghlt* for multiple comparisons of Means (Tukey contrasts). The 5 cups per treatment containing 10 mosquitoes each were considered as a co-variable. Statistical analysis was performed in R Studio© version 1.0.136 and the significance level was set at 5 %.

Concerning the CDC bottle test a 24h mortality rate < 100 % meant that the population tested was considered resistant to the tested insecticides. The interpretation of calibration data was based on the equations of 2 curves (for the susceptible strain Kisumu and the colony) of the proportion of mortality (Y) against increasing dose of deltamethrin (X). The level of deltamethrin resistance was estimated this way: lethal dose killing 50 % of the colony divided by the lethal dose killing 50 % of the susceptible mosquitoes[269].

3.4. Results

3.4.1. Adaptation in feeding success and fecundity during colony establishment

The mean success of blood feeding for mosquitoes exposed to an UTN, 43.0 ± 8.90 %, was always better than the feeding success for mosquitoes exposed to an LLIN, 19 ± 4.60 % ($F = 5.17$, $df = 2$, $p = 0.02$). Over time, the feeding success improved significantly for both treatments, with the proportion fed varying from 2 % to 76 % for mosquitoes exposed to an UTN, and from 2 % to 36 % for mosquitoes

exposed to an LLIN [linear fits : y_{LLIN} = - 22.24 + 6.21$e^{-9x}{}_{LLIN}$, R_{LLIN}^2: 0.32 ; y_{UTN} = - 39.06 + 1.094$e^{-8x}{}_{UTN}$, R_{UTN}^2: 0.26] (F = 5.33, df = 1, p = 0.04).

The mean number of eggs laid per fed female for mosquitoes exposed to an UTN was 84.1 ± 22.36, and it was not significantly different to mosquitoes exposed to an LLIN, 39.6 ± 24.49, (p > 0.05) and no change was measured over time (p > 0.05).

3.4.2. Insecticide susceptibility assays

9 replicates (5 tests x 10 mosquitoes x 2 exposure treatments) across time were tested for insecticide susceptibility. Mosquitoes were 4 times more knocked down 1 hour after the cone exposure when they contacted a PermaNet® 2.0 instead of an untreated net [LLIN: 9.9 (95 % Confidence Interval: 3.3 to 21.8); UTN: 1.9 (95 % CI: 0.03 to 10.8)] (F = 23.86, df = 1, p < 0.001) (Table 1). This knockdown effect induced by insecticide was dependent on the time of the year (F = 4.77, df = 1, p = 0.031).

There was no effect of treatment exposure (LLIN or UTN) in cones on survival (p > 0.05). For the first generation, the 24h mortality rate in the colony was extremely low when exposed to a PermaNet® 2.0 in WHO cones [8 (95 % CI: 2.22 to 19.23) % compared to 2.0 (95 % CI: 0.00 to 11) % for mosquitoes exposed to an UTN]. After one year of colony maintenance, the efficacy of the PermaNet® 2.0 was still negligible; 24h post exposure mosquito mortality was 6.5 (95 % CI: 1.32 to 18.04) % for mosquitoes exposed to an LLIN and 6.2 (95 % CI: 1.29 to 17.13) % for those exposed to an UTN (p > 0.05). However, the mean 24h mortality per experiment changed over time (and probably the season) (F = 5.18, df = 1, p = 0.02). For the period February to July, survival was higher in comparison to the period of September to January [95.2 (95 % CI: 92.86 to 96.94) % vs 91.0 (95 % CI: 88.12 to 93.37) %, respectively].

3.4.3. Resistance intensity assays CDC bottle

The results of the dose-response experiments comparing the colonized mosquitoes and the Kisumu susceptible strain are presented in table 1. The LD 50 for the colony was 22.6 µg/ml and 0.02 µg/ml for the Kisumu. After 1 year and 5 months of colonization the resistance ratio (RR) was 1250-fold in the colony in comparison to the Kisumu strain.

Deltamethrin	LD 50 (95 % CI)	LD 95 (95 % CI)	RR50 (95 % CI)
Kisumu	0.02 (0.01-0.03)	0.19 (0.11-0.38)	-
Colony	22.6 (14.01-31.66)	174.5 (111.77-223.57)	1257

Table 1: Intensity of resistance to deltamethrin in the colony after 1 year and 5 months of colonization measured with CDC bottle assays. LD 50: lethal dose leading to 50 % mortality in µg/mL; RR: resistance ratio; CI: confidence interval.

3.4.4. Species identification and target site resistance mechanisms

Anopheles gambiae M-form (*An. coluzzi*) was the only species detected in the colony. Among the 193 mosquitoes tested, only one specimen was an *An. gambiae* s.s. heterozygote for the ace-1^R G119S mutation in the second replicate (F1), corresponding to 1.6 % of the original population (considering the F0 and F1). This low frequency of ace-1^R mutation was not found in the next replicates.

However, the original population of mosquitoes collected from the field (F0) exhibited a high mutation frequency for kdr with 56.5 % homozygotes, 43.5 % heterozygotes, and no susceptible homozygotes (Table 2). Mosquitoes sampled at subsequent time points showed similar high mutation frequency. At 8 months mosquitoes were 61.1 % homozygotes, 33.3 % heterozygotes and 5.5 % susceptible for the kdr mutations. In our last PCR conducted after 1 year and 4 months of colonization, mosquitoes were found to be 68.4 % homozygotes, 31.6 % heterozygotes, with no susceptible. In total, 64.1 % were found homozygotes, 34.0 % were heterozygotes and 1.9 % susceptible for the kdr mutations.

Time after first test (in months)	Number of mosquitoes tested	KDR mutation		
		RR	RS	SS
0 (F0)	23	13	10	0
4	18	11	6	1
6	28	20	8	0
8	18	11	6	1
16	19	13	6	0

Table 2: Frequencies of kdr mutation in the colony. The first generation of mosquitoes was tested as well as mosquitoes 4, 6, 8 and 16 months later. RR: homozygote resistant; RS: heterozygote resistant; SS: homozygote susceptible; F(0): first generation.

3.5. Discussion

This study showed that we were able to establish a colony in the lab, with no loss of pyrethroid resistance (either resistance intensity or frequency of kdr mutation). Whether resistance was maintained because of the LLIN exposure protocol or because of periodic infusion of field mosquitoes is unclear. It could be that there are limited costs of resistance in this lab environment, providing little selective pressure for reversion to susceptibility. In general, the costs of insecticide resistance are extremely poorly quantified in *Anopheles* mosquitoes[19,238,275,276]. Work on *Aedes* and *Culex* spp do indicate costs of resistance on development, predation, longevity, mating competition and reproduction[277–282]. However, several highly resistant *Anopheles* lines have been in culture for a number of years and yet are maintained without any active insecticide selection[253,283].

Since its establishment the only species identified in this colony was *An. coluzzi* although based on the paper of Zoh and al., 2018, two molecular forms S (*An. gambiae*) and M (*An. coluzzi*) were identified around Bouaké[230]. It could be due to the fact that colonized mosquitoes came at first all from the same place in M'be (village with rice fields around Bouaké) and further addition of mosquitoes in the colony came from this place as well which is known to have high proportions of *An. coluzzi* during larval collection (unpublished data). Another option is that *An. coluzzi* may outcompete *An. gambiae* s.s. for mating or during larval rearing in our lab conditions, preventing any co-existence between both species even if we did introduce some *An. gambiae s.s.* in the colony[284,285].

The susceptibility tests performed with WHO cones and CDC bottle assays showed no change in phenotypic resistance status to pyrethroid. But the dry season and the colder temperatures might have affected the insecticide susceptibility of the colony overall since the 24h survival did vary with the time of year. From November 2017 to January 2017, the laboratory conditions were not optimal, the level of humidity was not constant and particularly low for mosquito rearing. An alternative may be that susceptibility tests were performed either prior to or just after an addition of wild-type mosquitoes in the colony, which could influence the adaptation to laboratory conditions and survival.

Overall productivity of the colony increased over time. There was an improvement in feeding success in the presence of an LLIN and in mosquito longevity depending on the time of the year. Even though more replicates are needed to assess whether the increase in feeding behavior depends on the season or the adaptability to the laboratory rearing condition, it appears that the colony adapted to the rearing condition over time. The time needed to feed a whole cage also decreased over time, as after 17 months of colonization only 20 min was needed to feed a whole cage against 2 times 1h in the

beginning (observation with no reported data). With this method, we may have selected not only for mosquitoes that are resistant to an insecticide exposure, but for those that are best at host seeking and feeding though an LLIN. It would be interesting to know if wild mosquitoes regularly added to the colony are more or less competitive at the larval stage than the colony and if they produce any eggs. Adding mosquitoes from the field does not mean they would be better competitors and be able to transmit their genes inside the colony. We suppose it happens but cannot be sure as the only genetic measure we have relate to insecticide resistance.

The number of eggs per female did not improve significantly over time. We observed only few eggs laid at the beginning of the colony, which does not necessarily relate to the fact that mosquitoes did not take blood meals, but rather that they laid eggs everywhere in the cage (cotton with sugar solution, water drop and condensation in the side of the plastic cage) rather than on the rearing petri dish meant for this purpose. Improved maintenance of the cages helped address this problem. The larval development and emergence rate also improved over time thanks to an optimized larval diet and larval density. The larvae grew faster and 100 % of the pupae emerged, a factor that was very unpredictable in the initial establishment phase. The Tetramin® baby fish food used worked alone without any addition. Yeast did not seem to improve the development, rather the opposite, but we do not have any empirical data to support that observation. Overall, the establishment of the colony was a success in terms of generating a reliable supply of quality-controlled mosquitoes for experimentation, whilst maintaining a high level of pyrethroid resistance.

CHAPTER FOUR

The presence of insecticide affects the vectorial capacity of insecticide-resistant mosquitoes

4.1. Abstract

Resistance threatens the reduction of malaria transmission as mosquitoes may survive a sub-lethal dose of insecticide. Neurotoxic are known to negatively affect mosquito's life history traits, but little is known about their effect on two important parameters for vectorial capacity, feeding behavior and longevity. The long-term impact of a Long-Lasting Insecticidal Net (LLIN) on host searching and feeding behaviors and its sublethal effect on survival was assessed using extremely resistant *Anopheles gambiae* s.l. females from Bouaké, Côte d'Ivoire.

One-way wind tunnel experiments were designed to evaluate behavioral responses of resistant mosquitoes to the presence of a human host protected by a LLIN (PermaNet® 2.0) or not, in three different scenario: (i) mosquitoes had every four days direct access to a foot enveloped in a net; (ii) mosquitoes had every day either direct access to the foot, indirect access through a damaged net, or no access at all; (iii) the maximum number of blood meals taken per individual mosquito over their lifetime was assessed with access to an host in the presence or absence of insecticide every day.

We found that: (i) females were estimated to visit the host twice and take 2 blood meals over their lifespan when the host was protected by an untreated net, which is twice the number of host searching and blood meals in the presence of insecticide. The average lifespan was 19 days long and insecticide exposures reduced it by 4.5 days; (ii) overall, the damaged bednets reduced host searching by 18 % and blood feeding by 17 % compared to direct access to the host. Mosquitoes lived on average 21 days and insecticide shortened the lifespan by 8 days for mosquitoes having access to the host through holes or no access at all. Mosquitoes with direct access to the host lived on average 24 days and the presence of insecticide reduced it by 4.8 days; (iii) 60 % of mosquitoes tested with the LLIN did not take a single blood meal over their whole lifespan against 18 % in the control group. Only 12 % of mosquitoes took 2 bloodmeals or more when tested with the LLIN against 34 % overall.

Delayed mortality and the important reduction in blood meals over the entire lifespan substantially reduce the vectorial capacity of extremely insecticide resistant mosquitoes and mitigate the threat imposed to malaria control by insecticide resistance. These results support the effectiveness of LLIN large-scale distribution and the importance of bed net usage throughout the year even when they are used and damaged.

4.2. Introduction

Long-lasting Insecticidal Nets (LLINs) impregnated with pyrethroids are the central pillar of malaria vector control. They work as a physical barrier, reduce human-vector interaction and kill host-seeking and susceptible mosquitoes at night[201]. However, with the widespread use of pyrethroid and in parallel the rapid adaptation of *Anopheles* mosquitoes to resist to its mode of action, there is a major concern that insecticide is no longer a successful strategy to control malaria vectors. Pyrethroid resistance is now widespread in many malaria endemic regions and the impact on pathogen transmission is still unclear[238]. The efficacy of LLINs against resistant mosquitoes is not well understood and it is only recently that research started focusing on the sublethal effects of pyrethroid on resistant malaria vectors[168,182,286].

Ultimately, insecticides could still work if they lowered the number of insecticide resistant survivors able to transmit infectious malaria parasites, as mosquitoes have both to survive 10-14 days post a first infectious blood meal and eventual insecticide exposures, and take a second blood meal to transmit the parasite[168]. Therefore, whether insecticide exposure directly kills mosquitoes or induces a delayed mortality, the potential for transmission might be negligible. A recent study showed that a delayed mortality effect of LLINs against highly resistant mosquitoes reduced malaria transmission potential by two third[168]. One or multiple exposures to insecticide may still work in regions classified by World Health Organization (WHO) as extremely high insecticide resistance areas[238], as resistance is here incomplete. Other recent papers do nuance this finding and only a minor sublethal effect of insecticide on longevity was found for highly resistant strains in Burkina Faso[286].

In addition to a life shortening impact, pyrethroids have been shown to directly affect mosquitoes' behavior around treated bednets, mosquitoes are either irritated upon contact or repelled before touching the treated surfaces[171,228,243,287–290]. Although the effects of LLINs exposure on host seeking behavior has already been studied, their influence on the vector potential of extremely insecticide resistant mosquitoes has been neglected. There is a pressing need to further investigate the direct and long-term effects of insecticide on mosquitoes that are seeking hosts protected by a bednet, whether there is no chance for them to take a blood meal on a protected host under an intact net, or when the effectiveness of LLINs diminish overtime due to physical damage such as rips and holes, giving the opportunity for insecticide resistant mosquitoes to go through and take a blood meal.

In the field, the high pyrethroid mutation rate in mosquitoes may increase the likelihood for humans to be bitten and therefore being infected by malaria[226]. However, malaria transmission models may

overestimate the vectorial capacity of mosquitoes in areas with extremely insecticide resistant mosquitoes and high LLIN or IRS coverage, as insecticide resistance may reduce host-seeking activity, reproduction and malaria infection[171,258,291] and insecticide exposure could negatively influence longevity[168].

To answer these questions, we used 1250-fold insecticide resistant mosquitoes colonized from M'be, Côte d'Ivoire (see Chapter 3). The first aim was to better understand whether LLIN are efficiently reducing mosquito host searching and feeding rate over the whole mosquito lifespan. The second aim was to assess whether variations in the quality of LLIN (intact or holed), accessibility to a human host (against an LLIN or not), and frequency of blood meal opportunities (every four days or every day) were affecting host searching and feeding rates.

4.3. Material and methods

A one-way wind tunnel was developed for this study to expose insecticide resistant mosquitoes to a human foot with or without the presence of insecticide (PermaNet® 2.0 or an untreated net) (Figure 1) following three experimental designs: (i) 500 mosquitoes had every four days direct access to a foot enveloped inside a sort of "sock" made from a piece of Long-Lasting Insecticidal Net (LLIN) or untreated net (UTN); (ii) 600 mosquitoes had every day either direct access to the foot, indirect access through a damaged LLIN or UTN (holed net fixed on the side of the cage), or no access at all (intact net fixed on the side of the cage); (iii) 400 mosquitoes had every day direct access to the foot either in the presence or absence of insecticide, and the maximum number of blood meals taken by individual mosquitoes over their lifetime was assessed.

4.3.1. Mosquito populations and rearing

All experiments were conducted on colonized *Anopheles gambiae* s.l. (1250-fold insecticide resistant) that originated from natural breeding habitats (-5.0303100 W longitude and 7.6938500 N latitude) from M'be, Côte d'Ivoire. This suburban village of Bouaké is dominated by 99 % M-form *An. coluzzi* that exhibits both kdr and metabolic resistance and breed in rice fields[165,230,242] (see Chapter 3 for further information on the establishment and maintenance of this colony of highly insecticide resistant mosquitoes).

All tested mosquitoes were maintained under standard insectary conditions (26 ± 1 °C, 70 ± 5 % RH) and natural light. Randomly selected larvae from the mosquito colony were reared in plastic boxes

with 1L deionized water and fed daily with Tetramin™ baby fish food following a standardized food regime per larvae described in Kulma and al.[266]: 1 day after hatching, 0.06 mg; day 2, 0.08 mg; day 3, 0.16 mg; day 4, 0.32 mg; day 5 and more, 0.6 mg.

7 to 10 days after hatching, rearing cages were filled with 120 pupae each. After emergence, mosquitoes were held in the same cage with access to 10 % glucose solution. 4 days after emergence, 50 females and 10 males were randomly kept in each cage until their death.

4.3.2. The preparation of the experimenters

Each experimenter was being monitored throughout the course of their participation to the study to avoid any malaria infection in mosquitoes and further transmission between experimenters. The experimenters washed their feet with unscented soap and rinsed them with water the day before each test and avoided the use of fragrance, repellent products, tobacco, and alcohol for 12 hours before as well as during testing. Experimenters were randomly rotated between treatments for every test.

4.3.3. Bednet evaluation against susceptible mosquitoes

Prior to experimentation, a WHO cone exposure of fully susceptible *An. gambiae* mosquitoes (Kisumu strain) was conducted to confirm the bio-efficacy of the netting material used in the study: a rectangular and an unwashed PermaNet® 2.0 (5 pieces tested: 4 side panels and one roof panel) manufactured by Vestergaard Frandsen SA (DK). The PermaNet® 2.0 (LLIN) is made of polyester, is green in color and is entirely coated with 55 mg/m^2 ± 25 % deltamethrin. A rectangular untreated ultra-fine 240 mesh polyester net distributed by Coghlan's green in color was used as control (UTN).

Cone tests were carried out in day light with non-blood fed females. Mosquitoes were exposed ten-by-ten in a plastic cone for 3 min, with cones angled at 40° to favor the contact between the mosquitoes and the net, and a ball of cotton used to close each cone's entry point. Following exposure, mosquitoes were removed to holding cups and monitored for mortality for 24h. The LLIN was effective at killing 100 % of mosquitoes, while the UTN killed none.

4.3.4. Behavioral assays

Before each behavioral test performed at dusk (around 5-6 pm), a connection between the Bugdorm© rearing cage (l = 32.5 cm^3) and the Bugdorm© experimental cage (same cage design; l = 32.5 cm^3) was made thanks to a Plexiglas® tube (L= 30 cm, d= 14,6 cm), and mosquitoes could fly freely from one

cage to the other during the test through the Plexiglas® tube. In the experimental cage, a volunteer inserted his foot as a blood source and the foot was protected by a piece of LLIN (l= 25 cm^2) or UTN (l= 25 cm^2) (Figure 1).

Mosquitoes were tested by cages, with 50 females and 10 males each per cage, every four days (experiment 1: 5 replicates x 2 cages = 2 net treatments) or every day (experiment 2: 2 replicates x 6 cages = 2 net treatments and 3 methods to access the host; experiment 3: 3 replicates x 2 cages = 2 net treatments). Each cage was randomly chosen for one treatment. For each experiment, the treatment was randomly allocated to one piece of net and one experimenter. Mosquitoes were starved 6h before each day of experiment.

A given rearing cage was tested with the same bednet treatment for the whole experiment (for example a LLIN "sock" around the host's foot in the experimental cage for each behavioral test). For each experiment, the behavioral tests were performed simultaneously for all the different treatments for 30 minutes (one wind-tunnel per treatment). Every 10 minutes, mosquitoes near the blood source in the experimental cage were considered host seeking and the number of blood fed mosquitoes was reported. After 30 minutes, the overall proportion of mosquitoes that successfully fed was reported (considering the whole tunnel this time and not only fed mosquitoes near a foot).

4.3.4.a. Direct access to a blood source against a LLIN

In the first experiment, 500 mosquitoes (50 x 2 treatment x 5 replicates) were tested every four days, to mimic gonotrophic cycles of fed mosquitoes[249], in a one-way tunnel to assess host-seeking and blood-feeding in the presence of a LLIN. In the experimental cage, the volunteer's foot was enveloped inside a sort of "sock" made either of a piece of LLIN or a piece of UTN. The foot was in direct contact with the netting to enable blood feeding through the net (Figure 1).

4.3.4.b. Indirect access to a blood source though a damaged LLIN

In the second experiment, we compared 3 different modes of insecticide exposures to mimic what happen in the field when mosquitoes are host searching and trying to feed around a host protected by an LLIN or an UTN. 600 mosquitoes (2 net treatments x 3 net protection scenario x 50 mosquitoes x 2 replicates) had every day either (a) a direct access to a foot like in the first experiment, or (b) an indirect access through holes on a damaged net or (c) no access at all. All pieces of net used in each treatment were of the same dimension (l= 25 cm^2). The damaged net had four holes (d= 1 cm^2). Each

hole was equidistant to the 3 other holes to form the 4 corners of a square (l= 8 cm^2) and its center was aligned with the center of the Plexiglas® tube opening.

In order to place the foot at the same distance from the net for all treatments, the experimenter's foot was introduced through an entry placed on the top of the cage. For treatment (b), in order to take a blood meal mosquitoes had to go through one of the 4 holes made in the net fixed on the tube at the entry of the experimental cage (the one with the experimenter's foot) (Figure 1). For treatment (c), the net was fixed at the entry of the experimental cage on the tube as well, but this time it was intact and there was no possibility for mosquitoes to go through the net and enter the experimental cage.

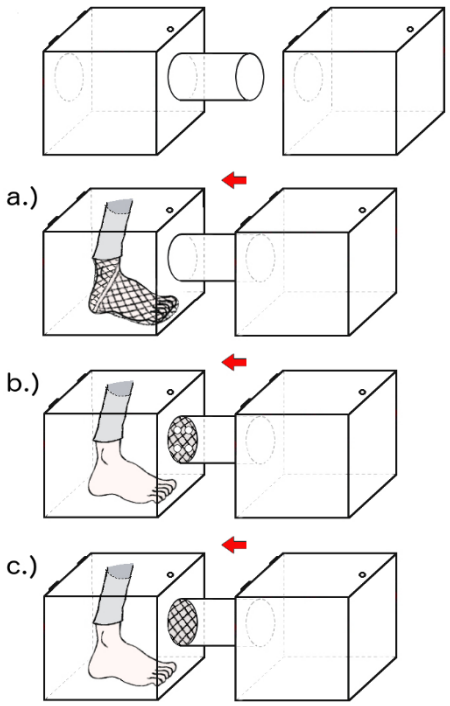

Figure 1: The one-way tunnel with one rearing cage (right), one Plexiglas® tube, and one experimental cage (left) with: a) direct access to an experimenter's foot enveloped in a Long-Lasting Insecticidal Net (LLIN) or an (un)-treated net (UTN); b) indirect access through a damaged net at the end of the tube; and c) no access at all with an intact net at the end of the tube blocking access to the experimenter's foot. The tunnels were assembled for behavioural test creating a connection between the rearing cage on the right (l = 32.5 cm^3) and the experimental cage on the left (l = 32.5 cm^3) thanks to a Plexiglas® tube (L= 30 cm, d= 14,6 cm). In the experimental cage, an experimenter introduced his foot as blood source and the foot was protected by a piece of LLIN (l= 25 cm^2) or UTN (l= 25 cm^2). In a) the experimental cage, the experimenter's foot was enveloped inside a sort of "sock" made of a piece of a LLIN or an UTN. The foot was in direct contact with the netting to enable blood feeding. In b) and c) the net was fixed on the Plexiglas® tube on the side of the experimental cage containing the foot. In b) the damaged net had four holes (d= 1 cm^2). Each hole was

equidistant to the 3 other holes to form the 4 corners of a square (l= 8 cm^2) and its centre corresponded to the centre of the diameter of the tube side. In c) the piece of net was intact.

4.3.4.c. Total number of blood meals taken per mosquitoes over the lifespan

In the third experiment, mosquitoes were given the opportunities to blood feed every day on an experimenter's foot. The exact number of meals taken during a lifetime was counted with 407 females (100 + 107 female tested against an UTN and an LLIN respectively in one replicate; 2 x 50 females in two other replicates) having a direct access to a blood source through an LLIN or UTN as in experiment 1. For this experiment, each time a mosquito took a blood meal during a daily behavioral test, this female mosquito was removed from its current rearing cage at the end of the test and placed into a new cage corresponding to the overall number of blood meals this mosquito took so far. For example, if a mosquito took a blood meal during its first occasion to feed, it would be placed in a second cage with others female that fed once until it feeds again and will then be placed in a third cage with others female that took 2 blood meals and so on.

4.3.4.d. Life history traits or survival, fecundity and mosquito wing size

Daily survival was measured for each cage in each behavioral experiment. In addition, in experiment 1, the number of eggs in each cage was reported daily (daily pictures of egg papers and then counted on the computer). Mosquito wings were also measured from the tip to the distal end of the allula by excluding the fringe, as an indication of mosquito's size. The wings were fixed into slides, scanned, and copied to a computer. Wings lengths were measured to the nearest 0.01 mm on the java-based application ImageJ 64 (*http://imagej.nih.gov/ij/*).

4.4. Statistical analysis

When needed, contrasts among treatments were assessed using the *multcomp* package version 1.4-10 and the function *glht* in the software R with a Tukey's honestly significant difference test (Tukey HSD) multiple-comparison test. It allowed simultaneous tests for General Linear Hypothesis and comparison differences between groups at p = 0.05. All statistical analysis and figures were performed in R© version 3.6.1 with the Rstudio interface version 1.2.5001.

4.4.1. Direct access to a blood source against a LLIN (experiment 1)

We investigated the host searching and blood feeding rates over time of 500 female that had direct access to a foot enveloped in an LLIN or an UTN every four days. Having done every four days observations per treatment:

_The host searching weighted means were calculated as the number of mosquitoes going to the other side of the tunnel (experimental cage) every four days, called “visits”, divided by the number of mosquitoes still alive each day of test, and multiplied by the overall number of mosquitoes tested in each treatment (number of female mosquitoes in the cage on the first day of experimentation). The number of “visits” per test was measured every 10 min during a 30 min test (a value of “visits” for 10, 20 and 30 minutes).

_The same was done for the blood feeding rate weighted means with the number of blood fed mosquitoes in the experimental cage, “blood meals”, at the end of the behavioral test each day.

The weighted numbers of visits and blood meals per day allowed the comparison over time while considering the unequal numbers of female mosquitoes in the cage each day in different replicates. To work with a Poisson distribution, we rounded the value of both the weighted number of visits per day and the weighted number of blood meals taken per day.

We used a Generalized Linear Mixed-Effects Models (GLMM) with a poisson distribution and log link distribution to analyze the weighted number of visits per day. The fixed effects were the net treatment (LLIN or UTN) and the time at which the measure was done (10 min, 20 min or 30 min after the beginning of each test). The random effects were the replicates, the day, and the observation-level random effect. The latter minimize overdispersion and correct for biased parameter estimates in poisson mixed effects models[292]. We compared our model two-by-two using measures of goodness of fit and tested for fixed and random effects significance.

We used a similar model to analyze the weighted number of blood meals per day.

In addition to the host searching (visits) and blood feeding (blood meals) weighted means, accounting for daily dynamics, another measure of host searching was analyzed, “the host searching performance” which estimates the average number of times a female visited the host during her lifespan. To obtain the “host searching performance” for a given cage, each day the maximum number of mosquitoes found near the foot out of the three observations (at 10, 20 or 30 minutes) was recorded, these daily maximum values where then summed, and this sum was divided by the initial number of mosquitoes present in the cage. This host searching performance was analyzed using an

Analyze of Variance (ANOVA) regarding the effect of the exposure treatment (LLIN or UTN), the replicate and their interaction (variability between replicate was high).

The same ANOVA model and estimation measure were used to measure the "feeding performance" (or the overall number of blood meals during the whole life of mosquitoes), the number of eggs per female, and the proportions of eggs laid per bloodmeal (for this particular model, we found no variability between replicates, therefore it was not in interaction with the insecticide exposure treatment). The Shapiro-Wilk normality tests of residual confirmed that the data were normally distributed.

Mosquito longevity was analyzed using a weighted Cox regression (using the R package *coxphw* due to the violation of the proportional hazards assumption in a Cox regression model) as a function of the exposure (LLIN or UTN), the replicate, and their interaction.

Using the same method but adding wing size to the model as co-variate, we analyzed the longevity of 282 mosquitoes in 3 replicates whose body size measure (wing length as a proxy) was available. To better understand how body size interacts with the effect of LLIN exposure on longevity, we also ran the model without the replicate 1 were mosquitoes did not move much from their rearing cage during their entire life, thus were never in direct contact with the insecticide.

4.4.2. Indirect access to a blood source through a damaged net (experiment 2)

In the second experiment, 600 mosquitoes had every day either direct access to the foot, indirect access through a holed net, or no access at all (tube blocked by intact netting). The 200 mosquitoes that did not have access to the host were removed from the analysis of behavior and only kept for the longevity analysis.

To obtain the "host searching performance" for a given cage, each day the maximum number of mosquitoes found near the foot out of the three observations (at 10, 20 or 30 minutes) was recorded, these daily maximum values when then summed, and this sum was divided by the initial number of mosquitoes present in the cage. The same was done for the "feeding performance".

We performed a gaussian GLMs to analyze the average number of visits per female to the other side of the tunnel ("host searching performance"). We looked at the effect of net treatment (LLIN or UTN),

net protection (direct access to the host through a net or indirect access to the host through a holed net) and their interaction. The replicate was considered a co-variate.

The same model was used to measure the feeding performance (or average number of blood meals taken per female).

We analyzed mosquito longevity with a weighted Cox regression the effect of net treatment (LLIN or UTN), net protection (direct access to the host through a net or indirect access to the host through a holed net) and their interaction. The replicates were considered as co-variates

4.4.3. Measures of maximum blood meal taken per mosquitoes (experiment 3)

We analyzed the number of bloodmeals per female (407 females) using a Generalized Linear model (GLM) with a Poisson distribution and a log link regarding the effect of the net treatment (LLIN or UTN), the replicate, and their interaction. There was a minor under-dispersion (0.96) of the Poisson distribution which could in principle mask significant relationships.

In addition, a binomial GLMs was performed to analyze what proportion of mosquitoes were able to take at least one meal during their life, considering the effect of the net treatment (LLIN or UTN) and the replicates. The same analysis was then performed to analyze what proportion of mosquitoes were able to take at least two blood meals during their life.

The longevity was only available for 2 replicates (307 females) and was analyzed using a Cox regression as a function of net treatment (LLIN or UTN), the number of blood meal taken, their interaction and the replicate.

4.4.4. Vectorial capacity of resistant mosquitoes

The dynamic of malaria transmission can be measured considering the vectorial capacity, a mathematical formula described in the Ross-MacDonald model as follows[293]:

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

Where *VC* is the vectorial capacity, m the mean number of mosquitoes per host, *a* is the daily mosquito's blood feeding rate, *b* is the probability of transmission from a vector to a host, *p* is mosquito survival probability, and *n* is the Extrinsic Incubation Period (EIP). Together these parameters

represent the number of infectious bites arising from one single infectious person for one day (also called reproductive number)[294]. We calculated vectorial capacity using mean parameter estimates quantified from our empirical data.

Adult vector density, *m*, was estimated using the number of mosquitoes alive on the first day of the test in each experiment (50 mosquitoes). The biting rate, *a*, was estimated for each net treatment using our empirical data of biting performance per female divided by the average longevity. Adult daily survival, *p*, was estimated using our empirical data of mosquito longevity. Mosquito daily mortality is measured as the reverse of longevity: x = 1 / (longevity); and the daily survival probability as follows: $p = 1 - x$. For the EIP of the parasite, *n*, we assumed the EIP for *Plasmodium falciparum* at 24 °C to be 12 days long for both treatment groups[18,295]. Probability of transmission from a vector to a host, *b*, is based on previous research[296].

4.5. Results

4.5.1. Direct access to a blood source against a LLIN (experiment 1)

The weighted average for the number of visits (mosquito visiting) per day was (mean ± standard error) 14.4 ± 0.76. The presence of insecticide reduced that average by 3 visits per day [12.0 ± 1.22 visits per day against UTN: 15.9 ± 0.96 visits per day of testing] ($\chi^2 = 38.56$, df = 1, $p < 0.001$). During the tests, the weighted average of visits per day increased every 10 min [10 min: 11.9 ± 1.14; 20 min: 14.5 ± 1.32; 30 min: 16.9 ± 1.44 blood meals per day of testing] ($\chi^2 = 7.75$, df = 1, $p = 0.005$). No interaction was found with the net treatment ($p > 0.05$).

The weighted average for the number of bloodmeals per day was 11.1 ± 0.74. The presence of insecticide reduced that average to 8.6 ± 1.14 blood meals per day of testing [against UTN: 12.7 ± 0.96 blood meals per day of testing] ($\chi^2 = 50.71$, df = 1, $p < 0.001$). During the tests, the weighted average of blood meals per day increased every 10 min [10 min: 8.7 ± 1.12; 20 min: 11.2 ± 1.28; 30 min: 13.5 ± 1.41 blood meals per day of testing] ($\chi^2 = 11.66$, df = 1, $p < 0.001$), and we almost found an interaction with the net treatment ($p = 0.08$), probably as for the LLIN group the weighted average seemed to increase and then decrease with time [LLIN, 10 min, 20 min and 30 min: respectively 7.5 ± 1.85 , 9.6 ± 2.17 and 8.7 ± 1.94 blood meals per day of testing].

The mean number of visits per female ("host searching performance") for mosquitoes tested in the tunnel with an untreated net was 2.3 times higher than for mosquitoes tested in the tunnel with a LLIN [Figure 2.1; UTN: 2.3 ± 0.53 visits per female over their lifetime; LLIN: 0.97 ± 0.28 visits per female over their lifetime] ($F = 22.75$, $df = 2$, $p = 0.003$). Overall, the mean number of visits per female ("host searching performance") was 1.6 ± 0.36 and it varied from 0.5 ± 0.13 to 2.5 ± 1.14 depending on the replicates ($F = 27.46$, $df = 1$, $p = 0.003$), but no interaction was found between the net treatment and the replicates ($p > 0.05$)

The feeding performance per female over their lifetime for mosquitoes tested in the tunnel with an untreated net was 3 times higher than mosquitoes tested in the tunnel with a LLIN [Figure 2.2; UTN: 2.1 ± 0.61 bloodmeals per female over their lifetime; LLIN: 0.70 ± 0.27 bloodmeals per female over their lifetime] ($F = 20.48$, $df = 2$, $p = 0.003$). The overall feeding performance per female over their lifetime was 1.4 ± 0.39 and it varied from 0.0 ± 0.02 to 2.29 ± 1.2 depending on the replicates ($F = 28{,}22$ $df = 1$, $p = 0.003$ but no interaction was found between the net treatment and the replicates ($p > 0.05$).

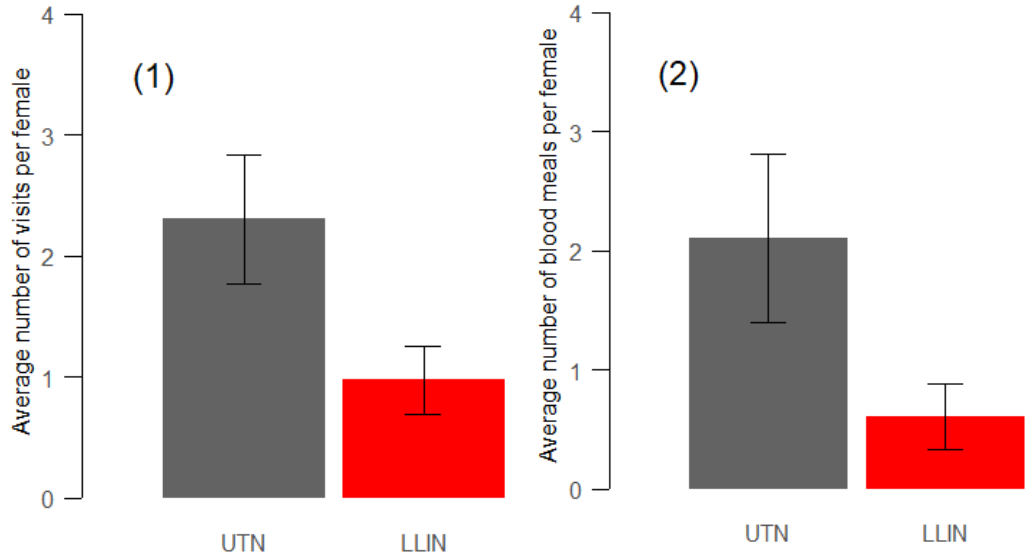

Figure 2: Average numbers of visits ("host searching performance") (1) and (2) blood meals ("feeding performance") per female mosquito in a one-way tunnel, with mosquitoes tested every four days for their behavioral responses to a human foot enveloped in a PermaNet® 2.0 (LLIN, in red) or an untreated net (UTN, in grey), Experiment 1. ± standard error bars are shown.

The mean number of eggs laid per blood meal (28.9 ± 6.80) did not depend on the net treatment (UTN or LLIN) and the replicates (all $p > 0.05$).

However, on average an estimated 59.0 ± 21.17 eggs were led per female, and twice more eggs were laid by mosquitoes tested in the tunnel with an UTN compared to the average [UTN : 100.6 ± 32.38; LLIN: 17.55 ± 10.40], as insecticide exposure decreased the average number of eggs laid per female ($F = 25.76$, $df = 2$, $p = 0.002$). The number of eggs laid in each replicate was significantly different [from 0 to 100.9 ± 62.48] ($F = 19.31$, $df = 1$, $p = 0.007$) and no significant difference were found in 3 replicates out of 5 ($F = 8.97$, $df = 1$, $p = 0.03$).

The average longevity for the mosquitoes tested was 18.8 ± 0.46 days and having the opportunity to take a blood meal every 4 days against a LLIN reduced the survival by around 4.5 days compared to the average [Figure 3: LLIN: 14.4 ± 0.37; UTN: 23.4 ± 0.74] ($\chi^2 = 130.5$, $df = 1$, $p < 0.001$). Mosquitoes longevity depended on the replicate [from 12.70 ± 0.40 to 22.45 ± 1.33] ($\chi^2 = 22.14$, $df = 1$, $p < 0.001$). In 4 replicates out of 5, mosquitoes tested in the tunnel with the UTN survived better than those tested in the tunnel with a LLIN, but in one replicate, we found no difference in longevity between the two exposure treatments. In that replicate only, mosquitoes did not host seek and did not take any blood meal during their entire life. Without this replicate, similar results are found.

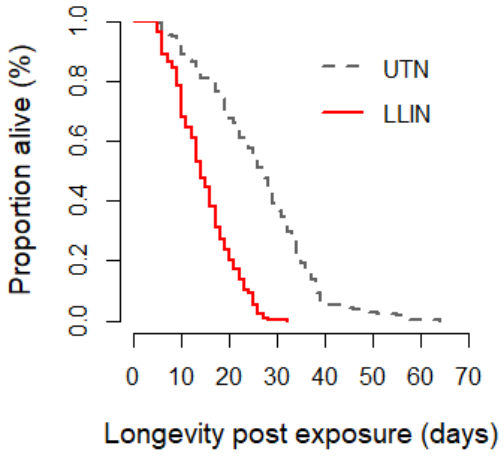

Figure 3: Survival curves for mosquitoes tested in a one-way tunnel that have the opportunity every four days to take a blood meal on a human foot protected by either a PermaNet® 2.0 (LLIN) or an untreated net (UTN), experiment 1. The grey dotted line represents the longevity of mosquitoes that were tested in a tunnel with an untreated net and the red solid line represents the longevity of mosquitoes that were tested in a tunnel with a LLIN.

In replicates were the wing length was available, mosquitoes survived on average 17.23 ± 0.51 days. Having the opportunity to take a blood meal every 4 days against a LLIN reduced the survival by 4.5 days compared to the average [LLIN: 14.22 ± 0.48; UTN: 20.48 ± 0.84] ($\chi^2 = 46.17$, $df = 1$, $p < 0.001$).

For the same reason detailed above (one replicate different from the others as mosquitoes did not host search or blood fed for their entire life), mosquito longevity depended on the replicate ($\chi^2 = 12.33$, df = 1, $p < 0.001$) and we also found an interaction between the replicate and the net treatment (UTN or LLIN) inside the tunnel ($\chi^2 = 8.55$, df = 1, p = 0.003). Overall, larger mosquitoes had a longer lifespan in comparison to smaller mosquitoes, independently of the insecticide treatment inside the one-way tunnel ($\chi^2 = 5.60$, df = 1, p = 0.018).

However, the same analysis without that different replicate was re-run and while the effect of the exposure treatment inside the tunnel was still influencing mosquito longevity ($\chi^2 = 59.5$, df = 1, $p < 0.001$), the effect of both replicates on longevity was not different ($p > 0.05$) and the body size did not influence mosquito longevity anymore ($p > 0.05$).

4.5.2. Indirect access to a blood source through a damaged net (experiment 2)

The estimated host searching performance per female was 3.0 ± 0.49. The presence of insecticide in the tunnel reduced by 45 % this average [Figure 4; LLIN: 1.9 ± 0.44; against 4.1 ± 0.36 for the control group UTN] (F = 70.92, df = 1, p = 0.03). The net protection also influenced this average and having access to a host through a damaged net reduced by 18 % the number of mosquitoes visiting the other side of the tunnel [against foot: 3.5 ± 0.60; damaged net: 2.5 ± 0.79] (F = 13.66, df = 1, p = 0.034).

The estimated feeding performance per female was 2.5 ± 0.43. The presence of insecticide in the tunnel reduced this average by 56 % [LLIN: 1.4 ± 0.26; against 3.5 ± 0.19 for the control group UTN] (F = 70.92, df = 1, p = 0.003). The net protection also influenced this average and having access to a host through a damaged net reduced by 17 % the number of mosquitoes taking blood meals [against foot: 2.8 ± 0.58; damaged net: 2.1 ± 0.66] (F = 13.66, df = 1, p = 0.03).

Both replicates were significantly different in terms of the overall number of visits and bloodmeals taken per female, but no interaction with other parameters was found (for both F = 10.24, df = 1, p = 0.049). The interaction between the net treatment (UTN or LLIN) and the net protection (against foot or damaged net) did not influence the overall number of visits and bloodmeals taken per female ($p > 0.05$).

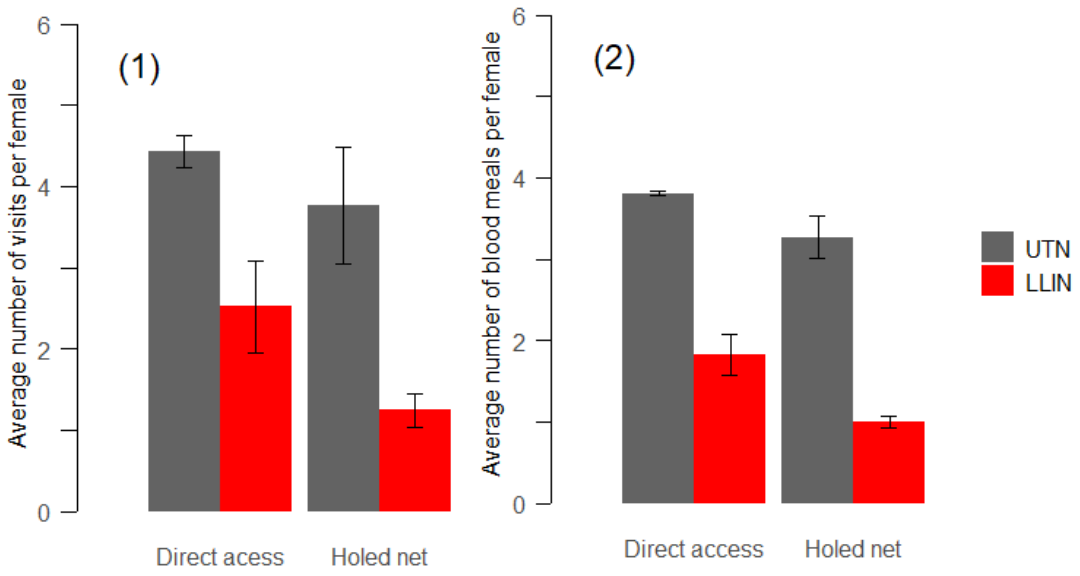

Figure 4: Average numbers of visits (1) and (2) blood meals per female mosquito tested in a one-way tunnel with a PermaNet® 2.0 (LLIN, in red) or an untreated net (UTN, in grey), experiment 2. Mosquitoes had an access every day to a host partially protected by a net laying against its foot ("direct access") or a damaged net ("holed net": piece of meeting placed on the end of the Plexiglas tube with four holes in the net and mosquitoes have to go through to access the foot, it is an indirect access). ± standard error bars are shown.

The mean mosquito longevity in experiment 2 was 20.8 ± 0.51 days. Mosquitoes having no access to the blood source (intact net) had a 3-days shorter lifespan than the average [intact net: 17.9 ± 0.81 days, damaged net: 21.1 ± 0.93 days, and direct access through the net: 23.5 ± 0.87 days] (χ^2 = 16.14, df = 1, p < 0.001) and a shorter lifespan than mosquitoes with an indirect (damaged net) or direct access (Tukey: p < 0.001 and p = 0.02 respectively).

Mosquitoes tested daily in the tunnel with the LLIN had a 6 days shorter lifespan (Figure 5; LLIN tunnel: 14.9 ± 0.49 days; UTN tunnel: 26.9 ± 0.76 days) (χ^2 = 167.01, df = 1, p < 0.001). Having direct access to the foot through a LLIN reduced the overall mean longevity by 2 days (18.9 ± 0.97 days) but the mean longevity for mosquitoes tested against an intact LLIN with no access to blood (intact net) was not significantly different from mosquitoes having an indirect access (damaged net), respectively around 9 days and 7 days lower than the average [intact net: 11.9 ± 0.71 days and damaged net: 14.08 ± 0.70 days] (χ^2 = 11.89, df = 2, p = 0.002; Tukey comparison between "intact net" and "damaged net" : p > 0.05).

In the control group, having the opportunity to take a blood meal increased the lifespan (direct access through an UTN: 28.5 ± 1.29 days and damaged net: 28.5 ± 1.39 days, against 23.9 ± 1.19 days for those having no access to the foot) and there was no difference between having a direct or indirect

access (Tukey comparison between “direct access” and “damaged net”: $p > 0.05$). No difference was found between the replicates ($p > 0.05$).

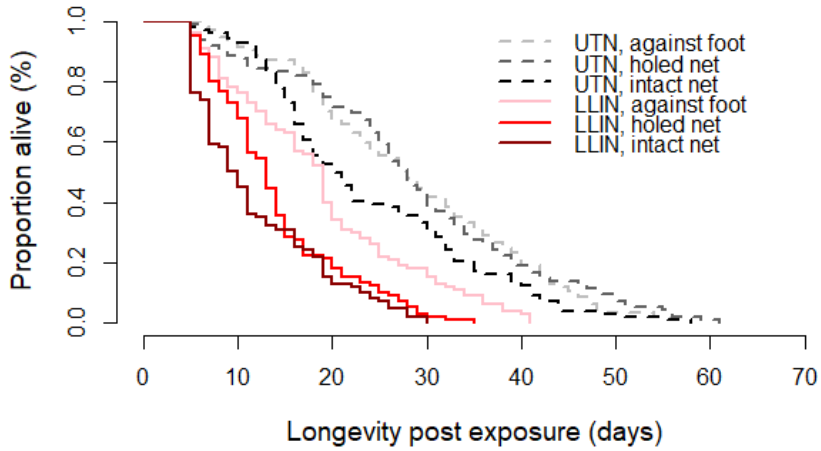

Figure 5: Survival curves for mosquitoes tested in a one-way tunnel that have the daily opportunity to take a blood meal on a human foot protected by either a PermaNet® 2.0 (LLIN) or an untreated net (UTN), experiment 2. The host was either completely protected by an intact net, or partially protected by a damaged net “holed net”, or a net laying against its foot. The dotted lines represent the longevity of mosquitoes that were tested in a tunnel with an untreated net, the solid lines represent the longevity of mosquitoes that were tested in a tunnel with a LLIN. The black and dark red colors represent the longevity of mosquitoes having no access to the host due to an intact net barrier. The grey and red colors represent the longevity of mosquitoes having indirect access to the host through a damaged “holed net”. The light grey and pink colors represent the longevity of mosquitoes having direct access to the host through a net.

4.5.3. Measures of maximum blood meal taken per mosquitoes (experiment 3)

The average number of blood meals per female was 1.2 ± 0.06. Mosquitoes tested in the tunnel in the presence of insecticide (LLIN) had a highly reduced average to 0.6 ± 0.05 blood meals per female taken over their lifespan [against UTN: 1.93 ± 0.09 blood meals per female] ($\chi^2 = 162.62$, df = 1, $p < 0.001$). The different replicates added variability to this average [replicates 1, 2, and 3: 1.2 ± 0.07; 0.5 ± 0.09; and 2.01 ± 0.14 blood meals per female respectively] ($\chi^2 = 17.39$, df = 1, $p < 0.001$).

63.1 (95 % Confidence Interval: 58.2 to 67.8) % of all mosquitoes took at least one blood meal during their life, but the presence of insecticide (LLIN) reduced that number to 41.5 (95 % CI: 34.7 to 48.6) % [Figure 6; against 85.5 (95 % CI: 79.8 to 90.0) % for mosquitoes tested in the control group] ($\chi^2 = 89.17$, df = 1, $p < 0.001$). On average only 33.9 (95 % CI: 29.3 to 38.7) % were able to take at least 2 bloodmeals and the presence of insecticide (LLIN) reduced that number to 11.6 ± 2.23 (95 % CI: 7.6 to 16.7) % [against 57.0 (95 % CI: 49.8 to 63.9) % for mosquitoes tested in the control group] (Figure 6; $\chi^2 = 99.44$,

df = 1, p < 0.001). The replicates added some variability (χ^2 = 6.07, df = 1, p = 0.01) but no interaction was found between the replicates and the net treatment (p > 0.05).

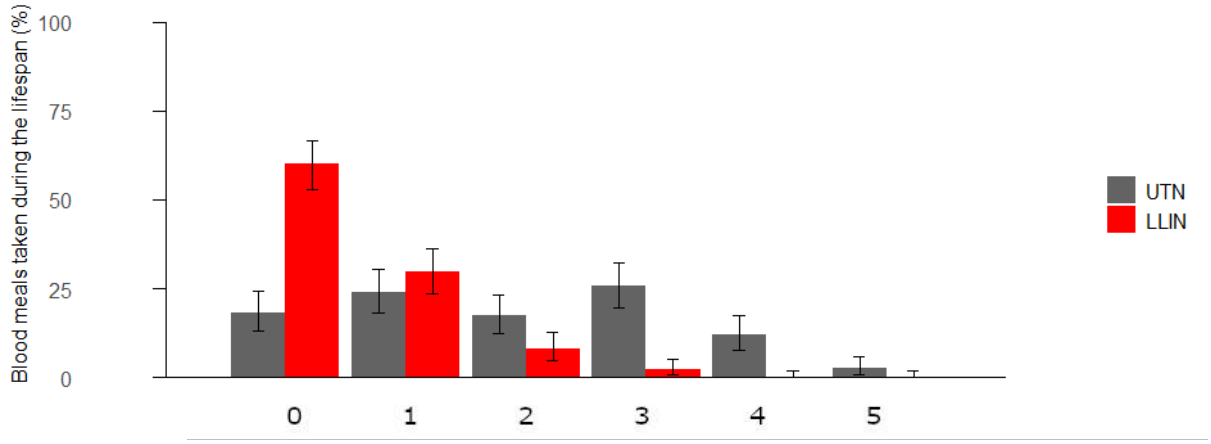

Figure 6: Percentage of mosquitoes having taken 0, 1, 2, 3, 4 or 5 bloodmeals over their lifespan when tested daily in a one-way tunnel with access to a human foot enveloped in piece of a PermaNet® 2.0 (LLIN, in red) or an untreated net (UTN, in grey), experiment 3. The blood feeding performance is expressed as the proportion of mosquitoes having taken 0, 1, 2, 3, 4 or 5 blood meals over their lifespan. 95 % Confidence Intervals are shown and represent the variability between the 3 replicates.

The average longevity for the mosquitoes tested in this experiment 3 was 21.7 ± 0.56 days and having the opportunity to take a blood meal daily against a LLIN reduced the longevity by around 4.5 days [Figure 7; LLIN: 17.2 ± 0.37; UTN: 26.4 ± 0.93] (χ^2 = 90.49, df = 1, p < 0.001). The more blood meals mosquitoes took the longer they lived [0 blood meal per female: 16.7 ± 0.51; 1 blood meal per female: 22.0 ± 0.88; 2 blood meals per female: 26.8 ± 1.35; 3 blood meals per female: 29.9 ± 3.05; 4 blood meals per female: 38.5 ± 2.58] (χ^2 = 38.45, df = 4, p < 0.001). We found no effect of the replicates (p > 0.05).

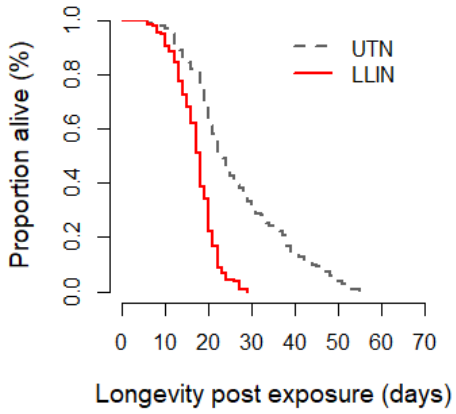

Figure 7: Survival curves for mosquitoes tested in a one-way tunnel that have the opportunity every day to take a blood meal on a human foot protected by either a PermaNet® 2.0 (LLIN) or an untreated net (UTN), experiment 3. Mosquitoes were grouped in cages regarding the number of bloodmeals taken since the start of the experiment and moved accordingly after each test. The host was partially protected by a net laying against its foot (a LLIN or a UTN). The grey dotted line represents the longevity of mosquitoes that were tested in a tunnel with an untreated net and the red solid line represents the longevity of mosquitoes that were tested in a tunnel with a LLIN.

4.5.4. Vectorial capacity of resistant mosquitoes

In the experiment 1, the parameter values yielded a vectorial capacity of $VC_{1,\ LLIN} = 0.21$ for mosquitoes tested in the tunnel with a LLIN (Table 1), and $VC_{1,\ UTN} = 1.64$ for those tested in the control tunnel (UTN).

In the experiment 2 with a direct access to a host, $VC_{2,\ LLIN} = 1.34$ for mosquitoes tested in the tunnel with a LLIN, and $VC_{2,\ UTN} = 4.89$ for those tested in the control tunnel (UTN). In the experiment 2 with an indirect access to a host through a damaged net, $VC_{2,\ LLIN} = 0.43$ for mosquitoes tested in the tunnel with a LLIN and $VC_{2,\ UTN} = 3.59$ for those tested in the control tunnel.

In the experiment 3, the parameter values yielded vectorial capacity of $VC_{3,\ LLIN} = 0.14$ for mosquitoes tested in the tunnel with a LLIN and $VC_{3,\ UTN} = 1.31$ for those tested in the control tunnel (UTN).

In the experiment 2, the vectorial capacity of mosquitoes having indirect access to a host and having to go through a damaged net before feeding is always estimated to be smaller compared to mosquitoes having direct access to the host. It is estimated to be 3 times smaller for mosquitoes tested in the presence of insecticide (LLIN) and 1.4 times smaller for mosquitoes tested in the absence of insecticide (UTN) [LLIN, direct access: $VC_{LLIN,\ direct} = 1.35$; LLIN, indirect access: $VC_{LLIN,\ indirect} = 0.43$; UTN, direct access: $VC_{LLIN,\ direct} = 4.89$; UTN, indirect access: $VC_{LLIN,\ indirect} = 3.59$].

Considering all experiments together, the vectorial capacity of mosquitoes having direct access to the host is estimated to be 4.6 times smaller for mosquitoes tested in the presence of insecticide compared to mosquitoes exposed to an untreated net [LLIN: VC_{LLIN} = 0.57; UTN: VC_{UTN} = 2.61].

Exp.	Treatment	Bites per female	Longevity	Vector density *(m)*	Biting rate *(a)*	Daily survival *(p)*	Proportion infectious *(b)*	EIP *(n)*	Vectorial capacity *(VC)*
1	LLIN	0.70	14.4	50	0.05	0.93	0.3	12	0.21
1	UTN	2.10	23.4	50	0.09	0.96	0.3	12	1.64
2	LLIN	1.83	18.9	50	0.10	0.95	0.3	12	1.35
2	Holed LLIN	1.01	14.1	50	0.07	0.93	0.3	12	0.43
2	UTN	3.81	28.5	50	0.13	0.96	0.3	12	4.89
2	Holed UTN	3.27	28.5	50	0.11	0.96	0.3	12	3.59
3	LLIN	0.60	17.2	50	0.03	0.94	0.3	12	0.15
3	UTN	1.93	26.4	50	0.07	0.96	0.3	12	1.31

Table 1: Output of vectorial capacity equation with experimental parameters (for experiments 1, 2 and 3). The parameters: adult vector density *m*, biting rate *a*, adult daily survival *p*, proportion infectious *b,* and extrinsic incubation period (EIP) *n* represent the number of infectious bites arising from one single infectious person for one day. We calculated vectorial capacity using mean parameter estimates quantified from our empirical data (for *m*, *a* and *p*). Probability of transmission from a vector to a host, *b*, and the estimate for the EIP of the parasite, *n*, were based on previous research for *Plasmodium falciparum* at 24 °C[18,295,296]. Exp. Means experiment.

4.6. Discussion

The standardized WHO discriminating dose assay is the principal method used to monitor resistance in Africa and its intensive 24h-mortality post-exposure data currently inform modeling about the threat of resistance[297]. However, insecticide resistance poses a threat to vector control only if mosquitoes can survive long enough to take a least two blood meals during their life while also surviving past the parasite extrinsic incubation period of the parasite (EIP) and the WHO assays miss this crucial information[238,249]. The one-way tunnel method developed in this study offers a great alternative to these methods as it gives the opportunity for mosquitoes to freely host seek and take blood meals with or without the presence of insecticide and during their entire life. It allowed a precise analysis of the impact of insecticide on the physiology and behavior of extremely resistant mosquitoes. Not only did insecticide exposures reduce the longevity, it also reduced the host searching and blood feeding behaviors even after correcting for daily mortality. We found no major effect of the net treatment on egg laying. Nonetheless, the reduction in egg laying over time due to less blood meals being taken per female exposed to insecticide added to those effects and could in nature affect the

age structure of the mosquito population and potentially adult mosquito density, both important parameters of the vectorial capacity.

The vectorial capacity of mosquitoes tested in the tunnel with insecticide (a LLIN) is extremely reduced compared to mosquitoes tested in the tunnel in the absence of insecticide (UTN). Overall, mosquitoes did go through at least one less gonotrophic cycle in their life when they are tested in the presence of insecticide. The delayed effect of insecticide on mosquito longevity helps reduce the vector-borne disease transmission potential of highly resistant mosquitoes. Based on our design where mosquitoes had their first opportunity to feed at 4-5 days old and given that the EIP for malaria parasites is around 10-14 days[79], we measured a reduction of 20-50 % transmission potential of vectors because of repeated insecticide exposures. This effect might be even more important if mosquitoes were older for their first opportunity to blood feed. We compared several LLIN coverage scenarios: no protection at all, partial, and complete protection. Still, several other coverage scenarios are possible, and mosquitoes could encounter a LLIN only once or multiple times during their entire life.

Bednets work first as a physical barrier to reduce vector-host interactions. However, mosquitoes can often find their way to the blood source through tears and holes on the net, or because it is misplaced against the host. Our experiments suggest realistic exposures to insecticide influence host searching and feeding behavior measured over time. When mosquitoes needed to navigate through holes in a damaged and permeable net in order to take a blood meal, host searching and feeding success were even more reduced. Our results corroborate those from other studies suggesting that sublethal exposure of resistant mosquitoes to pyrethroids can decrease host seeking efficiency and the capacity to find a way through a LLIN[161,165,171]. Resistance allowed a prolonged contact with an LLIN, but a diminished sensitivity to contact irritancy and repellency may have consequence for blood meal success[298].

Contact irritancy could have influenced feeding success by dissuading and reducing persistence to find a host and/or a hole on the net[162,247]. However, less mosquitoes were motivated to fly to the other side of the tunnel in the presence of insecticide. Therefore, we suggest that mosquitoes were able to detect LLINs over this short distance (the tunnel is around 1 meter long) and they deliberately chose to stay in the rearing cage instead of searching for a host. Some studies found that mosquitoes with knockdown resistance might increase orientation towards hosts in the presence of an LLIN compared with an untreated control net[290,299]. Even though we did not use dual-choice tunnels to properly assess bednet net preference, we compared host searching simultaneously in a relatively short-distance

setup and we always found mosquitoes host searching more in the tunnel with an untreated net. Therefore, our data do not confirm these findings. Yet, it is probable that mosquitoes somehow detect LLINs from a short distance.

The host searching and feeding behaviors increased during each behavioral assay from 10 to 30min. At the end of the 30 min, nearly all blood fed mosquitoes were still near the host. This suggests that mosquitoes would not necessarily go back to the rearing cage if they are interested by the host. In the presence of insecticide, this observation was less clear and the number of mosquitoes near the host did not increase from 20 min to 30 min. Besides, even with a LLIN, the number of fed mosquitoes inside the tunnel when the tests ended was still similar to the number of fed mosquitoes found near the host after 30 min. As, the excito-repellency properties of the LLIN did not increase the number of mosquitoes avoiding the presence of insecticide after feeding, we suggest either a possible faster foraging decision or a reduced motivation to feed over time during the 30 min of the test in the presence of insecticide.

The delayed mortality effects of insecticide exposure appear reduced if mosquitoes had opportunities to take blood meals. Nevertheless, in the damaged LLIN treatment mosquitoes had a reduced lifespan equivalent to mosquitoes having no access at all to the host, despite the opportunity to take blood meals. The time spent in contact with the net was probably higher in both these treatments (damaged or intact net) in comparison to the treatment where the host was immediately available through the LLIN (foot surrounded by the bednet sock), thus possibly leading to overexpression of detoxification enzymes which inactivate or sequester insecticide. Another explanation may be that longer flights deplete carbohydrate resources, requiring mosquitoes to free up additional energy stores[300].

We suggest that the foraging strategies may have been different in both treatments as in the first case (damaged net), extended periods of insecticide exposure may be necessary to achieve one or several successful blood meal(s) and egg laying. In the second case (intact net and no access), mosquitoes spend extended time in the contact with the LLIN until they get discouraged by the lack of access to the host. We would assume that this discouragement would then occur sooner, but they did not get a bloodmeal and so no extra energy. However, the data for mosquitoes exposed to a control net (UTN) revealed that the longevity for mosquitoes having access to a host was similar whether the net was damaged or placed against the foot (direct access). The energy spent host searching and finding holes did not influence the survival as it did for mosquitoes tested in the tunnel with a LLIN. It would have been interesting to separate mosquitoes that successfully found a hole through a LLIN and fed, from

the unsuccessful ones, to better understand whether only a small proportion of mosquitoes are successful every time or a large proportion of mosquitoes successfully feeds but will not often repeat this experience.

The individual feeding performance experiment (experiment 3) showed the variability in the numbers of blood meals taken per female over their lifetime. In the presence of insecticide (LLIN), the proportion of mosquitoes not feeding at all was very high, only 41 % of mosquitoes took at least one blood meal, and only 11 % took at least 2 blood meals during their life. The proportion of potential vectors was then low. In the control group (UTN), the variability was lower, with only 18 % not taking a blood meal and even 15 % taking 3 or 5 blood meals during their life. The observed variability in individual feeding performance in presence of insecticide, with some mosquitoes successfully taking several blood meals during their life while other will never take any, might be explained by a study showing that the effect of a sub-lethal exposure to permethrin during blood feeding of kdr resistant lab strain decreased the insecticide irritancy at the following exposure[163] which may increase the probability of taking another bloodmeal. This variability could also be quite specific to the kdr genotype, as our colony is composed of both kdr homozygotes and kdr heterozygotes (see Chapter 3) and one paper showed the reduced host-seeking activity in kdr homozygotes compared to kdr heterozygotes[171].

The number of blood meals was positively correlated with longevity but the relation with body length was unclear. The wing size measures were available only for the first experiment; therefore, we could not link the individual feeding performance with mosquito size, and this would constitute an interesting follow up experiment. We used a standardized method for our colony rearing and the variability in body size was expected to be smaller than in the field with reduced variability in larval environment.

Further differences in individual feeding performance might be found between mosquito strains with different resistance profiles, or using different types of LLIN and net quality[237]. Here, we only used pieces of the same new LLIN, a Permanet®2.0 (at least at the beginning of the experiment), which was unwashed in order to maximize its efficacy. Some pieces of it were damaged on purpose with holes for the design of the second experiment. We did not test a similar LLIN after several washes and we would expect the active ingredient (deltamethrin) to decay after 5-15 washes[301,302], although we do not expect it to dramatically lose efficacy with reasonable washings during the recommended time of use[303,304].

In continuity with this work, it would be interesting to study the variation in foraging strategies of mosquitoes infected with malaria parasites in response to the implementation of vector control tools. Transmission depends on the bite of an infectious mosquito, so the parasite may reduce the EIP length to adapt to shorter living vectors[79]. One study showed that the number of blood meals taken in *Aedes* mosquitoes was positively correlated with an acceleration of the Zika virus development[305]. For malaria vectors, the opposite may also be true and the increase in EIP length could be adaptive to vectors having difficulties to find hosts especially in high insecticide coverage areas where LLINs protect from bites and several feeding attempts may be needed before being successful.

While more information is needed on the sublethal effects of pyrethroid on insecticide resistant mosquito survivors able to transmit infectious malaria parasites[238], a better understanding of the variation in the impact of LLINs on field mosquitoes in different settings is of equivalent importance. Whether the presence of pyrethroid generate behavioral avoidance in mosquito population is not well reported in the field and our experiments do not capture it. It is nonetheless an important parameter to consider when studying the vectorial capacity of local *Anopheles* mosquitoes, especially because residual malaria transmission still persist despite high LLIN coverage and Indoor Residual Spraying IRS[306,307]. Behavioral adaptations to pesticides have been associated to changes in host-seeking behavior preferences such as biting time activity, level of anthropophily, endophily and endophagy[308]. Changes like these could potentially threaten LLIN and IRS effectiveness, as vectors feeding behavior evolve in order to avoid insecticide exposure[154,309].

4.7. Conclusion

Even though pyrethroids do not kill mosquitoes 24h after an exposure anymore in areas with very high insecticide resistance, they still provide physical protection during the night from mosquito that tries to bite people sleeping under them and remain more effective than untreated nets, regardless of resistance. Given the substantial effect of insecticide on feeding success and longevity, novel adequate assays are needed to characterize resistance. Net efficacy should not be assessed without considering patterns of exposure history, blood-feeding status, and ability for a female to take several blood meals over her whole lifespan. A standardization of methodologies used to study the long-term effects of vector control tools on blood feeding success over time could improve predictions of malaria transmission potential. Varying net quality and host availability would be important to further explore vector-host interactions.

CHAPTER FIVE

Nutritional stress decreases host searching and blood feeding rates in insecticide resistant *Anopheles* mosquitoes

5.1. Abstract

Nutrient deprivation and resource competition during larval development can influence malaria mosquito life history traits like longevity or vector competence which could be detrimental to vectorial capacity. Despite its importance, very little is known about how the larval environment may impact host searching and blood feeding success in presence of insecticide during a lifetime.

In this study, we used field collected 1700-fold insecticide resistant *Anopheles gambiae* s.l. whose larval environment was manipulated to induce nutritional stress. Mosquitoes were reared with one of two larval diets (standard insectary diet or half or it) and one of two larval densities (standard insectary density or a third of it). A one-way flight tunnel was used to evaluate the behavioral responses of adult mosquitoes in the presence of a human host protected by a PermaNet® 2.0.

Both the standard larval diet cut in half and the standard density (compared to a third of the standard density) increased time to pupation by one day, decreased pupation rate by respectively 6 to 12 %, and decreased adult body size. The standard density also decreased the emergence rate by 14 %. In terms of adult behavior, both the standard larval diet cut in half and the standard density (compared to a third of the standard density) decreased the daily number of mosquito host searching and blood feeding, but there was no interaction between the two treatments was found. When near the host, the proportion of mosquito successfully blood feeding was not dependent on the larval environment. Finally, larval diet cut in half marginally increased mosquito longevity by one day.

On one hand, laboratory studies may over-estimate the vectorial capacity by increasing the resources available to mosquito larvae (which is estimated to be lower in the field than our laboratory standard), thus increasing adult mosquito host searching and blood feeding rates. On the other hand, an opposite effect was found for larval density (which is estimated to be lower than our standard in the field), suggesting a possible under-estimation of the vectorial capacity in laboratory studies. Hence, this highlights the importance of integrating larval environment when studying the adult foraging strategy with potentially larger consequences on malaria transmission dynamics. Besides, in our study nutritional stress influences host searching and feeding rates but not feeding success once near the host. Therefore, we need to better understand how the resource available early in life influences the daily resource allocation for flight and host detection.

5.2. Introduction

Most malaria mosquitoes prefer to breed in temporary, open, and sunlit bodies of water[12]. Variation in temperature, resource availability, and population density in such unstable environments may have a uneven effect on larvae's development and their capacity to store reserves necessary for the next life stages[310,311]. Indeed, plastic developmental responses to cope with changes in larval environment[312–314] can induce carry over effects that shape adult phenotype including: body size, female fecundity, oviposition site selection, energetic reserves and vector competence, immune responses and longevity[78,83,84,89,90,315,316,317,318]. The impact of such life history traits changes on the vectorial capacity (defined as the average number of potentially infectious bites that would originate from one case of malaria[319]) or the measure of vector-borne pathogen transmission potential is well-documented and provide important data for transmission models[320,321].

With a first infectious meal mosquitoes acquire the pathogen and after the extrinsic incubation period disease transmission can occur upon further blood meals. Therefore, the biting rate strongly influences vectorial capacity and is an important parameter to understand diseases transmission dynamics[78,306]. Yet, nutritional stress during larval development is largely overlooked in experiments that measure host seeking and blood-feeding behaviors of wild-type mosquito populations over their lifetime. Some previous studies have studied the negative impact of larval conditions and weather patterns on flight capacity and mosquito dispersal[322–325] and larval and adult rearing conditions on host-seeking behavior[86,92,326–328]. But whether the biting frequency and feeding success changes over time due to variation in nutritional reserves is unclear despite its importance for transmission potential, especially when considering the whole lifespan and not just one or two biting events. One study with field-captured *Anopheles darlingi* shows a decrease in biting rate with age and positive correlations between both wing size and biting frequency, and the amount of larval food and wing size[82]. While the decrease in biting frequency with age was confirmed with *Aedes aegypti* captured from the field[329], a study with *An. gambiae* shows the opposite trend[87].

Another important parameter influencing mosquito feeding rate is longevity. The effects of larval conditions on larval and adult survival are both well-documented and complex. Often, larger mosquitoes are expected to live longer[328,330], but there is not always an effect of size on longevity[331,332] or even a negative relationship[81,84]. To illustrate this complexity, Barreaux and al. showed that wing length decreases with larval food levels, but the relationship between size and longevity ranges from positive to negative depending on the larval rearing temperature[89].

In areas of high insecticide coverage resistant mosquitoes are constantly exposed to pyrethroids when searching a blood source. We already know the importance of factors such as a reduced larval diet[173,174] and senescence[333,334] in the expression of resistance to insecticides in laboratory *Anopheles* s.l. strains. In this chapter, we would like to test whether the benefit of being able to host seek in presence of an insecticide-treated net may be less compelling depending on exposure to stress during larval development. Carry-over effect on adult life history traits may not only influence phenotype expression of insecticide resistance but also modify mosquito feeding rate with age, which ultimately could reduce the number of gonotrophic cycles and the vectorial capacity per individual.

We hypothesize here that density-dependent competition and food deprivation at the larval stage can decrease host seeking and blood feeding motivation and success over a lifetime. We used the F1 generation of pyrethroid-resistant field-collected *An. gambiae* s.l. mosquitoes. These F1 mosquitoes are reared with one of two larval diets (standard insectary diet or half or it) and one of two larval densities (standard insectary density or a third of it) and tested in a one-way tunnel to give them daily the opportunity to take a blood meal in the presence of insecticide.

5.3. Material and methods

5.3.1. Mosquito populations

The experiment uses the direct offspring (F1 generation) of field-collected *An. gambiae* s.l. mosquito collected as larvae in natural breeding habitats around Bouake (-5.0303100 W longitude and 7.6938500 N latitude) Côte d'Ivoire which are known to exhibit both kdr and metabolic resistance and breed in rice fields[165,230,242]. CDC bottle assays indicate more than 1700-fold resistance to deltamethrin relative to standard susceptible strains[165].

5.3.2. Mosquito rearing

All tested mosquitoes were maintained under standard insectary conditions (26 ± 1°C, 70 ± 5% RH) and natural light. Field collected first stadia mosquito larvae were reared by 300 in plastic boxes with 1L deionized water and fed daily following the standardized food diet per larvae described in Kulma and al.[266] for 1 larvae: day of hatching: 0.04 mg of Tetramin™ fish food per larva; day 1 after hatching: 0.06 mg; age 2: 0.08 mg; age 3: 0.16 mg; age 4: 0.32 mg; age 5 and more: 0.6 mg. The female emerging were then fed on a human foot and from these eggs, randomly selected first instar larvae were reared

in 4 separate plastic boxes, one per treatment, with 1L deionized water (surface: 20 x 15 cm) and fed daily with Tetramin™ baby fish food. The four treatments were:

Tray 1: standard food diet (Kulma and al.[266]) and 300 larvae (standard lab density= 1 larvae/ 3 ml)

Tray 2: half the standard diet and 300 larvae

Tray 3: standard food diet and 100 larvae (a third of the density= 1 larvae / 9 ml)

Tray 4: half the standard diet and 100 larvae

Seven to ten days after hatching, 4 Bugdorm© rearing cages (V = 32.5 cm^3) corresponding to the four larval treatments were filled with 120 pupae each (put in a cup). After emergence, mosquitoes were held in the same cage with access to 10 % glucose solution. 4-5 days after emergence 41-50 females and 10 males were randomly kept in each tested cage until their death.

5.3.2. Preparation of the experimenter

One experimenter was being monitored for malaria throughout the course of the study to avoid any malaria infection in mosquitoes. This experimenter washed his feet with unscented soap and rinsed them with water the day before each test. He also avoided the use of fragrance, repellent products, tobacco, and alcohol for 12 hours before as well as during testing.

5.3.3. Bednet evaluation against susceptible mosquitoes

Prior to experimentation, a WHO cone bioassays[267] of fully susceptible *An. gambiae* mosquitoes (Kisumu strain) was conducted to confirm the bio-efficacy of the netting material used in the study: a rectangular piece of unwashed PermaNet® 2.0 (5 pieces in total with 4 side panels pieces and one roof panel piece) manufactured by Vestergaard Frandsen SA (DK). The PermaNet® 2.0 (LLIN) is made of polyester, is green in color and is entirely coated with 55 mg/m^2 ± 25 % deltamethrin. A rectangular untreated ultra-fine 240 mesh polyester net distributed by Coghlan's green in color was used as control (untreated net, 5 pieces in total with 4 side panels pieces and one roof panel piece).

Cone tests were carried out in daylight with 4-5 days old non-blood fed female mosquitoes (*An. gambiae* Kisumu strain). Mosquitoes were exposed ten-by-ten in a plastic cone for 3 min, with cones angled at 40° to favor the contact between the mosquitoes and the net, and a ball of cotton used to close each cone's entry point. Following exposure, mosquitoes were removed to holding cups and monitored for mortality for 24h. The LLIN was effective at killing 100 % of mosquitoes, while the untreated net (UTN) killed none.

5.3.4. Behavioral assays

A one-way flight tunnel was used to expose daily 532 insecticide-resistant mosquitoes to the foot of the experimenter in the presence of insecticide (Figure 1). In replicate one, we tested 164 females (41 x 4 larval treatments), in replicate two 168 females (42 x 4 larval treatments) and replicate three 200 females (50 x 4 larval treatments). For each replicate, the 4 cages (41-50 females and 10 males each) were tested against a treated net every day in a randomized order (only one experimenter). Mosquitoes were starved 6 hours before each behavioral test.

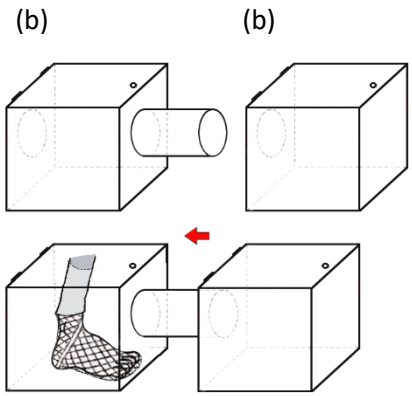

Figure 1: The one-way tunnel with one rearing cage (b), one Plexiglas® tube and an adjacent experimental cage (a) with a direct access to an experimenter's foot enveloped in a treated net. Mosquitoes pupated and stayed in the rearing cage (b) until they all died, except during the experimental tests in tunnel. Daily and before each behavioral test, the connection between the rearing cage (V = 32.5 cm^3) and the experimental cage (V = 32.5 cm^3) was made thanks to a transparent Plexiglas® tube (L= 30 cm, d= 14,6 cm). In the experimental cage, a volunteer put his foot in as blood source inside a sort of "sock" made of a piece of PermaNet 2.0 (l= 25 cm^2). The foot was in direct contact with the netting to enable blood feeding.

Before each behavioral test, the connection between the Bugdorm© rearing cage (V = 32.5 cm^3) and the experimental cage (V = 32.5 cm^3) was made thanks to a transparent Plexiglas® tube (L= 30 cm, d= 14,6 cm). In the experimental cage, an experimenter put his foot in as blood source inside a sort of "sock" made of a piece of LLIN (l= 25 cm^2). The foot was in direct contact with the netting to enable blood feeding (Figure 1). Mosquitoes were tested daily for 30 minutes in th presence of a LLIN (in separate cages corresponding to their larval diet and density treatments). Every 10 minutes, the position of all mosquitoes in the dispositive was determined (rearing cage or experimental cage), mosquitoes in the experimental cage were considered host seeking. After 30 minutes, the proportion of mosquitoes that successfully fed on the feet was reported.

5.3.5. Life history traits: survival, fecundity and mosquito size

Daily survival as well as the daily number of eggs collected were measured for each cage. Mosquito wings were measured upon mosquito death from the tip to the distal end of the allula by excluding the fringe, as an indication of mosquito body size. The wings were fixed into slides, scanned, and copied to a computer. Wings lengths were measured to the nearest 0.01 mm on the java-based application ImageJ 64 (*http://imagej.nih.gov/ij/*).

5.4. Statistical analyses

When needed, contrasts among treatments were assessed using the *multcomp* package version 1.4-10 and the function *glht* in the software R with a Tukey's honestly significant difference test (Tukey HSD) multiple-comparison test. It allowed simultaneous tests for General Linear Hypothesis and comparison differences between groups. All statistical analysis and figures were performed in R© version 3.6.1 with the Rstudio interface version 1.2.5001. The significance level was set at 5 %.

5.4.1. Effect of the larval environment on juvenile development

We analyzed the juvenile development of 4568 mosquitoes (replicate 1: 801, replicate 2: 2418, replicate 3: 1349). One binomial Generalized Linear Model (GLM) explored the pupation rate regarding the larval diet (standard insectary food and half or it), the larval density (standard insectary density and a third of it), the replicate and the effects of those parameters in interactions. One gaussian Generalized Linear Model (GLM) explored the emergence rate regarding the larval diet (standard insectary food and half or it), the larval density (standard insectary density and a third of it), the replicate and the effects of those parameters in interactions.

We also analyzed the time to pupation using a gaussian GLM to compare the pupation time of the 3912 larvae that became pupae, regarding larval diet, larval density, their interaction, and the effect of the replicate (the replicate was not kept in interaction with the other parameters for this model as it was not significant).

5.4.2. Effect of the larval environment on adult behavior and fecundity

We analyzed the adult behavior, host searching and blood feeding rates over time, of 527 female mosquitoes (replicate 1: 164, replicate 2: 168, replicate 3: 195) regarding the larval diet and larval density. Having done everyday observations per treatment:

_The host searching weighted mean was calculated as the number of mosquitoes going to the other side of the tunnel (from the rearing cage to the experimental cage), “visits”, everyday divided by the number of mosquitoes still alive each day, and multiplied by the number of female mosquitoes in the cage on the first day of experimentation. The overall number of “visits” per test (or daily behavioral assay of 30 min) and per treatment was the highest number of visits out of the 3 measures taken every 10 min during the 30 min test.

_The same was done for the blood feeding rate weighted mean with the number of blood fed mosquitoes in the experimental cage, “blood meals”, at the end of the behavioral test each day.

The weighted numbers of visits and blood meals per day allowed the comparison over time while taking into account the unequal numbers of female mosquitoes in the cage each day in different replicates. In order to work with a Poisson distribution, we rounded the value of both the weighted number of visits per day and the weighted number of blood meals taken per day.

We used a Generalized Linear Mixed-Effects Models (GLMM) with a Poisson distribution and log link distribution to analyze the weighted number of visits per day. The fixed effects were the larval diet, the larval density, and their interaction. The random effects were the replicate, the day and the observation-level random effect. The latter minimize overdispersion and recover unbiased parameter estimates in Poisson mixed effects models[292]. Measures of goodness of fit compared our model two-by-two with different models and tested for fixed and random effects significance.

We used a similar Poisson GLMM model to analyze the weighted number of blood meals per day.

The feeding success was calculated by dividing the total number of blood meals observed in a cage over the lifetime by the total number of mosquito visits to the cage with the host, the experimental cage. The feeding success was analyzed using a gaussian GLM considering larval diet and larval density treatments and their interaction. The effect of the replicates was also added to the model.

The average number of eggs laid per female over their lifetime was compared between treatments using an Analyze of Variance (ANOVA) considering the same parameters detailed for the gaussian GLM. The estimation for egg laying per female was calculated as the total number of eggs laid per cage divided by the number of individuals in each cage. The Shapiro-Wilk test confirmed that the residuals of the model were normally distributed.

5.4.3. Relationship between wing length and survival

First, we analyzed the longevity for 340 mosquitoes (those for which the wing length data was available) with a gaussian GLM that included the larval density, the larval diet, their interaction, and the replicate. The choice of the gaussian GLM instead of an ANOVA was based on the distribution of the longevity data being almost normal but not exactly and the GLM model being more robust to departure from normality.

Second, we analyzed the wing length of mosquitoes with an ANOVA that included the larval density, the larval diet, the replicates, and their interactions. The Shapiro-Wilk test confirmed that the residuals were normally distributed.

We then estimated the effects (direct and indirect) of larval density and food diet on longevity with a piece-wise structural equation model (SEM) following the method described in Barreaux and al[89], using the piecewiseSEM package 2.1.0[335]. We used a linear regression model to study the effect of larval diet, larval density, their interaction, and the replicate on wing length. This is expressed by the following equation

$$\text{wing length} \sim \text{larval diet} * \text{larval density} + \text{replicates} \quad (1)$$

We then used a Generalized Linear Model (GLM) with a gaussian distribution to study longevity regarding larval diet, larval density, wing length and the replicate as covariate. The interaction between the larval diet and the larval density in the linear model was not significant, therefore the interaction was removed. This is expressed by the following equation:

$$\text{longevity} \sim \text{larval diet} + \text{larval density} + \text{wing length} + \text{replicates} \quad (2)$$

5.4.4. Vectorial capacity of resistant mosquitoes

The dynamic of malaria transmission can be measured considering the vectorial capacity, a mathematical formula described by Ross-MacDonald as follows[293]:

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

Where *VC* is the vectorial capacity, *m* the mean number of mosquitoes per host, *a* is daily mosquito's blood feeding rate, *b* is the probability of transmission from a vector to a host, *p* is mosquito survival probability and *n* is the Extrinsic Incubation Period (EIP). Together these parameters represent the

number of infectious bites arising from one single infectious person for one day[294]. We calculated the vectorial capacity using mean parameter estimates quantified from our empirical data.

Adult vector density, *m*, was estimated using the mean number of mosquitoes alive on the first day of the test in each experiment (average total number of females tested in each replicate per treatment). The biting rate, *a*, was estimated for each net treatment using our empirical data of feeding success per female divided by the average longevity. Adult daily survival, *p*, was estimated using our empirical data of mosquito longevity. Mosquito daily mortality is measured as the reverse of longevity: x = 1 / (longevity); and the survival probability as follows: $p = 1 - x$. The EIP of the parasite, *n*, we assumed the EIP for *Plasmodium falciparum* at 24 °C to be 12 days for both treatment groups[18,295]. Probability of transmission from a vector to a host, *b*, are based on previous research[296].

5.5. Results

5.5.1. Effect of the larval environment on juvenile development

85.6 (95 % Confidence Interval: 84.6 to 86.6) % of the larvae pupated overall and 6 % more than the average when reared with the standard larval diet [Figure 2.1; 91.5 (95 % CI: 90.2 to 92.6) % compared to the low diet (half of the standard larval diet): 79.6 (95 % CI: 77.9 to 81.3) %] ($\chi^2 = 132.73$, df = 1, $p < 0.001$). 12 % more mosquitoes pupated in the low larval density environment (a third of the standard density([97.8 (95 % CI: 96.8 to 98.5) % compared to 81.1 (95 % CI: 79.8 to 82.5) % in the standard density environment] ($\chi^2 = 272.29$, df = 1, $p < 0.001$). The pupation rate in the different replicates was significantly different ($\chi^2 = 659.73$, df = 1, $p < 0.001$) and while we found significantly more mosquitoes pupating when given the standard larval diet in replicates 1 and 2, in the replicate 3 all mosquitoes pupated no matter the larval diet ($\chi^2 = 26.31$, df = 1, $p < 0.001$). The same observation was made with the larval density treatment added to this interaction between larval density and replicate [standard density + low diet: 73.5 (95 % CI: 71.3 to 75.6) %; standard density + standard diet: 88.6 (95 % CI: 87.0 to 90.1) %; low density + low diet: 96.2 (95% CI: 94.3 to 97.6) %; low density + standard diet: 99.3 (95 % CI: 98.3 to 99.8) %] ($\chi^2 = 5.22$, df = 1, $p = 0.02$).

The overall emergence rate in all replicates was 80.0 (95 % CI: 78.83 to 81.18) %. With the standard larval density cut in three (low density), the emergence rate increased by 14 % more important [standard density: 65.7 (95 % CI: 64.3 to 67.1) %; low density: 94.3 (95 % CI: 93.6 to 94.9) %] ($F = 33.7$, df = 1, $p = 0.004$). A similar trend was found for a standard larval diet instead of a diet cut in half (low diet) but it was not significant ($F = 4.69$, df = 1, ($p = 0.09$) nor was the interaction ($p > 0.05$) [Figure 2.2;

standard density + low diet: 59.0 (95 % CI: 56.6 to 61.4) %; standard density + standard diet: 72.39 (95 % CI: 70.2 to 74.5) %; low density + low diet: 90.3 (95 % CI: 87.7 to 92.6) %; low density + standard diet: 98.3 (95 % CI: 96.9 to 99.2) %]. Replicates had significantly different emergence rates ($p = 0.01$) but the trends between treatments did not change ($p > 0.05$) for all interactions between replicate and larval treatments).

On average, the pupation time was (mean ± standard error) 8.1 ± 0.02 days. Being fed with the standard larval diet accelerated the pupation time by almost half a day compared with the standard diet cut in half (low diet) (standard diet: 7.7 ± 0.03 days and low diet: 8.6 ± 0.04 days) ($F = 362.11$, $df = 1$, $p < 0.001$) (Figure 2.3). Mosquitoes reared in the low density (a third of standard density) treatment pupated 1 day quicker than mosquitoes reared in the standard, higher density environment (standard density: 8.6 ± 0.3 days and low density: 7.01 ± 0.3 days) ($F = 1004.05$, $df = 1$, $p < 0.001$). Larvae reared in the resource-rich environment (i.e. with a standard diet and low larval density) were the quickest to pupate (6.9 ± 0.04 days), while those in the most resource-limited (i.e. diet cut in half and a standard larval density) had the longest time to pupation (9.2 ± 0.04 days) ($F = 79.58$, $df = 1$, $p < 0.001$). The pupation time in the different replicates was not significantly different ($p > 0.05$).

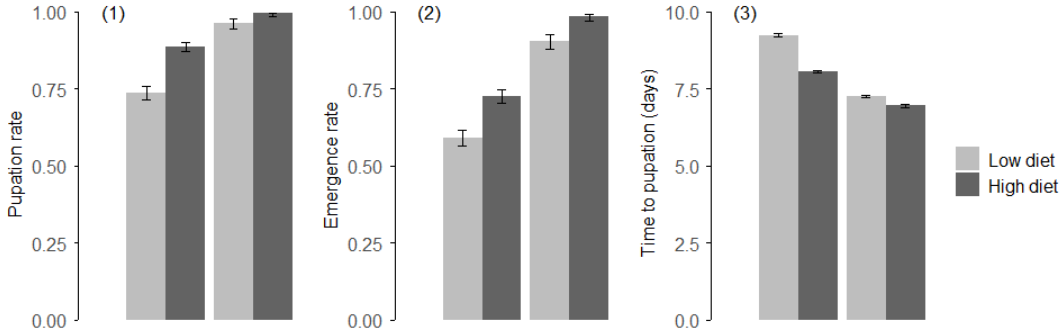

Figure 2: (1) Pupation rate, (2) emergence rate and (3) time to pupation of juvenile mosquitoes reared in different environments. Mosquitoes were reared with two different food regimes, a standard "high" diet (dark grey) or half of it, "low" diet (grey), either in a crowded environment (the standard "high" density) or three times less crowded ("low" density). ± standard error bars are shown. In graph (1) (pupation rate) and (2) (emergence rate) the confidence interval show the variability between individuals, whereas for the time to pupation (3) the error bars show the variability between the average time to pupation in the different replicates.

5.5.2. Effect of the larval environment on adult behavior

The weighted average of host visits (mosquitoes flying to the other side of the tunnel near the blood source) is 10.0 ± 0.51 mosquitoes near the host. Mosquitoes reared with more food (standard diet) as

larvae were 1.15 times more motivated over their lifespan to fly to the other side of the tunnel near the blood source than those reared as larvae with less food [Figure 3.1; low diet: 8.5 ± 0.67 visits; standard diet: 11.5 ± 0.77 visits] (χ^2 = 19.57, df = 1, p < 0.001). The weighted average of hosts visits of mosquitoes reared in the less crowded environment during their juvenile development was also 1.1 times higher than the weighted average of those reared as larvae in the more crowded environment [Figure 3.2; low density: 11.4 ± 0.85 visits; standard density: 8.6 ± 0.56 visits] (χ^2 = 4.37, df = 1, p = 0.04). There was no significant interaction between larval density and larval diet on host visits (p > 0.05).

The weighted average number of mosquitoes taking a blood meal per day was 6.6 ± 0.40. Mosquitoes reared with more food as larvae (standard diet) were blood feeding 1.1 times more over their lifespan than mosquitoes reared with the low diet [Figure 3.3; low diet: 5.8 ± 0.58 blood meals; high diet: 7.5 ± 0.56 blood meals] (χ^2 = 19.58, df = 1, p < 0.001). Mosquitoes reared in a less crowded environment during the larval stage (low density) took 1.2 more blood meals over their lifespan than the average [Figure 3.4; low density: 7.7 ± 0.67 blood meals; standard density: 5.5 ± 0.41 blood meals] (χ^2 = 6.31, df = 1, p = 0.01). There was no significant interaction between larval density and larval diet on the number of mosquitoes taking a blood meal (p > 0.05).

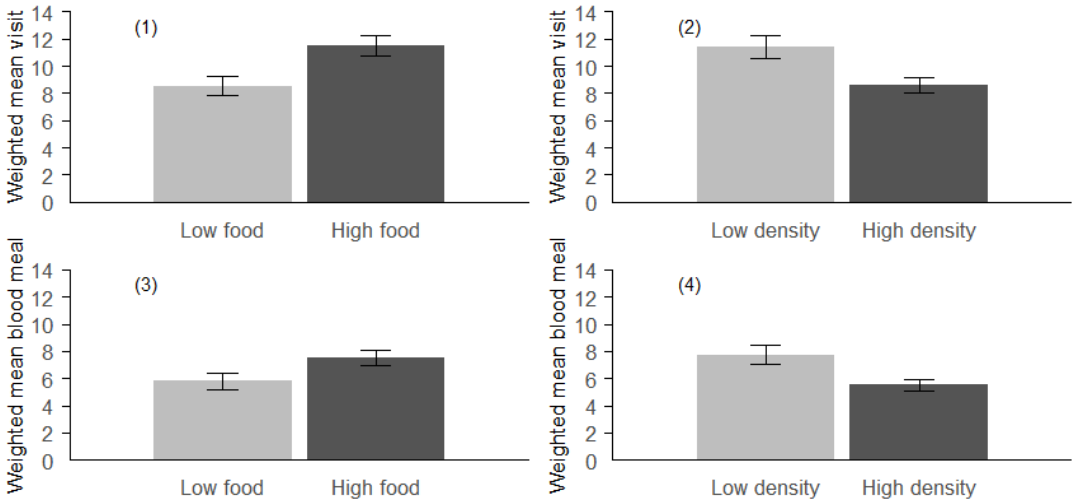

Figure 3: Estimated weighted mean for host seeking (1&2) and host feeding (3&4) of adult female mosquitoes reared in different larval environments. Mosquitoes were reared with two different larval food regimes (1 & 3), a standard "high" diet (dark grey) or half of it, "low" diet (grey); and two different larval densities (2 & 4), either in a crowded environment (the standard "high" density) (dark grey) or in a density three times less crowded environment ("low" density). We named "visit" mosquitoes motivated enough to fly from their rearing cage to the other side of the tunnel, in the experimental cage where a host's foot was present (see Figure 1). The weighted mean number of visits and blood meals were calculated as the number of mosquitoes going to the other side of the tunnel everyday (or the number of blood meals per day) divided by the number of mosquitoes still alive each day, and multiplied by the overall number of mosquitoes tested in each treatment (the number of female mosquitoes in the rearing cage on the first day of experimentation). The weighted numbers of visits and blood meals per day allowed the comparison over time. ± standard error bars are shown.

The proportion of mosquitoes successfully feeding out of the mosquitoes that went near the host in the experimental cage was on average 70.9 ± 1.76 (66.9 to 74.8) %. We found no statistical difference in average blood feeding success (successfully feeding once on the other side of the cage) regarding larval diet or density (all $p > 0.05$). The feeding success was higher in one replicate ($F= 24.85$, $df= 1$, $p < 0.001$).

The estimated number of eggs laid per female 43.10 ± 7.42 was not influenced by the larval environment or the replicate (all $p > 0.05$).

5.5.3. Relationship between wing length and longevity

Mosquitoes lived on average 16.8 ± 0.50 days. Mosquitoes lived a longer life when the larval diet where cut in half [Figure 4; low diet: 17.8 ± 0.68; standard diet: 15.7 ± 0.74] ($F = 378.03$, $df = 1$, $p = 0.03$), but there was no effect of density or the interaction, and no effect of the replicate ($p > 0.05$).

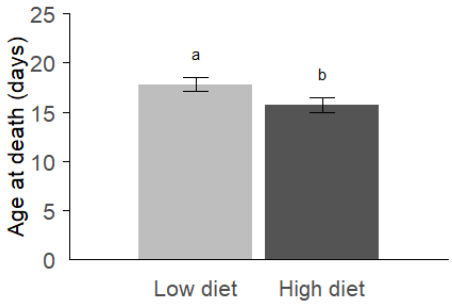

Figure 4: Mean longevity of mosquitoes reared with different larval diets. Mosquitoes were reared with two different food regimes, a standard "high" diet (dark grey, b) or half of it "low" diet (grey, a). Mosquitoes were given the opportunity to host seek and feed through a PermaNet 2.0 every day from when they were four days old until they all died. In the one-way tunnel (see Figure 1), mosquitoes had direct access to a human foot enveloped in a treated net. The longevity of mosquitoes was individually scored each day. ± standard error bars are shown and a is significantly different from b.

The average wing size was 0.34 ± 0.001 cm. When given half of the high larval diet, the average size of adult female was 0.01 cm smaller than the average size of those given the standard larval diet [low diet: 0.34 ± 0.001; standard diet: 0.35 ± 0.001] ($F = 27.05$ df = 1, $p < 0.001$). Similarly, the average size of adult female from the most crowded environment (standard density) was 0.01 cm smaller than the average size of those in the less crowded environment [standard density: 0.34 ± 0.002; low density: 0.35 ± 0.001] ($F = 30.91$, df = 1, $p < 0.001$). The mosquitoes emerging from the most crowded environments with the lowest diet were smaller than all other mosquitoes [standard density, low diet: 0.33 ± 0.002; other treatments: 0.35 ± 0.001] ($F = 7.47$, df = 1, $p = 0.006$). The average wing length of mosquitoes in one of the 3 replicates was significantly bigger than the average wing length in one of the two other replicates ($F = 11.10$ df = 1, $p = 0.001$). In this replicate, both the larval food and larval density treatments did not influence the wing length (respectively $F = 8.11$, df = 1, $p = 0.004$ and $F = 26.10$ df = 1, $p > 0.001$). There was no triple interaction ($p > 0.05$).

The piecewise structural equation model (SEM) confirmed the direct effects of larval density and the interaction between larval density and diet on body size (Table 1). There was also a difference in body size between replicates as seen above. The SEM confirmed the direct effects of larval diet on adult longevity even when correcting for indirect effects through body size (Table 1, Figure 4).

Response	Predictor	Estimate	SE	p-value
wing length	larval diet	-0.00	0.00	0.8890
wing length	larval density	-0.01	0.00	**0.0001**
wing length	replicate	0.00	0.00	**0.0006**
wing length	larval diet: larval density	0.00	0.00	**0.0094**
longevity	larval diet	-2.48	1.04	**0.0174**
longevity	larval density	0.42	0.52	0.4121
longevity	wing length	35.77	24.89	0.1516
longevity	replicate	0.69	0.06	0.2829

Table 1: Regression coefficients of the structural equation model. The estimate column gives the regression coefficient associated with the predictors, or their interactions. SE is the standard error of the coefficient value. The p-values in bold text are inferior to 0.05 and indicate significant predictors.

5.5.4. Effect of the larval environment on the vectorial capacity

The vectorial capacity $VC_{low.dens+stand.diet}$ = 0.70 for mosquitoes reared in the low density and with the standard diet, was more than 2 times higher than the vectorial capacity $VC_{low.dens+low.diet}$ = 0.30 for mosquitoes reared in the low larval density and with the standard diet cut in half (Table 2). We found the opposite effect of diet at standard density. The vectorial capacity $VC_{stand.dens+low.diet}$ = 0.45 for mosquitoes reared in the standard larval density and with the standard diet cut in two was 1.7 times higher than the vectorial capacity $VC_{stand.dens+stand.diet}$ = 0.26 for mosquitoes reared in the standard density and with the standard diet.

Larval density	Larval food	Bites per female	Lifespan (days)	Adult vector density (*m*)	Biting rate (*a*)	Adult daily survival (*p*)	Proportion infectious (*b*)	Parasite EIP (*n*)	Vectorial Capacity *(VC)*
low	low	2.26	17.65	42.67	0.05	0.94	0.3	12	0.30
low	high	3.15	19.82	44.33	0.07	0.95	0.3	12	0.70
high	low	2.68	18.62	44.33	0.06	0.95	0.3	12	0.45
high	high	2.41	15.37	44.33	0.05	0.93	0.3	12	0.26

Table 2: Output of vectorial capacity equation with experimental parameters. The parameters: adult vector density *m*, biting rate *a*, adult daily survival *p*, proportion infectious *b* and extrinsic incubation period (EIP) *n* represent the number of infectious bites arising from one single infectious person for one day. We calculated the vectorial capacity using mean parameter estimates quantified from our empirical data (for *m*, *a* and *p*). Probability of transmission from a vector to host, *b*, and the estimate for the EIP, *n*, were based on previous research for *Plasmodium falciparum* at 24 °C[18,295,296].

5.6. Discussion

The variation in larval environmental conditions influenced the juvenile development, as well as adult size, longevity, and blood feeding rate. First, a dietary restriction and an increase in larval density increased larval developmental time were detrimental to larval survival and led to smaller adult mosquitoes. Second, those stressful conditions during the larval stage reduced the motivation to find a host and take a blood meal over a lifetime. These mosquitoes having a larval diet cut in half ("low" diet) did live slightly longer but only a day on average. Consequently, the vectorial capacity increasing with both a higher biting rate and longevity favored mosquitoes reared with the lowest larval density and the standard diet and those reared with the standard density and the standard diet cut in half the most. Third, for mosquitoes that did approach the host there was no difference in feeding success whatever their larval environment, nor any differences in average number of eggs laid per female.

Our study confirms predictions from theory that low food availability leads to slow larval growth, late pupation and small adults (the theory includes also a low fecundity[336] but that did not happen with our mosquitoes) and findings of previous studies[78,81,336,337]. The food supply represents 25 % of the biomass needed for mosquito ecdysis (moulting of the cuticle) and the extended larval period was probably not enough to compensate for the low diet leading to the observed higher larval mortality rate[338]. There may be a limit in the plasticity of the duration of this sensitive period of development because of evolutionary constrains at other moments of the development or the life cycle, as well as an increased biological risk (predation, parasitism or competition) or habitat degradation risk (desiccation, vector control) over time[339].

Several studies have shown that larval competition can affect the evolution of parasite within-host growth strategies[68,79,84,340,341] and the maintenance of an effective immune response[296,342–345]. Here, the effects of the larval competition for food were visible in the motivation as an adult to find a host and to take a blood meal. Both a diet cut in half and a more crowded larval environment reduced the motivation to fly toward a host and take a blood meal over age. However, the blood feeding success (the number of mosquitoes blood feeding out of the number host searching) was not dependent on larval conditions. It may indicate that the larval environment had a bigger impact on the feeding decision rather than feeding abilities. Alternatively, it could also mean that the capacity to find a host and fly toward is affected by both larval and adult nutrition, as previous studies have shown the effect of nutrition on flight capacity[322,325,346]. One study showed that 22 % of the pre-flight lipid were found to be mobilized for flight when mosquitoes took a blood meal. Plus, after a blood meal mosquitoes have a longer flight activity and 61 % of the energy obtained from the nutrient are used for flight

(against 11 % for survival)[346]. Teneral reserves are important for flight and eventually mosquitoes that have less reserve will invest less energy for blood feeding[327] and more for egg laying and survival. This could explain why the proportion of blood meal taken out of the number of host visits (or feeding success) and the estimated number of eggs laid per female were not dependent on the larval environment conditions despite the reduced host-seeking and feeding activity and also why mosquitoes longevity was increased when larvae had a diet cut in half. These mosquitoes may have invested less on flying and more on fecundity and survival. It would have been interesting to follow maternal reserves and egg quality over time.

In addition, mosquitoes reared with a nutritional rich diet and in less crowded environment had a larger body size (i.e quantified as wing length). The opportunity to store high energy reserves and a higher change to become a healthy and bigger adult favorized host seeking and feeding behaviors. Previous research found that body size is strongly linked to a density-dependent larval development and access to food resources[78,331,347] and mosquitoes with larger body size were found to tolerate better immune challenge and competition[78,174,348,349]. For example, a recent study showed a positive relation between vector competence for malaria and food regime[78], and another study showed a positive correlation between melanization response and food regime[350]. In villages near rice irrigation fields where mosquito densities are estimated to be lower than in the laboratory (un-published data), higher access to nutritional resource could increase mosquito vectorial capacity and the number of infectious bites (note that we only quantified the number of bites without infection).

Longevity is also related to the teneral reserves that built up during the larval stage. Therefore, we hypothesized that larger mosquitoes would survive longer (we did not find any study showing how larval reserves influenced longevity in absence of sugar and water availability). Actually, larger mosquitoes in our study took blood meals more often which could have improved their longevity but did not. In several published studies as well as in Chapter 2, blood feeding was found to increase longevity even in the presence of insecticide[165,168,182,351,352] and the effects of age on insecticide susceptibility was reduced with multiple blood meals[353]. Similarly, we expected these large mosquitoes feeding more often to lay more eggs, especially as it has been shown that fecundity increases with body size[336]. But in our study the reproductive performance was not dependent on the rearing conditions at the larval stage. Smaller mosquitoes took less blood meal over time, laid as much eggs as bigger mosquitoes and lived a longer life when the larval diet was cut in half. Our data suggest that reducing the number of blood meals taken in the presence of insecticide to invest in egg laying and longevity may be a better strategy for smaller mosquitoes to avoid the cumulative metabolic costs

associated with digesting blood and insecticide exposure. Indeed, one study suggests that blood feeding comes with an anticipated metabolic cost (that occurs even before blood feeding), due to the up-regulation of gene involved in protein degradation, ovarian maturation and cell cycle activity[354]. In addition, taking more blood meals in that tunnel means being exposed more often to insecticide during one's lifespan, thus suffering from cumulative sublethal exposures to insecticide, which impact longevity[168,238]. Bigger mosquitoes are supposed to have more teneral reserves to detoxify both insecticide[181,355] and reactive oxygen species generated as a product of blood digestion[248]. For them, egg production can be spread out between more blood meals[81]. On a side note, we observed that in the more crowded environment with less available resources mosquitoes laid eggs later in life probably first storing energy thanks to the first bloodmeals or through sugar feeding, while mosquitoes reared in the best larval conditions were immediately laying eggs after their first blood meal and the number of eggs decreased with age.

Our study highlights that a longer life does not necessarily increase the number of gonotrophic cycles and biting rate, and thus the disease transmission potential[320]. Also, in regions with high insecticide coverage, mosquitoes may increase their risk of dying because of multiple insecticide exposures while trying to take blood meals[168,238]. The correlation between variable environmental conditions and the behavioral response of adult mosquitoes exposed to an insecticide-treated net baited host need further attention in *Anopheles*. As malaria transmission risk increases with biting rate and a denser population of vectors, this chapter is a first step towards better understanding the variation in feeding and reproductive performances of highly insecticide resistant mosquitoes collected in the field.

5.7. Conclusion

The aim of this study was to better understand how larval ecological settings, and more particularly density and diet, influence adult mosquito life history traits and feeding behavior in the presence of insecticide-treated nets. *Anopheles* mosquito larvae reared with food deprivation had less chance to become adult and their motivation to fly toward a host and take a blood meal over time (from four days old to their death) was reduced. As mosquito biting rate is one of the most important parameters included in vectorial capacity models, this study highlights the need to further study mosquito behavior and physiology under a range of environmental conditions, to better understand the link between disease transmission and vector control. We argue that to ensure sustainable and efficient vector control tools, a deeper comprehension of the ecological process leading to variation in foraging strategies and feeding success in anophelines is needed.

CHAPTER SIX

The effect of LLIN exposure on odor detection, feeding rate and longevity of pyrethroid-resistant *Anopheles gambiae* s.l.

6.1. Abstract

There is substantial concern that insecticide resistance will reduce the effectiveness Long-Lasting Insecticidal Nets (LLINs) for malaria mosquito control. However, even if resistant mosquitoes survive initial insecticide contact, exposure to a LLIN might still affect other traits such as host seeking, blood feeding and overall longevity. Here, we investigate how these more subtle consequences of LLIN contact might contribute to reducing transmission potential of a highly resistant strain of *Anopheles gambiae* s.l.

Presented here are three experiments on pyrethroid resistant *An. gambiae* s.l. exposed once to a PermaNet® 2.0 or an un-treated net (UTN): (i) mosquitoes were given a first chance to blood feed through a LLIN or an UTN and then again every other day until they died, directly through the netting on top of the rearing cup; (ii) a treatment was added with the possibility to blood feed through the netting only after an initial exposure against a LLIN or an UTN. In addition, 5 more opportunities were given for all mosquitoes to take a blood meal through a UTN, from 1h to 5h after the first occasion to feed; (iii) we measured how a LLIN influences electrophysiological responses to odor stimuli 10 min, 1h, and 24h post-exposure.

We found that: (i) a LLIN exposure affected initial blood meal success (84 % of mosquitoes took a blood meal through an untreated net but only 44 % through the LLIN), reduced the short-term propensity to feed, and reduced overall feeding rates across mosquito lifetime. Mosquitoes exposed to a LLIN took on average only 0.7 blood meals across their lifetime compared with 2.2 for those exposed to an untreated net. Those mosquitoes that were able to take an initial blood meal were more likely to take subsequent blood meals; nonetheless, initial exposure to an LLIN still constrained the average number of blood meals across mosquito lifetime to be below 2. Additional blood meals increased the chance of survival of larger mosquitoes exposed to a LLIN, but it was not the case for smaller mosquitoes exposed to a LLIN and all mosquitoes exposed to an UTN. (ii) on the first day of experimentation, the impact of insecticide, an exposure to a LLIN before the first opportunity to feed, on feeding success was even stronger as only 38 % of the mosquitoes took a blood meal that day against 92 % in the control group. While the exposure timing did not influence the maximum number of blood meals taken individually in the control group, mosquitoes exposed to a LLIN took 1.3 times less blood meals in their life if they were pre-exposed to insecticide before being able to feed compared to those exposed to a LLIN during their first feeding opportunity. (iii) Electrophysiological measures (electroantennograms, EAGs) indicated a short-term reduction in responsiveness of mosquitoes to host odours immediately following LLIN exposure, which could contribute to the transient decline in

feeding propensity observed in the feeding assays. However, other mechanisms appear to operate over the mosquito lifetime.

These results indicate that although highly resistant mosquitoes can survive exposure to an LLIN, there are still effects on initial blood feeding rates, lifetime feeding rates and longevity. Patterns are complicated by mosquito size and blood meal history. Further work is needed to better understand how such sub-lethal effects might impact transmission potential and influence the overall epidemiological impact of insecticide resistance.

6.2. Introduction

Long Lasting Insecticide Treated Nets (LLINs) are a cornerstone of contemporary malaria control[128]. Accordingly, there is substantial concern that increasing insecticide resistance in malaria vector populations will reduce the effectiveness of LLINs and lead to a resurgence of malaria in areas where LLIN use is widespread[224,225]. However, in spite of this threat, current evidence suggest that LLINs remain more effective than untreated nets, regardless of resistance[356–359].

There are several possible reasons why LLINs might continue to contribute to malaria control in areas of insecticide resistance. In the first instance, nets provide a physical barrier that can reduce vector-host contact rate even in the absence of a functional insecticide. Additionally, while LLINs might not cause high levels of immediate mortality of resistant mosquitoes, sub-lethal effects of insecticide exposure might still impact longevity and host seeking behavior[168,229]. Such effects could impact overall transmission potential and hence, the epidemiological consequences of resistance. For example, a recent study showed that the propensity of moderately resistant mosquitoes to search for a host and blood feed was reduced immediately following sub-lethal contact with an LLIN, but that mosquitoes recovered over the subsequent 6 to 24 hours, depending on the mosquito strain[165]. Other recent research suggests that resistant mosquitoes can suffer delayed mortality following LLIN exposure and that this impact on longevity can reduce overall malaria transmission potential[168]. In addition, pyrethroids have excito-repellency properties that could potentially affect the behavior of pyrethroid resistant mosquitoes and result in kinetic disengagement (i.e. reduction in the number of mosquitoes host seeking due to insecticide)[287,360] regardless of mortality.

Understanding how LLINs work in disrupting vector-host interactions and how sub-lethal effects of insecticide affect pyrethroid-resistant mosquitoes' life history is key to fully comprehend the functional significance of insecticide resistance. Here we conducted a series of assays exposing a wildtype strain of highly resistant *An. gambiae* s.l. from Bouaké, Côte d'Ivoire, to either a PermaNet® 2.0 LLIN or an Untreated Net (UTN). (i) In the first assay mosquitoes were placed in cups and exposed to either the LLIN or UTN, as they tried to feed on the arm of a human host (i.e. simulating the situation where a host might be sleeping up against a net). We recorded initial feeding success, daily feeding rates across the remainder of the mosquito lifespan, and longevity. (ii) The second assay followed a similar protocol but with half the mosquitoes exposed to the netting without access to a blood meal (i.e. simulating a host properly protected beneath the net). (iii) In addition, we monitored the short-term effect of exposure by evaluating feeding propensity over the first 6h, as well as the longer-term effects by monitoring daily feeding rate across the lifetime of the mosquitoes. To complement these

assays, we recorded the electrophysiological responses (electroantennograms, EAGs) of mosquitoes to human and plant odor stimuli 10min, 1h, and 24h following exposure to an LLIN.

6.3. Material and methods

6.3.1. Mosquito populations

Experiments were all conducted on field-collected *Anopheles gambiae* s.l. from natural breeding habitats. 60 % of the tested mosquitoes were collected in Yao Koffikro (-5.09000W longitude and 7.68000 N latitude) with mostly S-form *An. gambiae* s.s (un-published data) and 40 % of the tested mosquitoes were collected in M'be (5.209963 W longitude and 7. 970241 N latitude) with 99 % M-form *An. coluzzi*, in central Côte d'Ivoire[239–241]. These suburban villages of Bouaké are dominated by highly pyrethroid and DDT resistant *Anopheles gambiae* s.l. that breed in rice and vegetable fields[165,230,242]. Recent analysis using CDC-bottle assays indicates that these mosquitoes exhibit 1500-2000-fold resistance to deltamethrin relative to fully susceptible counterparts[165]. The field-collected *An. gambiae* larvae were reared using Tetramin™ baby fish food at 27 ± 2 °C and standard density (about 300 larvae) in metallic bowls with about 1 liter of deionized water. Adult mosquitoes were then housed in standard 32.5 cm^3 mosquito cages and maintained on 10 % sugar solution at 27 ± 2 °C, 60 ± 20 % RH and ambient light. All tested mosquitoes were 4-5 days old on their first day of experimentation and kept alive during the experiments in transparent hard-plastic cups (180 ml) in the lab at 27 ± 2 °C, 60 ± 20 % RH and ambient light. Each cup was covered with mosquito-proof netting with access to 10 % sugar solution using cotton wool pads.

6.3.2. Initial exposure to insecticide

Insecticide exposure bioassays were carried out on 4-5 days old and non-blood fed females using either a PermaNet® 2.0 (LLIN) for half of the tested mosquitoes, or an untreated net (UTN) for the other half of the mosquitoes. The unwashed LLIN manufactured by Vestergaard Frandsen SA (DK), was made of polyester, was green in color and was entirely coated with 55 mg/m^2 ± 25 % deltamethrin. An untreated ultra-fine mesh polyester net distributed by Coghlan's was used as the UTN control. A 3-min exposure of a fully susceptible Kisumu strain of *An. gambiae* assured that the LLIN caused 100% mortality within 24h, while the UTN caused essentially none.

In the behavioral assays, mosquitoes were individually exposed in transparent plastic cups covered with either a LLIN or an UTN, with the arm of a human host placed adjacent to the netting to attract

mosquitoes and bring them into contact with the netting. Mosquitoes were then kept two-by-two in cups covered with UTN, with a sugar-soaked cotton ball on top. These holding cups also contained a water saturated cotton ball as an oviposition substrate that was re-moistened every 2 days (although we did not count the number of eggs laid per female in order to minimize daily mosquito handling).

In the electrophysiological assay, mosquitoes were exposed ten-by-ten in plastic WHO cones for 3 min and then kept in mesh-covered plastic cups until further study. Cones were angled at 40° to encourage the contact between the mosquitoes and the net, and a ball of cotton used to close the entry point of the cone.

6.3.3. Human host preparation

Experiments were performed by a single experimenter who was monitored throughout the course of the study for malaria infection to avoid any contamination of the mosquitoes by malaria-infected blood. The experimenter avoided the use of fragrance, repellent products, tobacco, and alcohol for 12 hours before and during testing. The host's arm was washed with unscented soap and rinsed with water the day before a test.

6.3.4. Survival and mosquito size

Daily survival was measured in each experiment. After death of each mosquito the length of one wing was measured from the tip to the distal end of the allula by excluding the fringe, as an indication of mosquito size[53]. The wings were fixed onto glass slides, scanned, and copied to a computer. Wing lengths were measured to the nearest 0.01 mm on the java-based application ImageJ 64 (*http://imagej.nih.gov/ij/*).

6.3.5. Experimental designs

6.3.5.a. Effect of LLIN exposure on short- and long-term feeding rate

Mosquitoes were individually exposed in plastic cups to either a LLIN (98 mosquitoes) or a UTN (75 mosquitoes) as described above, with the host's arm placed in direct contact with the netting so that mosquitos had the capacity to blood feed through the net. The blood feeding status of mosquitoes was assessed, and they were transferred to holding cups. These mosquitoes were then offered blood meals in the holding cups (with no further contact with the LLIN) every subsequent day until death (Figure 1A). Mosquitoes with a visible amount of blood in their abdomen where considered as "fed" each day.

6.3.5.b. Effect of exposure timing on short- and long-term feeding rate

To study both the immediate effect of insecticide pre-exposure on the capacity and the motivation to take a blood meal, and the long-term effect on the subsequent blood feeding rate, mosquitoes were individually exposed in plastic cups to either a LLIN or UTN (approximately 180 mosquitoes each), but with the human arm placed in contact with netting for half of the exposures so that mosquitoes could blood-feed, or the harm held 1 cm above the netting so that mosquitoes could not access the host. All mosquitoes were transferred to holding cups, mosquitoes that could not access the host were given the opportunity to take a blood meal immediately after the exposure, and then all mosquitoes were provided the opportunity to blood feed every hour for the next 5h to look at short-term effects, and then daily thereafter to monitor long-term effects (Figure 1 B&C).

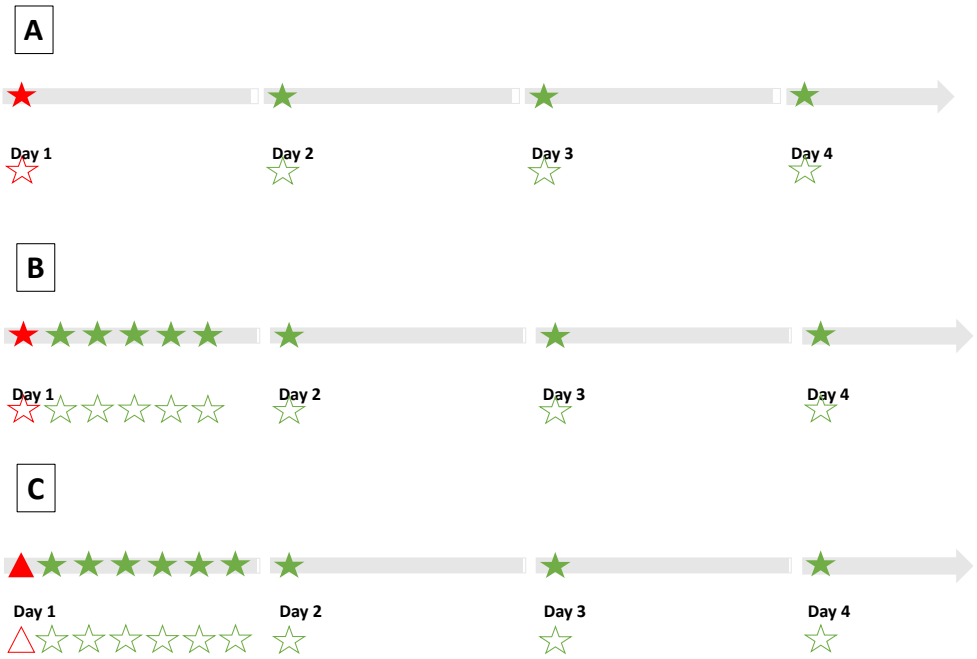

Figure 1: Schematic representation of Long-Lasting Insecticidal Net (LLIN) exposure and blood feeding opportunities of wild-type and insecticide resistant *Anopheles gambiae* s.l. female tested in experiments 1 and 2. In experiment 1, mosquitoes were exposed once to a LLIN while having the possibility to take a blood meal in a plastic cup (treatment A). In experiment 2 mosquitoes were either exposed once to an LLIN while having the possibility to take a blood meal in a plastic cup and then given 5 more occasions (1 per hour post exposure) to take a blood meal but in the absence of insecticide (treatment B), or pre-exposed to an LLIN and then offered a blood meal through an UTN during 6 occasions at hourly intervals (treatment C). After the first day, every mosquito was given the possibility to take a blood meal through an Untreated Net (UTN) on a daily basis until death. Red color = LLIN exposure; green color = UTN exposure; star = opportunity to take a blood meal; triangle = no opportunity to take a blood meal.

6.3.5.c. Effect of a sub-lethal exposure to a LLIN on odor detection

Electroantennograms (EAG) from single antennae of *An. gambiae* mosquitoes (38 in total: 20 from mosquitoes pre-exposed in WHO cones to a LLIN; 18 from mosquitoes pre-exposed to an UTN) were recorded to test the capacity of mosquitoes to detect odors after insecticide exposure. Mosquitoes were tested directly after the exposure (13 mosquitoes: 7 pre-exposed to a LLIN and 6 pre-exposed to an UTN), 1h post exposure (14 mosquitoes: 7 pre-exposed to a LLIN and 7 pre-exposed to an UTN), or 24h post exposure (11 mosquitoes: 6 pre-exposed to a LLIN and 5 pre-exposed to an UTN). Mosquitoes were randomly chosen for a given exposure treatment and EAG time post exposure.

Chemical stimuli: Seven selected essential oils: linalool oxide, sulcatone, 1-octen-3-ol, E-2-hexenal, 2-heptanone, acetophenone and heptanal were diluted in hexane at three different concentrations for each compound; $10E^{-3}$, $10E^{-2}$ and $10E^{-1}$ mg/µl. The 21 dilutions obtained were all tested on each antenna. This panel of volatile compounds is known to elicit antennal receptor responses in *Anopheles gambiae* during feeding behavior[43,361–366]. The chemical stimuli order was randomized for each mosquito. All compounds were obtained from Sigma Aldrich.

Clean 2x1 cm strips of filter papers were dotted with 10 µl of either hexane, as negative control, or one of the 21 dilutions, as a chemical stimulus. The solvent was evaporated for 1 min. Odors captured on filter paper were later injected into the air stream trough a 2 ml glass Pasteur pipette containing the stimulus (paper with the 10 µl of control or chemical stimuli). Compounds were randomized for each concentration and applied in ascending order of concentration:

1. empty > hexane > randomization of the compounds at $10E^{-3}$ mg/µl
2. hexane > randomization of the compounds at $10E^{-2}$ mg/µl
3. hexane > randomization of the compounds at $10E^{-1}$ mg/µl > hexane

All essential oils dilutions were tested on each mosquito.

Electroantennogram: Two micropipettes were filled with an electrically conductive and saline Ringer's solution (7.55 g NaCl, 0.64 g KCl, 0.22 g $CaCl_2$, 1.73 g $MgCl_2$, 0.86 g Na_2HCO3, and 0.61 g Na_3PO_4 l^{-1} water) and connected to a DC amplifier thanks to AgCl-coated silver wires inserted into them. The micropipettes were previously 2 mm glass tubes whose tip was drawn to a point using a microelectrode puller machine. Then the tip was broken off with forceps to enable the connection between the neck and the antenna thanks to an inner diameter roughly of the same size as the tip of the antennae. The indifferent micro-electrode, which was grounded, was softly enclosing the mosquito neck. A recording micro-electrode (~2 mm) was positioned at the tip of an antenna until

electrical contact was established thanks to the Ringer solution. In between treatments, the electrodes were changed to avoid any contamination. Before we started recording, we let the device stabilize for at least 1min and in between stimulations we waited 30 sec.

Through a glass tube, a continuous and humidified airflow was blown at a constant rate (90–100 % RH, 23 ± 2 °C delivered at 1 m.sec-1) 1 cm over the preparation (mosquito and electrodes). At 10 cm from the outlet of the tube, odor stimuli were injected into the air stream (one puff lasting for 1.5 seconds) using a Pasteur pipette containing an odor compound. The mounted mosquito head was viewed through an Olympus Stereo zoom microscope SZX7 which allowed for a magnified (120× with 2x objective) view of the antenna. Flexible entomological tweezers were needed during mosquito manipulation. Using a desktop computer with IDAC4 data acquisition board linked to the EAG apparatus (Syntech Ltd., Hilversum, The Netherlands), successful potential differences between the electrodes were imported, quantified, and analyzed using EAG software, AutoSpike 3 (Syntech). The spikes were discriminated based on amplitudes as well as the shape using the interactive AutoSpike procedures. The variable of response was adjusted to compensate for solvent and/or environmental sensory artefacts by subtracting the mean EAG response of the two nearest negative controls, or hexane stimuli. The EAG amplitude was also corrected to compensate for the reduction of antennal responsiveness during the experiment[367].

6.4. Statistical analyses

All statistical analysis and graphs were performed in R© version 3.6.1 with the Rstudio interface version 1.2.5001.

6.4.1. Effect of LLIN exposure on short- and long-term feeding rate

We first analyzed the direct effect of insecticide on mosquito blood feeding. One Generalized Linear Model (GLM) with a binomial error structure was performed to evaluate how blood feeding success on the first day of experimentation was influenced by the treatment (LLIN or UTN) with the effects of the replicate and mosquito wing size as covariates.

We then tested the long-term effect of insecticide on mosquito blood feeding. Using a gaussian GLM we analyzed the total number of bloodmeals taken per mosquito in their life regarding the treatment, the knock down status, the age at death, the feeding status after the first day of experimentation, the number of legs (mosquitoes can lose legs through insecticide exposure and generally through

handling, which could potentially impact feeding success[368]) and their interaction. The wing size was a covariate.

Regarding leg loss up until death, on the first day of experimentation we assumed that all mosquitoes had 6 legs and after that day we considered that the number of legs detached at death could have influenced mosquito behavior and survival, therefore we counted it as a fixed value upon death and a potential indicator of mosquito condition (we don't know the actual number of legs over time but just the final value).

Finally, we tested the sub-lethal effect of insecticide on mosquito survival. A weighted cox model was performed considering the effects on survival of treatment (type of bednet), the number of blood meals taken individually by mosquitoes, the proportion of blood fed mosquitoes on the first day of experimentation (out of the two mosquitoes in the cup), the number of legs, and the interaction between those factors.

In a further analysis we divided the data in two in order to obtain a category with the smaller-sized mosquitoes (wing lengths of 0.26 to 0.32 cm) and a second category with larger mosquitoes (0.32 to 0.37 cm) to analyze the variations in survival with respect to size. The assumption of proportional hazards assumption was tested with the cox.zph function from the survival library.

6.4.2. Effect of exposure timing on short- and long-term feeding rate

We first analyzed the direct effect of insecticide on mosquito blood feeding. A GLM with a binomial error structure was performed. The variable of response was blood feeding success on the first day of experimentation regarding the treatment (type of net), and the type of exposure (arm accessible or arm not accessible upon first exposure) and their interaction. The effects of the replicate and mosquito wing size were tested as covariates.

A GLM with a gaussian error structure was used to evaluate blood feeding success throughout the hours/opportunities on the first day of experimentation, consider again the treatment (type of net) and the type of exposure (arm accessible or arm not accessible upon first exposure) in interaction. The replicates and mosquito wing size were also added as covariates.

We wanted to see if the first insecticide exposure would influence the motivation and/or capacity to take a blood meal in the following days. No departure from normality was observed. A Linear Mixed-

Effects Model was performed using the lmer function in the lme4 package in R©. We analyzed the maximum number of blood meals taken individually by mosquitoes and tested the effects of the treatment, the type of exposure, leg loss upon death and wing size. The replicate was considered as random effect. Note that mosquitoes were placed two-by-two in cups after the first day of experimentation due to the lack of material and space in the lab. For that reason, every other day we counted the total number of blood meals in one cup and divided that number by 2 until the first mosquito in the cup died. Regarding leg loss up until death, on the first day of experimentation we assumed that all mosquitoes had 6 legs and after that day we considered that the number of legs detached at death could have influenced mosquito behavior and survival, therefore we counted it as a fixed value upon death and a potential indicator of mosquito condition (we don't know the actual number of legs over time but just the final value).

Finally, the sub-lethal effect of insecticide on mosquito survival was analyzed. Here, the Cox model violated the coz.zph (< 0.05), therefore a gaussian GLM was performed to analyze the effect of treatment, the number of blood meals taken individually by mosquitoes, the proportion of blood fed mosquitoes on the first day of experimentation, the number of legs, and the interaction between those factors on survival. The mean wings size was considered as co-variate and the replicates as categorical variable.

6.4.3. Effect of a sublethal exposure to a LLIN on odor detection

The corrected EAG response in millivolts (mV) was analyzed using a gaussian GLM regarding the treatment during the pre-exposure in WHO cones (LLIN or UTN), the compounds used, the different concentrations, the time post insecticide exposure and all the interactions. The individual mosquitoes were first considered as co-variate, but as we tested multiple compounds with different concentrations per mosquitoes, we also performed gaussian GLM analysis for each compound separately. Contrasts among treatments were assessed using the *multcomp* package and the function *glht* in the software R with a Tukey's honestly significant difference test (Tukey HSD) multiple-comparison test. It allowed simultaneous tests for General Linear Hypothesis and comparison differences between groups at p = 0.05.

6.5. Results

6.5.1. Effect of LLIN exposure on short- and long-term feeding rate

The LLIN significantly reduced the capacity of mosquitoes to blood feed (χ^2 = 28.6, df = 1, p < 0.01), with 84.0 (95 % Confidence Interval: 73.7 to 91.4) % successfully feeding through the UTN and only 43.9 (95 % CI: 33.9 to 54.3) % taking a blood meal through the LLIN (Figure 2.1).

The initial single exposure to an LLIN also impacted the number of subsequent blood meals (F = 216.32, df = 1, p < 0.001). Mosquitoes exposed to an UTN took on average 3 times more blood meals in their life than those exposed to a LLIN [2.2 ± 0.10 blood meals per mosquito compared with (mean ± standard error) 0.7 ± 0.06, respectively] (Figure 2.2).

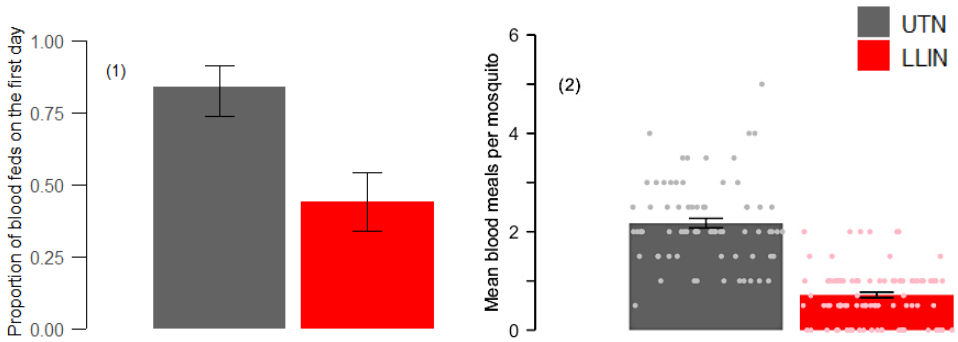

Figure 2: Blood feeding rate of insecticide resistant *Anopheles gambiae* s.l. measured in cups in a lab setting following exposure to either a Long-Lasting Insecticidal Net (LLIN) or an Untreated Net (UTN) in Experiment 1 (treatment A). (1) Proportion of mosquitoes taking a blood meal during the initial period on day 1 when mosquitoes were given the possibility to feed on the arm of a human host for 5 minutes through either a LLIN (grey bar) or an UTN (red bar). (2) Mean number of blood meals taken across the lifetime of mosquitoes following initial short-term exposure to either an LLIN (red bar and pink dots representing individuals) or UTN (red bar and light grey dots representing individuals). Mosquitoes were given the opportunity to feed once every day for 5 minutes through untreated netting directly from their rearing cups. In (1) the Confidence Interval is shown and in (2) the standard error is shown.

Independent of initial exposure treatment, mosquitoes that fed on the first day were more likely to feed on subsequent days than those that did not feed on day one (F = 14.70, df = 1, p < 0.001). However, the main effect of the LLIN remains; even those mosquitoes that took an initial blood meal still took half the average number of lifetime feeds compared to mosquitoes exposed to the UTN alone (Figure 3) (F = 13.13, df = 1, p < 0.001).

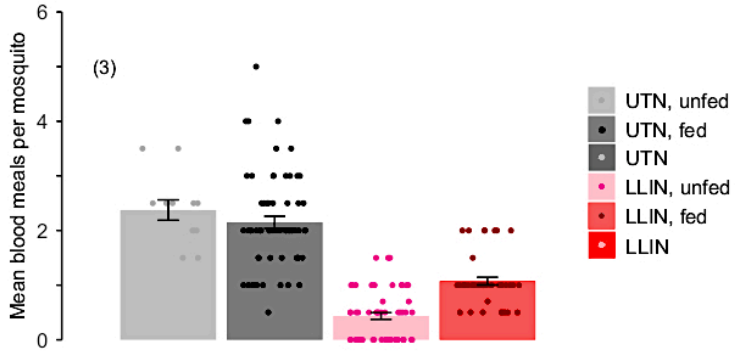

Figure 3: Blood feeding rate of insecticide resistant *Anopheles gambiae* s.l. measured in cups in a lab setting following exposure to either a Long-Lasting Insecticidal Net (LLIN) or an Untreated Net (UTN) in Experiment 1 (treatment A). Average number of bloodmeals per mosquito in Experiment 1 depending on the feeding status on the first day of experimentation. The light grey bar and light grey dots represent the unfed mosquitoes exposed to an UTN on day 1, the grey bar and dark grey dots represent fed mosquitoes exposed to an UTN on day 1, the pink bar and pink dots represent unfed mosquitoes exposed to a LLIN on day 1, the red bar and dark red dots represent fed mosquitoes exposed to a LLIN on day 1. ± standard error bars are shown.

Exposure to an LLIN reduced mean mosquito lifespan by 3.6 days [LLIN 16.6 ± 0.76 days; UTN 25.1 ± 1.07 days; $\chi^2 = 34.9$, df = 1] ($p < 0.05$; Figure 4). However, this overall pattern was nuanced by blood feeding history and size. For example, mosquitoes that took a bloodmeal on day 1 had an overall increased longevity of 1.4 days [fed, 21.6 ± 0.92 days; unfed, 18.2 ± 1.06 days] ($\chi^2 = 11.60$, df = 3, $p = 0.01$) and multiple feeding increased this further, with one additional blood meal per mosquito adding 4.0 days to the predicted average lifespan [$y_A = 0.3975 + 0.04708x_A$, $R_A^2 = 0.189$, $F_{1,171} = 39.93$}($p < 0.001$; $\chi_A^2 = 17.78$, df = 1, $p = 0.04$).

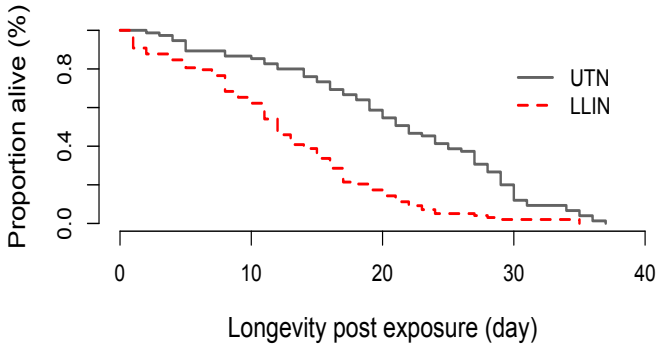

Figure 4: Survival curves of insecticide resistant *Anopheles gambiae* s.l. following initial short-term exposure to either a Long-Lasting Insecticidal Net (LLIN) or an untreated net (UTN) in Experiment 1 (treatment A). Day 1: mosquitoes were either exposed to a UTN (grey and solid line) or a LLIN (red and dotted line). Day 2-mosquito death: one opportunity per day to take a blood meal through the netting on top of the rearing plastic cup (untreated netting).

Additionally, bigger mosquitoes had a longer lifespan if they blood fed on the first day of experimentation [fed, 22.4 ± 1.23 days; unfed, 17.2 ± 1.53 days], but this was not the case for smaller mosquitoes [fed, 20.4 ± 1.53 days; unfed 18.8 ± 1.61 days] (χ^2 = 5.06, df = 1, p = 0.024). When comparing the unfed mosquitoes on day 1, larger mosquitoes exposed to a LLIN had the shortest mean lifespan but no significant difference in survival was found between exposure treatments for the smaller mosquitoes [LLIN, larger-sized: 14.96 ± 1.32 days; LLIN, smaller-sized: 16.96 ± 1.47 days, UTN: 28.5 ± 2.39 days] (χ^2 = 4.22, df = 1, p = 0.04).

In comparison to smaller mosquitoes, bigger mosquitoes did gain in average 1.6 times more days of longevity per blood meal [$y_{BIG} = 14.21 + 4.717x_{BIG}$, $R_{BIG}^2 = 0.27$, $F_{1,78} = 32.52$, $p < 0.001$; $Y_{SMALL} = 15.83 + 2.857x_{SMALL}$, $R_{SMALL}^2 = 0.08$, $F_{1,78} = 6.06$, p = 0.01 respectively] (χ^2 = 27.03, df = 8, p < 0.01). While this gain did benefit larger mosquitoes exposed to a LLIN [with an increase of 4.56 days of longevity per blood meal; $y_{B.LLIN} = 13.22 + 4.556x_{B.LLIN}$; $R_{B.LLIN}^2 = 0.14$, $F_{1,52} = 8.27$, p = 0.006] (χ^2 = 7.49, df = 2, p = 0.02), additional blood meals did not increase the chance of survival of neither the smaller mosquitoes exposed to a LLIN [$y_S = 15.83 + 2.857x_S$, $R_S^2 = 0.08$, $F_{1,69} = 6.06$, p = 0.02] nor the chance of survival of all mosquitoes exposed to an UTN on day 1 [$y_{L,UTN} = 23.94 + 1.166x_{L,UTN}$, $R_{L,UTN}^2 = 0.01$, $F_{1,33} = 0.42$ p = 0.52].

We also found a significant effect of the interaction of the maximum number of blood meals taken by individual mosquitoes, the number of legs and the wing size, on longevity (χ^2 = 24.34, df = 5, p < 0.01) and that interaction was also dependent on the exposure treatments (χ^2 = 23.10, df = 10, p = 0.01).

6.5.2. Effect of exposure timing on short- and long-term feeding rate

The main effects in this experiment were consistent with those of the first experiment. Across all treatments, by the end of day one, 51.9 ± 3.64 (95 % CI: 44.5 to 59.2) % of mosquitoes had taken a blood meal through the LLIN compared with 86.6 ± 2.55 (95 % CI: 80.7 to 91.2) % through an UTN (χ^2 = 45.48, df = 1, p < 0.001). Between the different exposure methods, an average of 64.65 ± 4.85 (95 % CI: 54.4 to 74.0) % of mosquitoes exposed directly to the LLIN during feeding were able to blood feed, compared with 80.0 ± 4.50 (CI: 69.6 to 88.1) % in the control group. Where mosquitoes were pre-exposed to netting before being able to access the host, 37.8 ± 5.14 (CI: 27.8 to 48.6) % of mosquitoes exposed to the LLIN were able to feed compared with 91.9 ± 2.75 (CI: 84.7 to 96.4) % in the control group (χ^2 = 17.14, df = 1, p < 0.001).

The cumulative feeding success over the 5h following initial exposure (Figure 5.1) was always greater for the control groups ($F = 30.92$, $df = 1$, $p < 0.001$). All treatments show a saturating relationship with most feeding occurring over the first one or two feeding opportunities and very little additional feeding by the fourth opportunity (Figure 5.1 and 5.2). While mosquitoes pre-exposed to an UTN used the further hourly opportunities to feed after the initial exposure, mosquitoes pre-exposed to a LLIN were less motivated to feed and ended up with an average of 1.7 times less blood meals than those having the opportunity to feed during the initial exposure to a LLIN ($F = 11.95$, $df = 1$, $p < 0.001$).

The patterns of long-term feeding are shown in Figures 5.3 and 5.4. Mosquitoes exposed once to an LLIN (either exposure method) took an average of 1.3 ± 0.08 blood meals over their lifetime, whereas those exposed to a UTN took 2.5 ± 0.10 blood meals ($F_{1,306} = 7.15$, $df = 1$, $p = 0.007$). Separating by exposure method, mosquitoes that were directly exposed to the LLIN at the first feeding attempt took on average 1.5 ± 0.10 blood meals in their lifetime, compared with 1.2 ± 0.13 in those that were pre-exposed to the LLIN ($F_{1,306} = 3.16$, $df = 1$, $p = 0.07$). The paired control treatments were 2.5 ± 0.14, and 2.5 ± 0.15, respectively. As in the first experiment, those mosquitoes that fed successfully on day 1 were more likely to take blood meals on subsequent days (mosquitoes that took a blood meal on the first day of experimentation took 2.35 times more blood meals during their life than mosquitoes that did not feed on the first day ($F_{1,306} = 33.70$, $df = 1$, $p < 0.001$). Mosquitoes that lived longer were also likely to take more blood meals [$y_{B+C} = 0.03694 + 0.07477x_{B+C}$, $R_{B+C}^2 = 0.285$, $F_{1,364} = 145.01$, $p < 0.001$] ($F_{1,161} = 65.75$, $df = 1$, $p < 0.001$). We also found an interaction between the effect of mosquito longevity and the exposure method on the number of blood meals taken in one's life ($F_{1,306} = 4.11$, $df = 1$, $p = 0.04$).

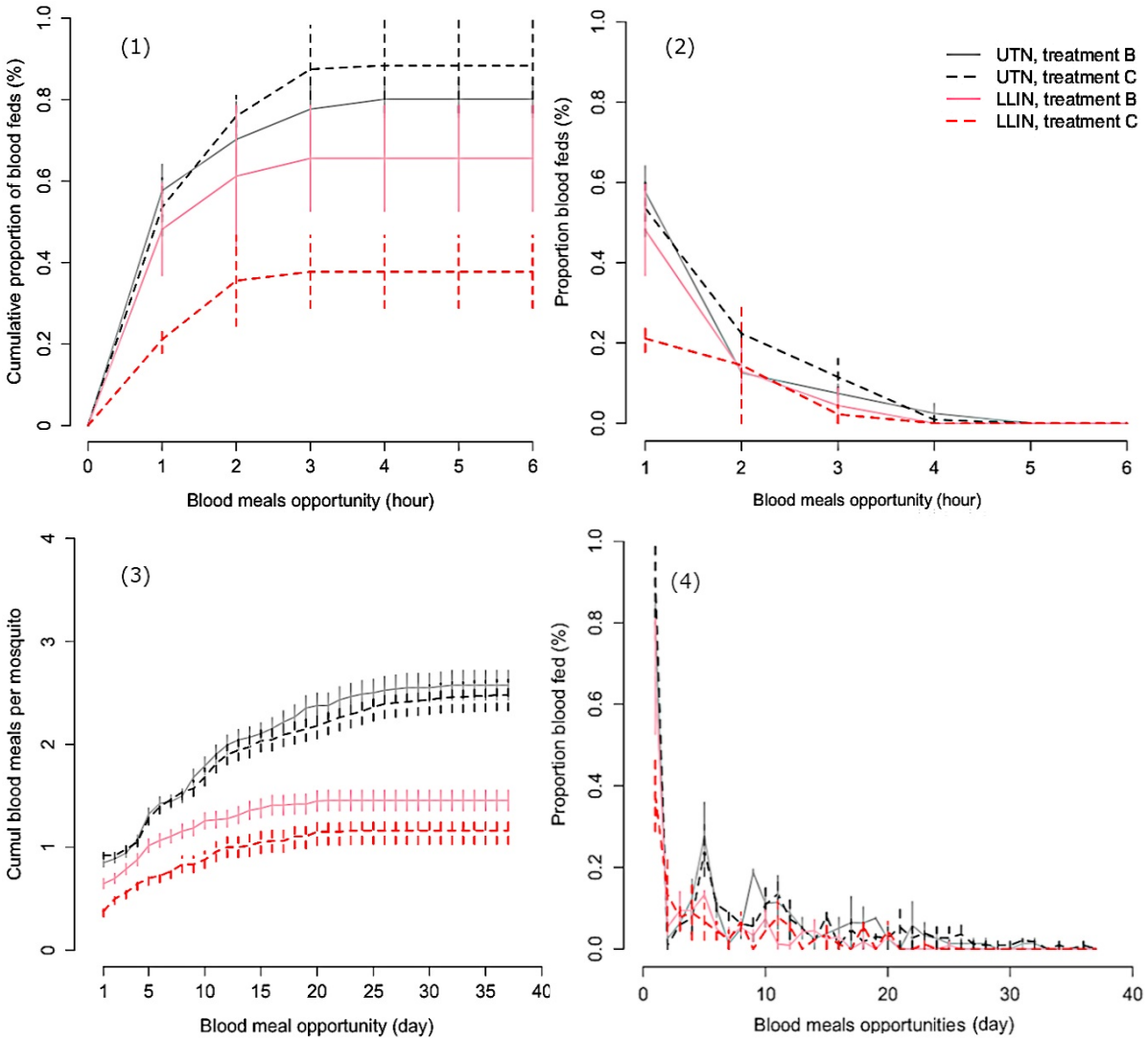

Figure 5: Over the first day of experimentation: (1) Cumulative proportion and (2) Proportion of blood fed insecticide resistant *Anopheles gambiae* s.l. tested in Experiment 2 (treatments B&C) when given 6 hourly opportunities to feed. Over the entire lifespan: (3) Estimation of the cumulative proportion of bloodmeals taken per female and (4) Proportion of blood feds per day when given one daily opportunity (from day 2 until death + proportion of blood fed at the end of day 1) to take a blood meal. Mosquitoes represented with a solid line and respectively in pink and grey colors were on day 1 either exposed once to an Long-Lasting Insecticidal Net (LLIN) or an untreated net (UTN) while having the possibility to take a blood meal in a plastic cup and then given 5 more occasions (1 per hour post initial exposure) to take a blood meal but in the absence of insecticide (treatment B). Mosquitoes represented with a dotted line and respectively in red and black were on day 1 pre-exposed to an LLIN or an UTN and then offered a blood meal through an UTN during 6 more occasions at hourly intervals (treatment C).

LLIN exposure reduced mosquito longevity by an average of 4.1 days [UTN, 24.0 ± 0.82 days; LLIN, 16.6 ± 0.52 days; F = 85.11, df = 1, p < 0.001] (Figure 6). There was no difference in survival between mosquitoes directly exposed to the LIIN during feeding and those pre-exposed (p > 0.05). Mosquitoes that took blood meals lived longer (F = 85.86, df=1, p < 0.001), and theoretically, having a wing length 0.05 cm longer than others gives a mosquito more chance to live 3.7 days longer [y = -1.52 + 73.86x, R^2 = 0.024, $F_{1,340}$ = 8.39, p = 0.004] (F = 6.42, df = 1, p = 0.012). For one replicate, the longevity was 5.7 days shorter than the longevity in the other replicate [17. ± 0.70 days and 23.3 ± 0.73 days, respectively] (F = 35.84, df = 1, p < 0.001).

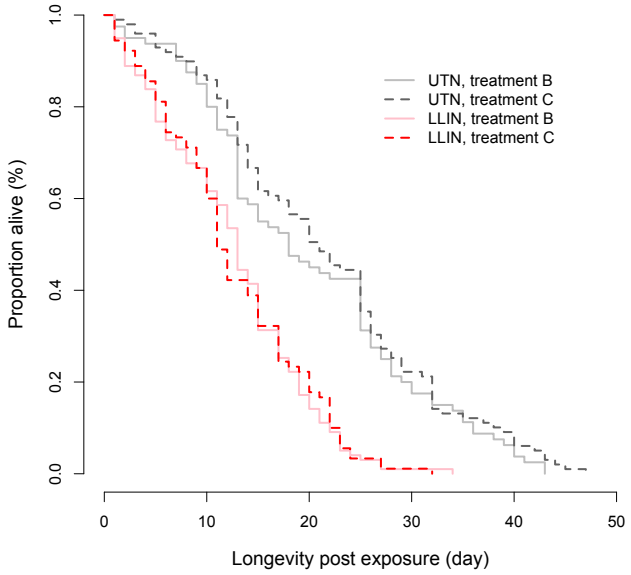

Figure 6: Survival curves for insecticide resistant *Anopheles gambiae* in Experiment 2 (treatments B&C). On the first day of experimentation, mosquitoes were either exposed once to an insecticide-treated net (LLIN; red colors) or an untreated net (UTN; grey colors) while having the first opportunity to take a blood meal (treatment B; solid line) or they were pre-exposed once to an LLIN or an UTN before having the first chance to take a blood meal through untreated netting on top of the rearing cup (treatment C; dotted line). On day 1, each female had a maximum of six opportunities to take a blood meal, once through an LLIN and five others time through the cup netting (treatment B) or six times through netting after an exposure to LLIN (treatment C). From day 2 until death, each mosquito had one opportunity to take a blood meal through the cup netting.

6.5.3. Effect of LLIN exposure on odor detection

In general, the EAG responses of mosquitoes increased in amplitude as the concentration of the odorants increased (F = 54.82, df = 1, p < 0.001) (Figure 7, Table 1). Regardless of concentration, there was a consistent temporal pattern in the mean EAGs following exposure to the LLIN (F = 5.51, df = 1, p = 0.02) (Figure 7). EAGs were significantly depressed in the LLIN-exposed mosquitoes compared with controls 10 minutes after exposure (F = 24.52, df = 1, p < 0.001; Tukey post-hoc test: p < 0.001) This effect appeared to dissipate by 1 hour resulting in no significant difference in EAGs between

treated (LLIN) and controls (UTN) (Tukey post-hoc test: p = 0.99). At 24h after exposure, the LLIN-exposed mosquitoes appeared to show enhanced responsiveness to the odors, with elevated EAGs relative to control mosquitoes exposed to the UTN (Tukey post-hoc test: p = 0.07). This effect was clear with the compounds at the highest concentration (F = 12.82, df = 1, p < 0.001; Tukey post-hoc test: p < 0.001) There was no significant temporal variation in the EAG responses of control mosquitoes across the monitoring period (10 min-1h Tukey post-hoc test: p = 0.99; 10 min-1 day Tukey post-hoc test: p = 0.29).

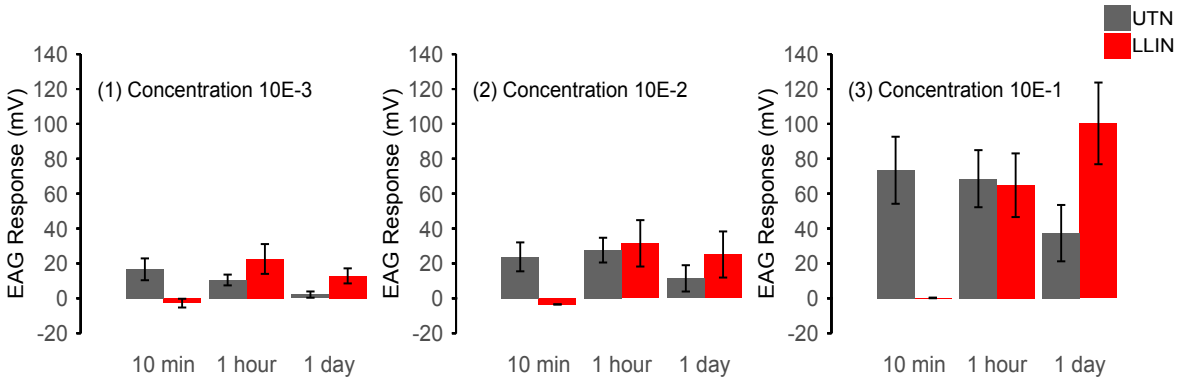

Figure 7: Corrected mean of electrophysiological responses (EAG) post-exposure to a PermaNet 2.0 (LLIN) or an untreated net (UTN) in World Organization Health 3-min cones test, 10 min, 1 hour and 1 day later, for seven selected essential oils (linalool oxide, hept-5-en-2-one (sulcatone), 1-octen-3-ol (octenol), E-2-hexenal, 2-heptanone, acetophenone and heptanal) diluted in hexane at concentrations (1) 10E-3, (2) 10E-2, and (3) 10E-1 mg/µl. The EAG responses are expressed in mV. Red = LLIN pre-exposure, grey = UTN pre-exposure. ± standard error bars are shown.

The pattern of the EAG responses averaged across all the odor sources was generally mirrored in the responses to the individual odorants. This was particularly so for compounds that elicited the strongest EAG responses, such as 1-octen-3-ol and the linalool oxide (F = 6.61, df = 6, p < 0.01). Mosquitoes tested 10 minutes after LLIN exposure showed less response to these odors compared with mosquitoes tested 1h and 24h after exposure (F = 4.40, df = 1, p = 0.04; F = 4.40, df = 1, p = 0.05, respectively). For the compounds that elicited the smallest EAG responses (i.e. heptanal, 2-heptanone and E-2-hexenal) the temporal variation in response was not significant but followed a similar trend. Besides, these compounds did not elicit larger EAG amplitudes with increasing concentrations like the best detected compounds (F = 2.57, df = 6, p = 0.02).

	Treatment	UTN			LLIN		
Corrected mean EAG response in mV (SE) for each compound	**EAG time**	**10 min**	**1 hour**	**1 day**	**10 min**	**1 hour**	**1 day**
	1-octen-3-ol	37.50	45.36	25.16	0.19	50.60	71.56
		(24.51)	(19.45)	(18.78)	(0.10)	(25.80)	(29.78)
	Linalool oxide	70.48	61.80	37.22	-0.53	44.46	85.35
		(35.53)	(28.48)	(30.20)	(0.72)	(22.29)	(37.01
	Acetophenone	67.19	67.54	35.69	0.30	76.93	93.60
		(25.85)	(22.41)	(22.46)	(0.04)	(37.02)	(30.43)
	Sulcatone	40.12	40.61	13.94	0.12	39.68	50.47
		(19.00)	(16.02)	(10.62)	(0.04)	(19.05)	(15.70)
	2-heptanone	22.02	13.09	4.604	-6.98	22.47	7.09
		(9.22)	(5.20)	(4.54)	(7.05)	(12.51)	(14.60)
	E-2-hexenal	22.60	15.47	4.09	-5.89	30.05	10.42
		(10.71)	(7.71)	(3.25)	(6.01)	(20.25)	(22.26)
	Heptanal	19.63	14.19	1.62	-1.26	23.2	9.76
		(9.60)	(6.59)	(6.45)	(1.29)	(12.80)	(15.47)

Table 1: Corrected electrophysiology (EAG) response in mV with Standard Error (SE) for each compound tested. Mosquitoes were randomly chosen for one pre-exposure treatment (deltamethrin treated net or untreated net) and EAG time after the exposure (10 min, 1h or 1-day post exposure). We highlighted in red the EAG responses that are significantly affected directly after the pre-exposure to insecticide.

6.6. Discussion

Our lab-based behavioural assays show that extremely deltamethrin-resistant mosquitoes[165] are not readily killed by exposure to an LLIN. Such low immediate mortality clearly raises concerns over the efficacy of LLINs, especially considering that the fully susceptible mosquito strain we tested suffered 100 % mortality. However, consistent with previous studies[168] the resistant mosquitoes appear to suffer delayed mortality that leads to an overall shortening of lifespan. In principle such effects could lead to reductions in transmission potential since shorter-lived mosquitoes likely have fewer opportunities to feed once the malaria parasites have completed the extrinsic incubation period[165,168].

Potentially more important are the impacts of LLIN exposure on blood feeding. Mosquitoes that attempted to feed directly through the LLIN showed an approximate 30 % reduction in feeding success compared with those that fed through a UTN. Pre-exposure to a LLIN followed by a blood feeding

opportunity via a UTN caused a way more pronounced 80 % reduction. In addition to this reduction in initial feeding success, mosquitoes exposed to an LLIN never fully recovered and continued to exhibit reduced feeding rate. Importantly, LLIN-exposed mosquitoes took on average 0.7-1.5 blood meals across their lifespan, and only 30 % of the mosquitoes completed two or more blood meals. The mean number of blood meals for the UTN-exposed mosquitoes was 2.2-2.5, with 70 % completing two meals or more. Given that a mosquito must feed at least twice to transmit malaria (once to acquire the parasite and once to pass it on once the extrinsic incubation period has been completed), this sublethal effect of LLIN exposure could be of considerable significance for malaria control. In *Aedes* mosquitoes, it has been shown that successive blood meals enhance Zika virus dissemination from the midgut by significantly reducing the extrinsic incubation period (EIP) of the parasite[305]. Increasing the probability of mortality due to an exposure to insecticide could drive evolutionary change in EIP and favor the spread of parasite clones with shorter development period[79].

The electrophysiology studies might provide partial explanation for the effects on feeding rate during, or immediately after, LLIN contact. The EAG measures indicated a short-term reduction in sensitivity to host odor cues after exposure. An earlier study by Glunt et al.[165] showed that resistant mosquitoes exhibit a transient depression in blood seeking and feeding following LLIN exposure, with a gradual recovery over 6-24h depending on mosquito strain. It is possible that the post-exposure decline in the EAG olfactory responses contribute to this transient impact (note in the current study the mosquitoes in the EAG assays were exposed to the LLIN using a WHO cone, which is different to the exposure method used in our behavioral assays so it is difficult to map the EAG responses onto the behavioral responses directly). However, it appears that other mechanisms are likely responsible for the longer-term sublethal effects since the EAG responses recover within 1h. Indeed, at 24h the LLIN-exposed mosquitoes exhibited elevated EAG responses yet the impacts on feeding are long term.

Pyrethroids interfere with sodium ion channels, causing not only death in susceptible insects but also temporary paralysis[369]. The mechanisms behind the chronic (lifetime) effects in our highly resistant mosquitoes are less clear. These mosquitoes have a combination of both target site and metabolic resistance (Un-published data, Achille Oumboucke) so the effects we see could be a consequence of costs of detoxification[370,371]. That the impact of LLIN exposure was influenced by mosquito size and blood feeding history (larger mosquitoes were better at taking an initial blood meal and tended to survive longer, especially if blood-fed), suggests that mosquito condition and energetics might be important.

Additionally, contact irritancy of deltamethrin might directly affect feeding success[287] leading to contact avoidance[247,372]. The longer-term effects on feeding might then conceivably result from 'learned' behavior whereby insects can avoid certain odors if they are associated with negative fitness effects[373–375]. Insecticide exposure might also lead to neurologic dysfunction in the integration centers for odors and/or motor disorder[376–378], or a general damaging malaise not linked to olfaction[379,380].

6.7. Conclusion

Lab assays revealed that LLINs can also impact the short- and long-term feeding rate of highly resistant mosquitoes from the field, and that impact is more important when mosquitoes are pre-exposed to insecticide before having an opportunity to take a bloodmeal. The highly resistant mosquitoes suffered negligible immediate mortality from exposure to an LLIN. However, the sub-lethal exposure reduced overall longevity, although the magnitude of this effect varied depending on whether the mosquitoes had successfully blood-fed and was further influenced by mosquito size. This study provides novel insights to potentially help explain the continued effectiveness of LLINs despite insecticide resistance[227]. The assays were conducted in controlled lab settings so how these effects play out in natural field settings is unclear. Further work is needed to better understand how such sub-lethal effects might impact transmission potential and influence the overall epidemiological impact of insecticide resistance in different mosquito strains and species across diverse field settings.

CHAPTER SEVEN

Semi-field evaluation of the effects of LLINs on the host-searching behavior of highly insecticide-resistant *Anopheles gambiae* s.l.

7.1. Abstract

Pyrethroids remain the major insecticide class used for malaria vector control despite widespread pyrethroid resistance in many malaria endemic regions. While Long-Lasting Insecticidal Nets (LLINs) do not directly kill insecticide-resistant mosquitoes, they could still influence host seeking, host choice and blood feeding due to the repellency and irritancy properties of pyrethroids. In the present study, we investigate the effect of a pre-exposure to a LLIN on behavioral avoidance and mosquito feeding behavior. After a pre-exposure in WHO cone tests, mosquitoes were given the choice in an enclosure in Ivory Coast between a hut with a deltamethrin-treated net protecting a sleeper and a hut with an untreated net protecting another sleeper.

Overall, an average of 33 % of insecticide-resistant mosquitoes entered huts in our enclosure each night and a pre-exposure to insecticide increased this host-seeking rate by 3 %. In addition, 4 % more mosquitoes entered in the hut with the LLIN compared to the average. In our enclosure, LLINs did not offer personal protection as the overall proportion of blood fed mosquitoes was similar in both huts, suggesting only a minimal irritancy effect induced by insecticide. 97 % of fed mosquitoes were re-captured in the net and none of them were found in the veranda. Overall, 10 % more mosquitoes were recaptured in the veranda, in the hut with the LLIN, compared to the hut with the UTN. Mosquitoes lived around 12.5 days and a blood meal increased that number by about 2.5 days.

These results show that the effectiveness of LLINs is substantially compromised by the level of insecticide resistance in Côte d'Ivoire. One pre-exposure to insecticide does not stop hungry host-seeking mosquitoes to take a blood meal through a damaged LLIN and resistant mosquitoes might detect LLINs from a distance as more of them chose to enter the hut with the LLIN. However, LLIN still retain excito-repellency and mild irritancy properties as more unfed mosquitoes were recaptured in the veranda in the LLIN hut compared to the UTN hut. To better understand the discrepancy between the overall feeding rates in our previous laboratory studies and in this study, there is a need for more comparative studies across Africa that evaluate the direct and sublethal effect of insecticide on foraging strategies decisions (i.e. host seeking persistence, energy-state-based choice, host choice), feeding compliancy and behavioral avoidance in natural settings.

7.2. Introduction

Control of malaria vectors relies extensively on the use of long-lasting insecticide-treated nets (LLINs)[1]. LLINs work first and foremost by creating a physical barrier to reduce host-mosquito contact. This physical barrier is then augmented by a lethal insecticide that potentially kills mosquitoes as they contact the net and/or disrupts their host searching ability making them less able to sit on the net and feed on a host that is inadvertently touching part of the net, or to find holes in the net where they can gain access to the host. The vast majority of LLINs used to date have been treated with pyrethroid insecticides and there are now major concerns over the wide spread of pyrethroid resistance[2–5]. Numerous studies show that pyrethroid resistant mosquitoes suffer less mortality during standard exposures to LLINs compared with susceptible mosquitoes[6,7]. Reduced mortality is clearly worrying since it potentially increases the probability for resistant mosquitoes to transmit malaria across their lifetime[8], and also reduces the community level protection resulting from high levels of bed net coverage[9]. What is less clear is whether and how resistance alters the extent to which LLINs impact mosquito host searching and blood feeding behaviors. In principle, LLINs could still be highly effective against resistant mosquitoes even in the absence of mortality, if the sub-lethal insecticide exposure reduces blooding feeding rates[10,11].

The data on the spatial and contact repellency of LLINs against resistant mosquitoes is rather mixed. Some lab-based studies suggest that mosquitoes with knockdown resistance might actually increase orientation towards hosts (or at least host-related odors) in the presence of an LLIN compared with an untreated control net[12,13]. Other studies, however, suggest sublethal exposure of resistant mosquitoes to pyrethroids can decrease host seeking efficiency and the capacity to navigate through holes in a damaged net in order to take a blood meal[14–16]. In addition, the diminished sensitivity to contact irritancy and repellency due to resistance can lead to a prolonged contact with an LLIN resulting in higher insecticide exposure that ultimately reduces blood meal success[17]. How these potentially positive and negative effects play out under more realistic field conditions also remains unclear. Some semi-field studies report no evidence for spatial repellency of LLINs[18], others suggest the potential for LLINs to provide some attraction for resistant mosquitoes[19], while others show the opposite effect with reduced entry of mosquitoes into experimental huts with LLINs relative to huts with untreated nets[20].

Understanding how LLINs work (or do not work) in blocking vector-host interactions with pyrethroid-resistant mosquitoes is key to fully understanding the functional significance of insecticide resistance. Here, we present the results of two semi-field experiments that aimed to examine whether LLINs

impacted host searching, blood feeding and survival of highly pyrethroid-resistant field-derived mosquitoes, and whether previous exposure to an LLIN affected these responses.

7.3. Material and method

7.3.1. Overall study design

The study aimed to examine whether LLINs affected the capacity of resistant mosquitoes to host search and blood feed under semi-realistic field conditions. The approach used two experimental huts housed within a large enclosure. Mosquitoes were released into the enclosure in the evening and the number that had successfully recruited into the huts and taken a blood meal from human hosts sleeping within the huts was recorded the following morning. We ran two experiments with this same basic design.

In the first experiment we used new nets that were intact, with one hut housing a LLIN and the neighbouring hut housing an untreated net (UTN), essentially providing a choice test. We also examined whether pre-exposure to an LLIN affected searching and blood-feeding by comparing nights of release-recaptures where mosquitoes were exposed to an LLIN in the lab prior to introduction in the enclosure, against nights where mosquitoes were pre-exposed to a UTN. Thus, we had four treatment combinations in total: mosquitoes pre-exposed to an LLIN before release and subsequently recaptured from a hut with an LLIN or the paired hut with a UTN; and mosquitoes pre-exposed to an UTN before release and subsequently recaptured from a hut with an LLIN or the hut with a UTN. This experiment was implemented over a total of 17 nights giving respectively 9 and 8 nights per pre-exposure treatment. Nets and sleepers were rotated between huts each night.

In the second experiment we repeated this same basic design but damaged the nets to allow mosquitoes a direct access to the sleepers through holes in the net. This resulted in a further four treatment combinations: mosquitoes pre-exposed to an LLIN before release and subsequently recaptured from within a hut with a damaged LLIN, or the paired hut with a damaged UTN; and mosquitoes pre-exposed to an UTN before release and subsequently recaptured from the hut with a damaged LLIN or the hut with a damaged UTN. This experiment was implemented over a total of 23 nights giving respectively 11 and 12 nights per pre-exposure treatment. Nets and sleepers were rotated between huts each night.

Across experiments 1 and 2, a total of 4147 mosquitoes were released into the enclosure over 40 nights, with 98 % (4068 mosquitoes) recovered.

7.3.2. Mosquito population

Experiments were all conducted on field-collected *Anopheles gambiae* s.l. from natural breeding habitats in M'be (5.209963 W longitude and 7. 970241 N latitude) with 99 % M-form *An. coluzzi*, in central Côte d'Ivoire[21–23]. This suburban village of Bouaké is dominated by highly pyrethroids and DDT resistant *Anopheles gambiae* s.l. that breed in rice and vegetable fields[16,24,25]. The field-collected *An. gambiae* larvae were reared using Tetramin™ baby fish food at 27 ± 2 °C and standard density (300 larvae) in metallic bowls with 1 litre of deionized water. Adult mosquitoes were then housed in standard mosquito 32.5 cm^2 cages and maintained on 10 % sugar solution at 27 ± 2 °C, 60 ± 20 % RH and ambient light. All tested mosquitoes were 4-5 days old on their first day of experimentation and kept alive during the experiments (before and after recapture) in transparent hard-plastic cups in the lab at 27 ± 2 °C, 60 ± 20 % RH and ambient light. Each cup was covered with mosquito-proof netting with access to 10 % sugar solution using cotton wool pads.

7.3.3. Human host preparation

Volunteers human hosts were monitored throughout the course of the study for malaria infection to avoid any contamination of the mosquitoes by malaria-infected blood. The volunteers avoided the use of fragrance, repellent products, tobacco and alcohol for 12 hours before and during testing.

7.3.4. Pre-exposure of mosquitoes to an LLIN or UTN

Insecticide exposures were carried out on 4-5-day old, non-blood-fed female mosquitoes using either a PermaNet® 2.0 (LLIN), or an untreated net (UTN). The LLIN is a polyester net coated with 55 mg/m^2 ± 25 % deltamethrin. An untreated ultra-fine mesh polyester net distributed by Coghlan's was used as the UTN control. A 3-min exposure in WHO-cones of a fully susceptible Kisumu mosquito strain assured that the LLIN and the UTN killed 100 and 0 % of mosquitoes, respectively.

Mosquitoes (4147 mosquitoes in total) were pre-exposed for 3 min either against the LLIN or UTN, ten- by ten in plastic WHO cones in day light and then kept in mesh-covered plastic cups with access to cotton pads soaked in water until release in the enclosures. Cones were angled at 40° to favor the contact between the mosquitoes and the net. Because these mosquitoes exhibit extremely high levels of resistance, there was negligible knockdown or mortality (about 1 % knockdown and no mortality

1h post-exposure) from this initial exposure. Six hours after the pre-exposure the mosquitoes were transported to the field site and released in the semi-field enclosure. Six hours was selected to represent the scenario reported in Glunt et al.[26], which showed in a small-scale lab assay that resistant mosquitoes recovered their capacity to host search within 6h of sub-lethal contact with a LLIN. Here we aimed to see whether there were any "hangover" effects of LLIN exposure after 6h in a more realistic host-searching environment.

7.3.5. The enclosure and West African huts

A large screen house (5 m wide, 13 m long and about 4 m high) was erected to enclose 2 standard West African experimental huts that were modified with 12 holes (15 cm diameter) per hut at eave level to simulate a house with open eaves and maximize mosquito entry[21]. Each hut had four metallic windows with a horizontal window slit in each (two sheets of metal form a funnel inside the window frame with a narrow opening enabling mosquito entry but preventing mosquito exit) and a metallic shutter that can be closed. However, the holes in the eaves and the windows were left open during the night to maximize mosquito recruitment [27].

Huts were assigned one of two treatments that ran in parallel each night: (i) control, in which the volunteer slept under an UTN; (ii) treated, in which the volunteer slept under a LLIN. In all cases, windows and eaves were open, the doors of the huts were closed, and a sleeper was present in each hut. The treatments and sleepers were rotated over the two huts on sequential nights.

In the initial run of the experiment the nets were intact. In the second experiment the bed nets were damaged following the standard WHO testing guidelines whereby 6 square holes of 4 x 4 cm were made in the sides of each bed net[28]. The holes increase the possibility of mosquitoes being able to blood feed and so enable measurements of blood-feeding inhibition in addition to measures of overall host searching rate.

7.3.6. Mosquito release and recapture inside the enclosure

As described in Barreaux et al., 2018[21], the sleepers entered the huts at 20:00 and the windows and the eaves were opened by the supervisor. At 20:15 the supervisor then released 70-100 female *An. gambiae* in the central area of the enclosure. At 05:00 the following morning, the windows and the eaves were closed by the supervisor (the experimental period from 20:00 to 05:00 is representative of the period when household members are likely to be indoors and is typical for experimental hut

studies[22,29]). Mosquitoes (4068 in total) were then collected back by the sleepers with their position recorded (i.e., whether they were inside one hut or the other, or outside the huts in the enclosure). Mosquitoes were hand-captured one-by-one inside the experimental huts and enclosure using individual glass hemolysis tubes and a flashlight. Tubes were plugged with a small piece of cotton and labelled, prior to transportation to the laboratory at the Institut Pierre Richet research centre in Bouaké, Côte d'Ivoire. Mosquitoes were then identified to species level using a binocular microscope (40×) and their status (live or dead; blood-fed or not) assessed. Mosquitoes alive at recapture were kept for observation in the insectary on 10 % sugar solution, at 27 ± 2 °C, 60 ± 20 % RH and ambient light. Their longevity was monitored until death.

7.3.7. Statistical analysis

All statistical analysis and figures were performed in R studio© version 3.6.1. Where needed, contrasts among treatments were assessed using the *multcomp* package and the function *glht* in the software R with a Tukey's honestly significant difference test (Tukey HSD) multiple-comparison test. It allowed simultaneous tests for General Linear Hypothesis and comparison differences between groups.

First, a binomial Generalized Linear model (GLM) compared the choice of mosquitoes to enter or not any hut once released inside the enclosure, regarding the treatment imposed during WHO cone exposure in the laboratory and the type of experiment (damaged or intact nets in huts). The effect of the nights of release-recapture was also considered as the mosquitoes released from one night to another may came from different larval collections and rearing cages.

Second, considering only mosquitoes captured inside huts, a binomial GLM was performed to analyse the proportion of mosquitoes recaptured in a given hut out of all mosquitoes that entered any huts depending on the effect of the pre-exposure in cone (LLIN or UTN) and the bednet inside the hut (LLIN or UTN). The nights of release-recapture and the effect of the sleeper were also considered. This analysis helped investigate the spatial deterrence of LLINs or how likely mosquitoes are prevented from entering a house with a LLIN.

Third, considering only the experiment 2 (as the probability to get bitten under an intact net in experiment 1 was nearly zero), the blood feeding success was analysed using a binomial GLM regarding the treatment imposed during cone exposure in the laboratory, the insecticide treatment of the bednet inside the chosen hut (LLIN or UTN), and the effects of those parameters in interaction. The effect of the nights of release-recapture and the effect of the sleeper were also considered.

Considering all the mosquitoes released in the enclosure in the experiment 2, we also analysed the proportion of mosquitoes blood fed out of mosquitoes released using an Analysis of Variance (ANOVA) regarding the treatment imposed during the cone exposure in the laboratory, the insecticide treatment of the bednet inside the huts and the effects of those parameters in interaction. The effect of the nights of release-recapture and the effect of the sleeper were also considered. The proportion of blood fed mosquitoes was square root transformed so that the residuals of the model followed a normal distribution.

Fourth, the proportion of mosquitoes found in the veranda out of the proportion of mosquitoes entering huts in experiments 1 and 2 was analysed using a binomial GLM regarding the treatment imposed during the cone exposure in the laboratory, the insecticide treatment of the bednet inside the chosen hut, and the effects of those parameters in interaction. Capture in the veranda area of the hut is used as a measure excito-repellency in standard WHO assessments[30]. In experiment 2, the blood feeding status parameter was added in the model. The effects of the nights of release-recapture and the effect of the sleeper were also considered.

Finally, mosquito survival (considering mosquitoes dead in the huts and the survival post-release for mosquitoes recaptured and brought back to the lab) was analysed separately for both experiments using a gaussian GLM. With intact bednets (first experiment), survival was analysed regarding the treatment imposed during cone pre-exposure in the laboratory, the hut choice (LLIN and UTN huts) and their interaction. The effect of the nights of release-recapture was also considered. Mosquito survival for the holed net experiment was analysed the same way with the addition of the feeding success parameter in the model. In this model, we did not look at the interactions between the location of mosquitoes at recapture and the blood feeding status, as no blood fed mosquitoes were recaptured in the enclosure.

7.4. Results

7.4.1. Recapture into experimental huts

Recapture rates across both experiments were extremely high, with an average of 98 % of mosquitoes (alive or dead) collected back each morning after release. On average 33.4 (95 % Confidence Interval: 31.9 to 34.8) % of mosquitoes were motivated enough to enter one of the two huts in search of a human host; 30.3 (95 % CI: 28.2 to 32.5) % in the experiment 1 with intact nets and around 5 % more, 35.7 (95 % CI: 33.7 to 37.7) %, in experiment 2 with damaged nets (χ^2 = 13.7, df = 1, $p < 0.001$). The

pre-exposure to insecticide in a WHO cone with a LLIN marginally increased hut entry by 3 % [LLIN pre-exposure: 35.0 (95 % CI: 32.8 to 37.1 %); UTN pre-exposure: 31.9 (95 % CI: 29.9 to 33.9 %)] (χ^2 = 6.5, df = 2, p = 0.039) (Figure 1). There was significant variation in mosquito entry between nights of release/recapture (χ^2 = 351.5, df = 37, p < 0.001).

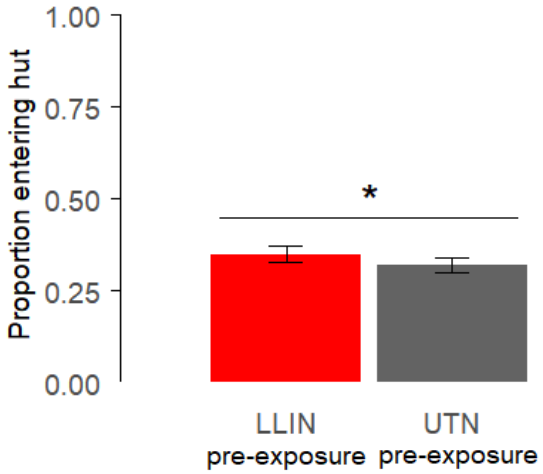

Figure 1: Proportion of mosquitoes entering experimental huts overnight in a semi-field enclosure depending on whether the mosquitoes were pre-exposed to a LLIN (PermaNet 2.0) or an untreated net (UTN) prior to release in the enclosure. Mosquitoes were pre-exposed to a LLIN or an UTN in a WHO cone 6 hours before release in the enclosure. Results are shown for the combined data from experiments 1 and 2, which used intact bed nets or damaged bed nets within the huts, respectively. Mosquitoes in the enclosure had the choice between two huts with a sleeper either protected by a LLIN or an UTN. Mosquitoes recaptured inside one of the two huts the day after the release were considered as host seeking mosquitoes and mosquitoes found outside the huts were not. The red color corresponds to mosquitoes pre-exposed to a LLIN (PermaNet 2.0) in a WHO cone and the grey color to mosquitoes pre-exposed to an UTN in a WHO cone. UTN pre-exposure: pre-exposition to an UTN in cones; LLIN pre-exposure: pre-exposition to a PermaNet 2.0 in cones. *: significant difference 0.001< p < 0.05. 95 % confidence interval bars are shown.

Considering only mosquitoes recaptured inside huts, there were 4 % more mosquitoes found in huts in which hosts were protected with a LLIN compared to the average [Figure 2; LLIN: 54.0 (95 % CI: 51.3 to 56.7) %; UTN: 45.9 (95 % CI: 43.3 to 48.6) %] (χ^2 = 5.2, df = 1, p = 0.02). The nights of release/recapture and the sleepers influenced the choice as well (χ^2 = 5.0, df = 1, p = 0.02 and χ^2 = 6.5, df = 1, p = 0.01 respectively). We found no effect of the pre-exposure in WHO cones (p > 0.05).

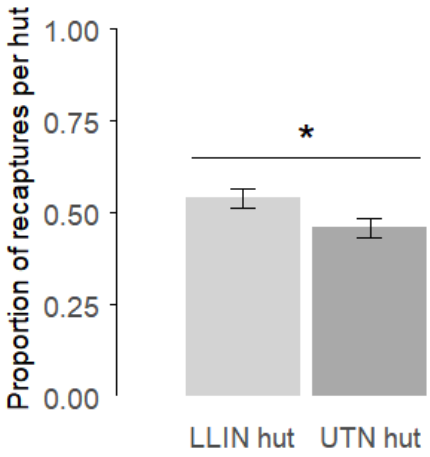

Figure 2: Proportion of mosquitoes re-captured either in a hut with an untreated net (UTN) or a hut with a long-lasting insecticidal net (LLIN). The light grey color corresponds to mosquitoes that were recaptured in the hut with the LLIN (on the left) and the dark grey color (on the right) corresponds to mosquitoes that were recaptured in the hut with the UTN. Results are shown for the combined data from experiment 1 and 2, which used intact bed nets or damaged bed nets within the huts, respectively. UTN hut: mosquitoes re-captured in the hut with an UTN; LLIN hut: mosquitoes re-captured in the hut with a LLIN**.** *: significant difference $0.001 < p < 0.05$. 95 % confidence interval bars are shown.

7.4.2. No blood feeding inhibition

Considering only mosquitoes recaptured within a hut, there was a small but non-significant reduction in blood feeding rate for mosquitoes attempting to access a host via a LLIN [24.2 (95 % CI: 20.2 to 28.6) %] compared with an UTN [28.6 (95 % CI: 24.1 to 33.3) %] ($\chi^2 = 4.0$, df = 1, $p = 0.15$) (Figure 3.1). There was some variability between nights of release/recapture ($\chi^2 = 76.34$, df = 21, $p < 0.001$). When considering the total pool of mosquitoes, the average feeding rate was 9.4 (95 % CI: 8.2 to 10.7) %, and there was also no significant difference between LLIN and UTN huts [4.5 (95 % CI: 3.7 to 5.4) % and 4.9 (95 % CI: 4.0 to 5.8) %, respectively; $p > 0.05$] (Figure 3.2). There was no effect of pre-exposure to insecticide and of the sleepers on the blood feeding success in experiment 2 ($p > 0.05$).

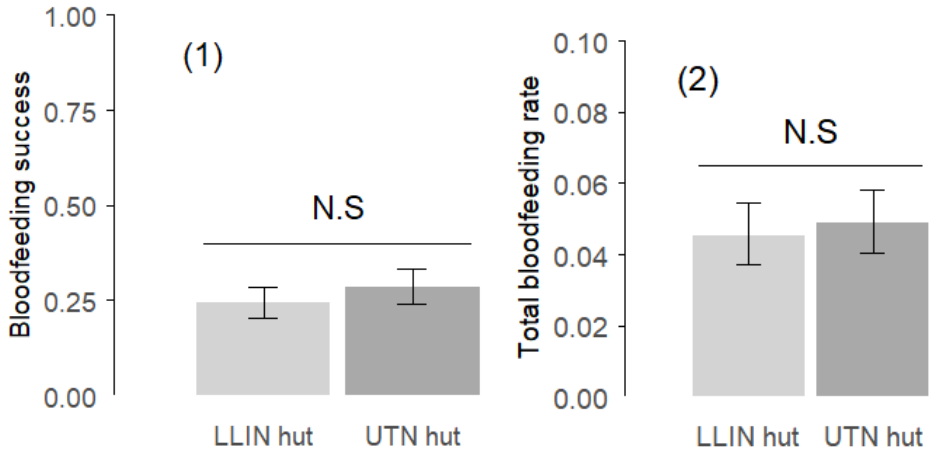

Figure 3: This panel represents the proportion of blood-fed mosquitoes re-captured in each hut regarding the type of bednet protecting the volunteer in each hut (untreated net, UTN, or long-lasting insecticidal net, LLIN). In (1) the number of blood fed mosquitoes is divided by the number of mosquitoes re-captured inside each hut and in (2) the proportion of blood fed mosquitoes in each hut is divided by the overall number of mosquitoes released in the enclosure. The light grey color corresponds to mosquitoes that were recaptured in the hut with the LLIN (on the left) and the grey color (on the right) corresponds to mosquitoes that were recaptured in the hut with the UTN. The figure shows the results from experiment 2 that used damaged nets. UTN hut: mosquitoes re-captured in the hut with an UTN; LLIN hut: mosquitoes re-captured in the hut with a LLIN**.** N.S.: non-significant difference as $p > 0.05$. Please note the difference in scale in the x-axis. The different scales help to visualize the total blood feeding rate which is small in comparison to the blood feeding success which accounts for mosquitoes that are host-seeking only. 95 % confidence interval bars are shown.

7.4.3. Excito-repellency of LLIN

In the initial experiment with intact bednets (experiment 1), the proportion of mosquitoes recaptured in the veranda [42.3 (95 % CI: 38.05 to 46.6) %] did not depend on the pre-exposure treatment, whether they were collected from a hut with a LLIN or UTN, nor any interaction (all P-values > 0.05). There was some variability between nights of release/recapture (χ^2 = 59.77, df = 15, $p < 0.001$) but there was no effect of the sleepers ($p > 0.05$).

In the second experiment with damaged nets, 35.7 (95 % CI: 32.4 to 39.1) % of the mosquitoes recaptured were found in the veranda. In the hut with a LLIN, around 10 % more mosquitoes were recaptured in the veranda [39.4 (95 % CI: 34.7 to 44.2) %] relative to mosquitoes in the hut with an UTN [31.6 (95 % CI: 27.05 to 0.36) %] (χ^2 = 3.93, df = 1, p = 0.047) (Figure 4). No blood feed mosquito and 48.4 (95 % CI: 44.4 to 52.5) % of all unfed mosquitoes were found in this area (χ^2 = 231.82, df = 1, $p < 0.001$). The recaptures there did not depend on the pre-exposure treatment ($p > 0.05$).

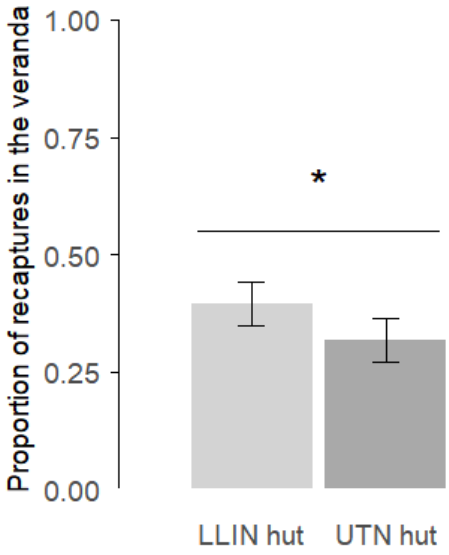

Figure 4: Proportion of mosquitoes re-captured in the veranda either in a hut with an untreated net (UTN) or a hut with a long-lasting insecticidal net (LLIN). The light grey color corresponds to mosquitoes that were recaptured in the veranda next to the hut with the LLIN (on the left) and the dark grey color (on the right) corresponds to mosquitoes that were recaptured in the veranda next to the hut with the UTN. The figure shows the results from experiment 2 using damaged nets. UTN hut: mosquitoes re-captured in the hut with an UTN; LLIN hut: mosquitoes re-captured in the hut with a LLIN**.** *: significant difference $0.001 < p < 0.05$. 95 % confidence interval bars are shown.

7.4.4. Mosquito survival time following release

In the initial experiment with intact bednets, the mean survival time of released mosquitoes was (mean ± se) 7.9 ± 0.15 days (note that mosquitoes were 4-5 days old when released in the enclosure, but we report here the survival rate post-release, not the age of mosquitoes at death). The survival rate did not depend on the pre-exposure treatment, whether the mosquitoes were collected from a hut with a LLIN or UTN, nor any interaction (all P-values > 0.05).

In the second experiment with damaged nets, the overall average survival time post-release was 8.4 ± 0.14 days, and again there was no effect of pre-exposure treatment ($p = 0.08$) or whether the mosquitoes were collected from a hut with a LLIN or UTN ($p > 0.05$). There was a significant interaction whereby mosquitoes potentially exposed two times to insecticide (i.e. in the initial pre-exposure and then to a LLIN in a hut) had a small (0.5 day) statistically significant reduction in average survival time compared to the other treatments overall but the data are not sufficient to make statement about pair-wise differences ($F = 4.0$, $df = 1$, $p = 0.04$; Tukey post-hoc test: $p > 0.05$). There were effects of blood feeding, with mosquitoes that had obtained a blood meal surviving 2.4 days longer than those that did not (average survival time of 10.8 ± 0.27 days for fed mosquitoes compared with 7.6 ± 0.14

for unfed mosquitoes; F = 164.8, df = 1, p < 0.001). Blood feeding increased relative survival more in mosquitoes pre-exposed to an UTN compared with mosquitoes pre-exposed to a LLIN (blood-fed UTN pre-exposure: 11.5 ± 0.37 days; unfed UTN pre-exposure: 7.4 ± 0.19 days; blood-fed LLIN pre-exposure: 10.02 ± 0.40 days; unfed LLIN pre-exposure: 7.7 ± 0.21; F = 3.7, df = 1, p = 0.05; Tukey post-hoc test: respectively p = 0.04 and p = 0.22 for the comparison between fed and unfed mosquitoes for both pre-exposure treatment and all other comparison p > 0.001) (Figure 5). In both experiment 1 and 2, mosquito survival post-release was influenced by the release-recapture night (F = 6.6, df = 38, p < 0.001 and F = 6.9, df = 38, p < 0.001, respectively).

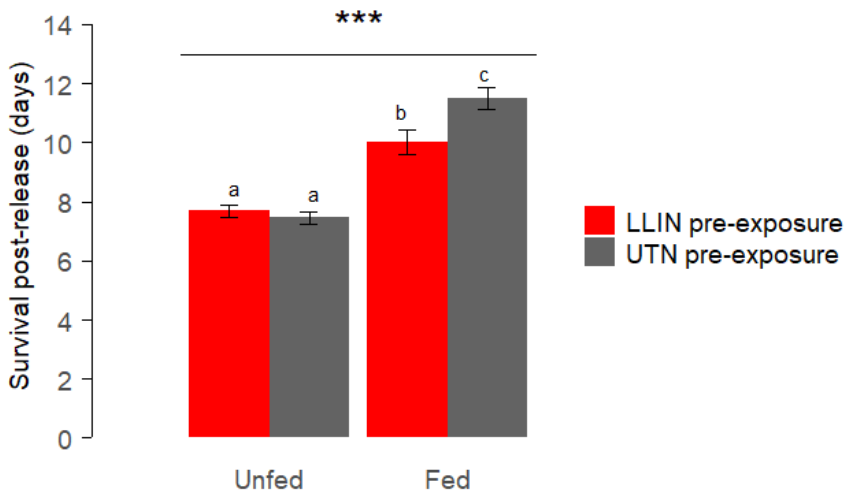

Figure 5: Average survival time of mosquitoes following their release into the semi-field enclosure and subsequent recapture and return to the laboratory. Mosquitoes were characterized based on blood feeding status (unfed or fed) and whether they were pre-exposed to a LLIN or UTN prior to introduction into the enclosure. Mosquitoes were pre-exposed to (PermaNet 2.0 or untreated net) 6 hours before release in the enclosure. On the left are unfed mosquitoes and, on the right, the fed mosquitoes. The red color corresponds to mosquitoes pre-exposed to a PermaNet 2.0 in a WHO cone and the grey color to mosquitoes pre-exposed to an untreated net. These are results from experiment 2 using damaged nets. Treatment a, b and c are significantly different; ***: significant difference, p < 0.001. ± standard error bars are shown.

7.5. Discussion

This chapter aimed at investigating the effects of an LLIN on host searching, blood-feeding and post-exposure mortality of highly pyrethroid resistant field mosquitoes. The approach used two experimental huts within a semi-field enclosure to provide a choice test between LLIN and UTN, and also considered whether an initial pre-exposure to an LLIN had any hangover effects on mosquito behavior. Overall, there were no substantial biological effects or interactions between treatments. These results suggest little difference between the LLIN and UTN, indicating that resistance has largely

nullified the effects of the pyrethroid insecticide. However, there were a number of results that warrant further discussion.

First, while there was no evidence of deterrence or spatial repellency by the LLIN (i.e. slightly more recruitment to huts with an LLIN vs UTN), a pre-exposure to insecticide appeared to increase host-searching (Figure 1). Previous studies have shown that sub-lethal exposure to an LLIN can result in a transient disruption of host searching ability[26,31] and this is potentially borne out by the feeding rates reported in the current study (discussed below). However, the current data suggest that once these transient effects have worn off, host searching might potentially be enhanced. This counter-intuitive result might be due to an adaptation to insecticide exposure in areas with high-insecticide coverage[32]. The stimulatory effects of low-dose of insecticide exposure has been widely studied in invasive species using the term "insecticide-induced hormesis"[33–36]. Stress-tolerance and adaptative stress (which involve genetic assimilation) are evolutionary consequences of sublethal exposures to an environmental stress, such as the exposure to insecticide[37]. Insecticide-induced hormesis has been shown to stimulate fecundity, survival and behavioral responses to odor stimuli in insects such as aphids and moths following a sublethal exposure to insecticide[35,38,39]. Although high doses of insecticide are clearly harmful to mosquitoes[10,40–43], less is known about the functional effects of insecticide on biological processes in highly resistant mosquitoes[32]. In Chapter 6, we show that the odor detection performance after a pre-exposure to a LLIN first goes down before going back up again and increases until 24h post-exposure. The odor detection of mosquitoes pre-exposed to insecticide is then somewhat better compared to the odor detection of mosquitoes pre-exposed to an un-treated net. This could explain why, in the enclosure, slightly more mosquitoes were host searching when pre-exposed against a LLIN in a WHO cone before being released in the semi-field setup a few hours later. A better odor detection performance could not only help find a host more easily but could also help find holes in a damaged net more efficiently.

Second, there was a small but significant increase in excito-repellency due to the LLIN (as measured by the proportion of mosquitoes attempting to exit the hut and captured in the veranda). These data suggest that some direct effects of the insecticide remain and that the LLIN is not yet functionally identical to an UTN. However, no effect of insecticide on blood feeding success was measured in the enclosure, there was no difference between the LLIN and UTN. Probably with more nights a reduced feeding could have been observed, however in this study the extent of repellency and blood feeding inhibition are small (even negligible maybe) compared with those that would be expected with a susceptible mosquito population[45,46]. The transient electrophysiological impairment following contact

with an LLIN shown in Chapter 6 did not seem to have affected the movement coordination, the flight ability, and capacity to find holes in the net over the night[44].

Third, mosquitoes that were successful in taking a blood meal survived significantly longer than those that did not, but this effect was reduced in mosquitoes that had been pre-exposed to a LLIN. This result suggests some functional effect of the LLIN but also indicates the complexity in evaluating the effects on survival since there was no direct effect of a single LLIN exposure (either from the initial exposure in the lab or in mosquitoes captured from LLIN huts in the absence of pre-exposure), and no differences in mortality between LLIN and UTN treatments when mosquitoes were not blood fed. The cause of the interaction with blood feeding is unclear. Blood feeding enhances survival of control mosquitoes presumably by boosting energy reserves beyond simple sugar water, and in the lab setting there is minimal energetic expenditure or mortality risk associated with searching for oviposition sites compared with those that might occur in nature[47]. The fact that mosquitoes pre-exposed to an LLIN gain less benefit suggests there could be significant energetic costs associated with detoxification of the pyrethroid[48,49].

7.5. Conclusion

These results mitigate previous studies evaluating the effectiveness of LLINs as they are likely substantially compromised by this level of insecticide resistance. Although they work as efficient barrier against mosquito bites, how the direct insecticidal and excito-repellency effects of LLINs against insecticide resistant mosquitoes play out in the field is still unclear. First, mosquitoes were more motivated to search for a host after a pre-exposure to insecticide and were slightly more interested by the hut with the LLIN, which could increase the risk of potential infectious feed for an un-protected host sleeping next to a host protected with an intact net in the same or in the next household. Second, LLINs retained some killing and excito-repellency properties in this study which could have help reduce the blood feeding as in our previous laboratory studies (c.f. chapters 2,4 and 6 of this thesis). However, in this semi-field setting mosquito blood feeding success and the overall feeding rate were not dependent on the presence of insecticide. LLINs seemed unable to provide better personal and wide-community protection than untreated nets, as overall feeding rates (which are a key endpoint measure of whether transmission is likely) were not impacted by the LLIN (directly or via pre-exposure). All these results stress out the need to study mosquito foraging decisions and feeding compliancy in larger and more natural settings and under different conditions to make more general predictions about the impact of insecticide resistance for malaria control.

CHAPTER EIGHT

General conclusion

8.1. Summary of the results

Long-Lasting Insecticidal Nets (LLINs) continue to protect better than untreated net against mosquito bites despite insecticide resistance. In this summary of the results, we are discussing both the direct effect of insecticide on odor detection, host searching, host choice and feeding success and the long-term effect of one or multiple exposure to insecticide on longevity and the capacity to take multiple blood meals over a lifetime. We worked with both mosquito larvae collected directly in the field and colonized mosquitoes from the field. Both the collected mosquitoes and the colony we built and maintained throughout this thesis were extremely resistant to pyrethroids[230] (from 1250-fold to 1700-fold resistant to deltamethrin). The 24h survival post-exposure was not different between mosquitoes exposed to a deltamethrin-treated net and mosquitoes exposed to an untreated net.

Tested in the laboratory, LLINS did not work only as a physical barrier, as the irritant properties of LLINs directly lowered the host seeking and feeding success of extremely insecticide-resistant mosquitoes around LLINs. Mosquitoes exposed to insecticide compared to those exposed to an untreated net spent less time in contact with the net and feeding. In addition, using electroantennograms we detected that insecticide induced a short-term odor detection disturbance following an exposure, a mechanism potentially linked to the reduction in behavioral response of mosquitoes to a LLIN-baited host. Though, this effect of the insecticide on sensory responses to attractive odors fades after one hour. Unfed mosquitoes after the first opportunity to take a blood meal through a LLIN did not use the multiple opportunities to feed in the absence of insecticide in the hours following the exposure to increase blood meal success in comparison to mosquitoes pre-exposed to an untreated net.

However, in the semi-field experiments the evidence for direct LLIN efficacy against young and unfed mosquito bite was less clear and LLIN efficacy seems to be largely compromised by resistance. First, LLINs did not reduce long-range host seeking behavior, and we found no spatial repellency of pyrethroid-LLINs against insecticide-resistant mosquitoes (rather a slight LLIN attractiveness). Second, we found poor evidence that overall feeding rates (which are a key endpoint measure of whether transmission is likely) are directly impacted by the LLIN in these more realistic settings. Insecticide-induced hormesis (biphasic phenomenon allowing low dose stimulation and high dose inhibition[391]) which assure mosquito survival in unfavorable environments could potentially stimulate host-seeking and feeding behaviors to increase exposure tolerance. However, as the level of stress increases, deleterious effects of pyrethroids will heavily affect life history traits of insecticide-resistant mosquitoes on the long-term. Besides, after being attracted to a host smell and having travelled a long

distance to access it, the motivation to take the blood meal is probably different from that of a mosquito sitting 10 cm below a host's arm placed directly against a LLIN.

The key message throughout this thesis is that successfully taking a bloodmeal once does not mean it will happen twice. In several chapters, sub-lethal effects of insecticides were shown to reduce the feeding compliancy over the lifetime and the longevity of mosquitoes, reducing the proportion of older resistant mosquitoes alive and strong enough to bite a second time in the presence of insecticide. Therefore, sub-lethal effects of LLINs have the potential to alter mosquito population age structure and repeated insecticide exposures for mosquito trying to feed multiple times at each gonotrophic cycles may help further maintain the efficacy of vector control as well as the reduction in vectorial capacity. The data obtain in this thesis suggest that insecticide coverage (from partial coverage to intense coverage) work better than un-treated bednets distribution to cut down the probability of malaria transmission.

In addition, the host searching and feeding rates over age are decreasing in mosquitoes reared in more stressful environments (for example with a diet cut in half and in a more crowded environment). A nutritional stress during the larval development probably occurs regularly in the field, hence the number of blood meal taken in one life could be even lower than what is observed in the laboratory. Our data highlights the urgent need to better understand the feeding strategies of mosquitoes depending on their physiology and their environment to find the limits of what is achievable with the current tools (from brand new LLINs to older LLINs). LLINs target mosquitoes feeding and resting indoors, and therefore potentially select for mosquitoes feeding and resting outside before people go to bed or after they wake up. The behavioral adaptations of mosquitoes in responses to LLINs mass-distributions could be a strategy adopted by mosquitoes to reduce the sub-lethal effect of insecticides[9,156,308]. As LLINs seem not efficient enough to target residual malaria transmission, the vector control tools box could benefit from complementary strategies used against vectors exhibiting more diverse behaviors, feeding on other hosts, feeding and resting outdoors, and/or feeding in the early evening (vs. feeding and resting indoors and strong preference for human blood feeding during nighttime)[211].

8.1.1. Chapter 2

For this first set of experiments we worked on better understanding mosquito behavior around LLINs. We found that insecticide resistant mosquitoes spent less time in contact with the net when it is insecticide-treated, and both the host seeking, and blood feeding behavior activities were reduced.

There were also long-term effect of insecticide leading to a delayed mortality with reduced mosquito's longevity with a single exposure and an even further reduction in longevity with repeated exposures. Insecticide exposure time (short period of time from 1 minute to 5 minutes) did not influence insecticide susceptibility and longevity, except when mosquitoes fed on a host. In this case, a blood meal helped overcome the sublethal effect of insecticide, and even more so when the time spent blood feeding was longer.

The experiments described in this chapter provided evidence that the performance of LLINs against more than 1700-fold resistant mosquitoes are less worrying than what the WHO test procedures let appear by measuring only the mortality 24h post-exposure. This delayed mortality and impact of repeated exposures could help explain the continued efficacy of LLINs in high malaria transmission settings. Less strong results were found in Burkina Faso, another country with high intensity pyrethroid-resistance[286], but as significant differences can be found in the longevity of mosquitoes exposed to an un-treated net from one study to another (due to un-controlled parameters), the delayed effects of insecticide might be diluted in some cases, like it seems to be the case in the study conducted in Burkina Faso. Further comparative studies between countries could be an interesting way to understand variation in the effect of insecticides, whether is it mostly driven by environmental changes, the microbiome, and/or different methodologies.

In this chapter, we also highlighted the fact that the standard susceptibility tests for insecticide resistance monitoring does not reflect the way mosquitoes interact with LLINs in real malaria transmission settings. This is a major issue when it comes to translate results from clinical trials into normative guidance and understand how a vector control product works in each setting and against different mosquito populations. The current WHO standard bioassays may not be as robust entomological indicators as expected and they do not allow epidemiological prediction about the impact of LLINs. The method of exposure of mosquitoes pushed against a net for an arbitrary exposure times (i.e. 3 min, 1 hour) does not represent a natural interaction between a LLIN and a thirsty mosquito host seeking around it. Whether the tested mosquitoes spend the whole time flying in the device or in contact with the net is an un-controlled yet important parameter. In absence of a bait, there is no motivation to host seek and probe on the net and thus the exposure method does not reflect how much mosquitoes are exposed, how long the exposure last and what part of the body is in contact with the LLIN. The individual 'cup assay' help get over those problems as that method allows mosquitoes that are trying to take a blood meal on the baited arm to freely sit on the net. It considers important parameters of malaria transmission, namely host seeking (and therefore also exposure

time), feeding behavior (time to take a blood meal and feeding success) and longevity (long-term survival post-exposure). The disadvantage of that method is that, experimenter handling may have a strong effect on survival. Thereby, like WHO standard bioassays, young females are tested only once which omit older mosquitoes that are potentially exposed several times to insecticide. The potential of malaria transmission mainly depends on that proportion of mosquitoes and the development of bioassays allowing these measures (insecticide resistance monitoring and sublethal pipeline) are needed to demonstrate the full efficacy of new vector control products.

8.1.2. Chapters 3 and 4

In the third chapter, we describe how we successfully built and maintained a colony of insecticide resistant mosquitoes originally collected in rice-fields around Bouake in Côte d'Ivoire. Using our colonized mosquitoes in chapter 4, we evaluated the efficacy of insecticide-treated nets (LLIN) on mosquito behavior and developed a method that limits the experimenter handling and maximize the feeding opportunities. For each replicate, we were exposing mosquitoes inside a one-way tunnel every four days (as a proxy of one gonotrophic cycle) or every day. To expose mosquitoes, we gave them (each four days or each day) the opportunity to freely host seek and feed on a human foot enveloped inside a LLIN (or an untreated net), no forced exposures to insecticide were involved. Overall, the presence of insecticide reduced the host searching and feeding behaviors over time. We calculated the individual feeding rate per mosquito to be less than two bloodmeals per female when mosquitoes were tested in the presence of insecticide, which would lead on average to a highly reduced vectorial capacity. That result (< 2 blood meal per female) is interesting in term of disease transmission but given the variability in overall feeding rate between the different replicates and experiments, we suggest focusing on the difference in feeding performance between bednet treatments. In fact, 2 to 3 times less blood meal were taken per female when they could take blood meals all their life in the presence of insecticide (compared to those tested in the absence on insecticide).

In addition, we found a delayed mortality effect with exposure to insecticide. Resistant mosquitoes were still alive 24h after insecticide exposure but their longevity was reduced, which increased the chance they would die before a potential completion of the parasite development (extrinsic incubation period, EIP). In this experimental setup parameters relevant to transmission, like longevity and feeding behavior, may be impacted by one or multiple lethal or sublethal exposures to insecticide. Repeated insecticide exposures could reduce the probability of the completion of the EIP post infectious blood meal if female mosquitoes engage in three to five gonotrophic cycles before being able to transmit malaria[168,238].

We have such data with a direct access to the blood source with a foot against the bednet and we have also compared scenario where the host is partially protected by a damaged net or completely protected by an intact net. It helped better understand mosquito behavior around a LLIN (or an untreated net) and measure transmission potential in three different scenarios that reflect what could happen in areas where bednet are distributed: either the blood source is directly available as the host in direct contact against a LLIN, the host is accessible but through a damaged net or the net is intact and host-seeking mosquitoes try to find a way through it but cannot. The first scenario gave mosquitoes the best chance to survive an exposure to insecticide and one explanation may be that mosquitoes did spend less time host seeking and potentially reduced their contact with insecticide before taking a blood meal. The opportunity to go through the insecticide barrier through holes and the feeding post-exposure did not help mosquitoes to survive better than those that had no access to a host. In these scenarios, the contact time with the net was maximized until a way was finally found through the net or mosquitoes lost the motivation to find a way. A damaged net reduced feeding success in comparison to an intact net placed directly against a host, which could suggest that a pre-exposure to insecticide confuses mosquitoes enough to reduce their host searching and feeding performance and/or increased energy demand because of the energy needed to find a hole in the presence of insecticide and/or increased insecticide detoxification-induced metabolic changes[403].

In addition, we measured the individual feeding performance by separating fed mosquitoes from unfed ones everyday day after testing. This provided insights in individual feeding strategy and survival instead of a mean feeding rate and overall mortality rate of groups of mosquitoes and further detail on biting rate and gonotrophic cycles data. Overall, 36 % of the mosquitoes never took any bloodmeals and the total number of blood meals per female varied from 0 to 5. The proportion of mosquitoes taking 2 bloodmeals over their lifetime was low (34 %) and even lower in the presence of insecticide (11 %). The data suggests that only a small proportion of mosquitoes would be responsible for malaria transmission and further work must been done to understand what parameters are involved in such variability. In these experiments, mosquitoes were reared under a standardized regime. It is unclear whether the body size played a role in the number of blood meals taken over the life. Although we would expect a great variability in size in field collected mosquitoes and therefore a potential effect on individual feeding performance, we would not expect a major impact of size in our colony. Besides, body size did not influence longevity. Other parameters could vary from one individual to another and could influence feeding performance. For example, a possible physiological divergence with different metabolic rates may be found in the *Anopheles gambiae* complex[404,405] as in our colony and at different

period during this thesis, both the M and S molecular forms of *An. gambiae* s.s were found. A recent paper compared *An. arabiensis* and *An. gambiae s.s* and found that change in behavior and feeding activity was a strategy to reduce metabolic rates[404]. For example, estivation (summer dormancy) could not only increase longevity and allow higher desiccation resistance but also reduce both nutritional needs and metabolic rate. As we regularly infused the colony with mosquitoes prospected from the field at different time of the year, the eggs used to create our adults could have very distinct genetic background[230] and the mother investment for their offspring may be different due to the adaption to potentially different environments[406]. Besides, while relationship between body size and metabolic rate exist, they are rarely linear and most of time logarithmic[407].

8.1.3. Chapter 5

In continuity with the chapters 3 and 4 and using the same method to expose mosquitoes against insecticide, we assessed in chapter 5 the consequences of larval environment stressors on mosquito behavior around bednet. Using two different food diets and two different mosquito densities at the larval stage, the goal was to stimulate larval competition and create difference in physiological needs which could affect juvenile energy reserves and with carry-over effects on host seeking, blood feeding and egg laying as adults. Both a higher diet and a less crowded larval environment accelerated larval development and survival, and at the adult stage, improved the motivation for host seeking over the lifespan and led to more blood fed mosquitoes overall. However, the blood feeding success rate for mosquitoes that successfully flied to the host, measured as the number mosquitoes blood feeding out of the number host searching near the host, was independent of larval conditions. It may indicate that the larval environment had a bigger impact on foraging decision rather than feeding capacities, and/or that the capacity to find a host and fly toward it was affected by larval conditions. Previous studies have shown the effect of nutrition on flight capacity[322,325,346] and one study showed that 22 % of the pre-flight lipid were found to be mobilized for flight when mosquitoes decided to take a blood meal and after a blood meal, mosquitoes had a longer flight activity and 61 % of the energy obtained from the nutrient were used for flight (against 11 % for survival)[346]. Teneral reserves are important for flight and eventually mosquitoes that have less reserve will invest less energy for blood feeding[327] and more for egg laying and survival. This could explain why the proportion of blood meal taken out of the number of host visits (or feeding success) and the estimated number of eggs laid per female were not dependent on the larval environment conditions despite the reduced host-seeking and feeding activity and also why mosquitoes longevity was increased when larvae had a diet cut in half.

An important parameter involved in host searching and feeding that we did not investigate is temperature and the thermoregulatory behavior of mosquitoes. The transmission potential is influenced by temperature and humidity[408] and more particularly temperature could influence the time to first bite (not always[409]) and potentially the biting frequency which could delay the parasite development in colder environment[92]. How realistic temperature and humidity affect the modulation of the antennal organs (via currents and internal pressure of the hemolymph) and induce desiccation stress is an understudied component of mosquito biology[410,411] despite its importance for a deeper understanding of the physiology oh host searching and thermoregulation. Recently, a study published the importance of temperature fluctuation on vector competence, as diurnal temperature fluctuation increased the vector competence of evening biting mosquitoes but a decrease for morning biting mosquitoes. Such data emphasize the role of temperature as a major ecological driver of vectorial capacity.

It would be interesting to repeat this experiment with variation in larval conditions to compare the feeding decision and allocation of reserves depending on the presence or absence of insecticide. Unfortunately for this chapter, the data presented do not have a control for the exposure to a LLIN (exposure to an untreated net) at the adult stage due to the lack of material at disposition and space in the lab in Côte d'Ivoire. Only some larval treatment where tested with an untreated net and WHO cones tests where carried on 4-5 days old males and females after emergence in the different larval environment. In order to have similar sample size, only mosquitoes exposed to insecticide were kept for analysis. A malaria transmission model is currently in construction and will incorporate the missing data for further publications.

8.1.4. Chapter 6

In chapter 6, we investigated the effect of one single insecticide exposure in cups on subsequent blood meal opportunities (this time in the absence of insecticide post first exposure) and longevity in extremely insecticide resistant mosquitoes. We found that mosquitoes died more rapidly and were less competent blood feeders once they have been exposed to insecticide, even though they were not visually knock down from the exposure and continued being active and feeding in the following hours. Remarkably, one single exposure at the beginning of mosquito life influenced the rest of mosquito's existence and more particularly it impacted its ability to start new gonotrophic cycles. Yet, a successful blood meal put the potential of vector into perspective as it helped reduce the susceptibility to insecticide.

In this chapter, the electrophysiological responses of mosquitoes pre-exposed to insecticide in cones showed differences in odor detection depending on the time mosquitoes were tested post-exposure and the type of net used to expose them (insecticide treated or untreated net). It turned out that mosquitoes previously exposed to insecticide were less able to detect odors directly after the exposure, but their odor detection came back to the same level as for mosquitoes pre-exposed to an untreated net one hour following the exposure and it was still the same one day later, so there was no obvious long-term detection disturbance. The mechanisms behind this are still unclear and more work must be done to find the reason for such a difference of odor detection. However, we suggest that mosquito feeding behavior may be greatly influenced by a pre-exposure to insecticide. In the field, mosquitoes can be exposed to insecticide before being able to feed when they are host seeking around a damaged net or when they are contacting an intact net and trying to find another less protected host later that night. Further work needs to focus on olfactory perception when mosquitoes are infected with malaria and with wild-type strains from different parts of the world, to better understand the vectorial capacity and competence of resistant mosquitoes across multiple settings. EAG measures are extremely helpful for quickly assessing electrical activity across the antennal flagellum. However, for more precise quantitative measurement of the sensitivity and selectivity of individual olfactory sensory neurons, single sensillum recording is recommended for further studies of mosquito response to odors post-insecticide exposure. This was not available in the field station in Bouake during my thesis.

8.1.5. Chapter 7

In this last chapter, the semi-field experiment in an enclosure with two experimental huts inside helped better understand mosquito behavior around LLIN after a sub-lethal exposure to insecticide in a setting closer to real life. The data obtained confirmed that mosquitoes are not knock-down after exposure and are host-seeking in the hours following an insecticide exposure. Mosquitoes were not found to be repelled by the presence of insecticide, neither the insecticide pre-exposed ones nor the control pre-exposed ones. At a long range, we thought that only the volunteers' odors were detected and not the LLIN as deltamethrin is unlikely to volatilize too far due to its low vapor pressure (1.5×10^{-8} mmHg at 25°C)[412]. However, the data suggests otherwise as mosquitoes were attracted to LLINs in the enclosure (marginal increase of 3 %). In addition, mosquitoes were more motivated to find a host when pre-exposed to a LLIN. Whether this observation was due to insecticide-induced hormesis is unclear and needs further investigation. The stimulatory effects of low-dose of insecticide exposure has been widely studied in invasive species and have been linked to benefices in keys life-history traits[391,393–395]. In mosquitoes, the fact that a high dose of insecticide is harmful to them is well-

documented[168,287,396–398], but less is known about the effects of low doses of insecticide on their biological processes. One of the mechanisms that could be involved in hormetic response of mosquitoes exposed to pyrethroid despite resistance is oxidative stress[413]. Pyrethroids are known to induce oxidative stress[392], which in turn, are associated with tolerance to insecticide[414,415], higher fecundity[416] and increased immunity against bacteria and malaria parasite[417–419].

Anyway, given the marginal preference for the LLIN hut instead of the UTN hut and an overall null effect of insecticide on feeding rate in the enclosure, the efficacy of LLINs was not as convincing as observed in the previous chapters, at least for the mosquito's first bite. How these effects play out exactly in the field is unclear, but this study suggests that LLINs are likely substantially compromised by the level of resistance of the mosquito population as mentioned in several studies[224,225] and the results were not convincing enough to conclude that LLINs offer more personal and community-wide protections than untreated net (based on the data from this chapter). However, the data obtained in the enclosure did not capture the effect of an exposure to insecticide on cumulative blood feeding attempts. As exposures to insecticide negatively affected longevity, we suggest that host-seeking and feeding success over multiple attempts would still be compromised by the presence of a LLIN.

These results help broaden the possibility for novel vector control tool strategies as clearly mosquito response in the face of pyrethroids show no clear sign of spatial repellency, but irritancy which reduce the feeding success (the effect of insecticide on blood feeding success against the proportion of mosquitoes host searching). Further studies need to investigate the long-term effect of insecticide exposure in semi-field and field setups to validate our results in multiple settings. Ultimately, new insights on insecticide-induced deleterious and hormetic effects and mosquito behavioral adaptations associated with insecticide exposure could help find new ways to target disease vectors on various fronts[308,392,394]. This chapter also highlights the importance to distinguish between the qualitative and quantitative effects of LLINs. In fact, the capacity to host-seek and take a blood meal in the presence of insecticide in a short-distance seems unlikely to translate 100 % of the efficacy of LLINs in field settings.

8.2. Further perspectives of research

The establishment of a field-based colony had the advantage to lead to a more reliable and constant source of mosquitoes in comparison to larval collections from the field that may be impacted by a whole range of environmental factors (temperature, pollution, insecticide, predation, nutrients, shade, and so forth).

For this thesis, mosquitoes from the field were regularly added to the insecticide resistant colony and finally maintained under selection to keep the diversity and level of resistance similar to the local populations. That situation drew attention to the fact that mosquitoes adapt to their environment and therefore could potentially lose their resistance status rapidly (if not fixed) in the absence of any selection pressure. One interesting research could be to measure how plastic the insecticide resistance status is depending on the selection pressure in each environment. It would be interesting to measure how fast a colony loses or regains its resistance status and intensity, and which mechanisms of resistance would first disappear if we vary the selection pressure. We could study selection, de-selection or variation in selection pressure. The 're-selection' process could help understand the rapid adaptation to both larval and adult selections (for example insecticide mixed in the larval environment and use of LLIN at the adult stage), which could help clarify the respective impact of agricultural pesticides and larval control in aquatic environments, and vector control tools targeting adult mosquitoes. That would give interesting insights on the evolution of resistance, genetic contribution to phenotypic variation and behavioral adaptation to vector control tools. Additional studies could be performed thanks to different colonies obtained with variable resistance status but with a similar genetic background. These new mosquito lines would represent a great basis for evolutionary experiment focusing on the fitness cost of insecticide resistance.

Talking about adaptation to the environment, the work done with mosquitoes from the field rapidly showed the difficulty to get constant results between replicates. In fact, these mosquitoes come from different environments and are collected at different times of the year. Whether mosquitoes are collected in muddy water in rice fields with high concentrations of insecticide or in clear water, and whether mosquitoes were collected during the dry season or wet season could makes a huge difference in terms of larval development and resistance status. In this thesis, I worked on the effect of the larval environment on mosquito behavior, but more work must be done to understand how realistic changes in the larval environment influences phenotypic expression of resistance. Insecticide resistance monitoring does rarely include data such as time of the year or season, larval environment specificities such as the presence of insecticide, larval density and larval stages collected. Such information could inform models focusing on the variation in the phenotypic expression of resistance and behavioral variation could be link to resistance level from one season to another or one larval collection place to another. More work has also to be done on the effect of larval environment on mosquito behavior in general[420] and in link with vector control tools in particular, very little

information is found in literature despite its huge implication in epidemiology and vector control management.

The amplitude of the electrophysiological (EAG) response and time to respond for each mosquito provide information on the selectivity and sensitivity of the receptor cells responding to an odor. One study did evaluate sensory responses to human odors in *Anopheles* mosquitoes to investigate whether malaria parasites influences the olfactory system or not. It showed evidence that infection can influence the physiology of *Anopheles* with non-transmissible parasites and lower the sensitivity to specific compounds differently depending on the mosquito and parasite species[421]. As parasite stage-specific changes are expected to correlate with behavioral changes in mosquitoes that take an infectious blood meal[53,93], we suggest that the momentary "shut down" effect of insecticide on the olfactory system as described in chapter 6 may vary depending on the infection stage to allow the detection of host attractants when the parasites are ready to be transmitted. This kind of experiment could be linked with behavioral studies on the motivation to feed (and re-feed when interrupted) for malaria-infected mosquitoes exhibiting different insecticide resistant status to study how different is the host seeking behavior in the presence of an untreated net or LLIN. Besides, it has been shown recently that mosquito tissues can be different between resistant and susceptible mosquitoes with a variation in the thickness/composition of the legs' cuticle[160,422]. Are such morphological and physiological modifications linked to potential modifications in behavior? In some extremely resistant populations, mosquitoes exhibit target site resistance through the knockdown resistance mutations as well as metabolic resistance[230]. Despite the opportunity to detoxify residual insecticide thanks to several DNA markers encoding enzymes that break down insecticide[423], we observed a long-term impact of LLIN exposure on mosquito longevity and behavior in different bioassays setups. So, what are the mechanisms behind these changes in physiology and behavior and can we use those to target the malaria vector accordingly?

Another area of research to pursue is the learning capacity and memory of mosquitoes. In fact, mosquitoes interact with LLIN in a way that is still largely misunderstood. For example, do mosquitoes associate the presence of an LLIN with the potential to take a blood meal, overstepping the insecticidal and irritant effects of LLINs? Do mosquito experience long-term associative learning, a process which associate a stimulus with a specific response. And if so, does a previous exposure to insecticide motivate mosquitoes to choose a host protected by an LLIN over a host protected by an untreated net? Does a mosquito remember the irritant effect of insecticide when its legs or gustatory organs contacted a LLIN? If so, would that result in a reduction in blood feeding during subsequent feeding

opportunities? When mosquitoes find a way through a holed net before a blood meal, do they find the way out more easily? A whole new area of research could be to investigate how LLIN are detected and whether mosquitoes learn to become better at finding a host protected under an LLIN. Also, if kdr mutation modulated the host choice in *An. gambiae*[290], it urges to better understand how changes in mosquito behavior relates to changes in physiological responses and genetic modification.

8.3. Thesis conclusion

Overall, the synthesis of this thesis is that insecticide exposure affects life history traits of extremely insecticide resistant mosquitoes, by reducing both blood-feeding compliancy and longevity. Even in region with high insecticide resistance, pyrethroid nets might still work, not only as physical barriers but by targeting different parameters of mosquito vectorial capacity. Our lab results confirm LLIN efficacy and could help explain why the increase of resistance is not yet linked to more malaria transmission[227]. However, this thesis makes a strong case for moving beyond standard laboratory conditions to further study mosquito basic biology and its interaction with human hosts and more data are needed to fully understand LLIN efficacy in the field. Currently, the basic bioassays for insecticide resistance and evaluation of vector control tools are somewhat arbitrary when considering mosquito behavior and biology and many questions remain unanswered, they do not correspond (and/or are not comparable) to what is happening in the field. The natural environment is extremely complex, and one cannot control all the factors involved. Though we can investigate environmentally mediated changes by isolating and controlling one or two variables in the laboratory and in semi-field experiments. Generally, we need to integrate more mosquito physiology and behavior parameters in the evaluation of vector control tools to better understand their efficacy.

To conclude, a stronger effort needs to be made to prevent malaria transmission and maintain the efficacy of current vector control tools as well as further develop resistance management plan[208]. In this thesis, we focused on mosquito behavior around LLIN and the efficacy of that specific vector control tool. But the development of complementary insecticide-based control tools is needed, and their evaluation should integrate parameters involved in the vectorial capacity of mosquitoes to achieve relevant regulatory approvals. The goal is to develop appropriate implementation strategies so that individual technologies can be tailored to local ecological and socio-economic contexts and combined into optimum integrated vector management strategies. Progress towards this approach will help sustain the downward trajectory in malaria burden and provide the platform for the next

generation of tools (e.g. transgenic mosquitoes) and approaches (e.g. drug and vaccine strategies) to deliver on the ultimate goals of malaria elimination and eradication.

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Opinion

Priorities for Broadening the Malaria Vector Control Tool Kit

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Long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) have contributed substantially to reductions in the burden of malaria in the past 15 years. Building on this foundation, the goal is now to drive malaria towards elimination. Vector control remains central to this goal, but there are limitations to what is achievable with the current tools. Here we highlight how a broader appreciation of adult mosquito behavior is yielding a number of supplementary approaches to bolster the vector-control tool kit. We emphasize tools that offer new modes of control and could realistically contribute to operational control in the next 5 years. Promoting complementary tools that are close to field-ready is a priority for achieving the global malaria-control targets.

Trends

The past decade has seen a dramatic decline in the burden of malaria, with vector control playing a central role. The aim is now to build on this recent success and progress towards elimination.

Current core vector-control tools alone are insufficient to achieve this goal, as they fail to target all adult mosquitoes, and emerging insecticide resistance is threating their effectiveness.

By considering the full range of adult mosquito behaviors, a number of supplementary tools, now under development, complement the core tools and create opportunities for tackling resistance and improving overall control.

Vector Control and Malaria

The World Health Organization (WHO) recently published its *Global Technical Strategy for Malaria 2016–2030*, which sets out a vision and strategic framework for reducing malaria transmission by at least 90% over the next 15 years, and preventing its re-establishment in countries that are currently free of malaria [1]. Vector control is a central pillar within this Global Technical Strategy, reflecting the fact that wide-scale deployment of long-lasting insecticide-treated bed nets (**LLINs**, see Glossary) and indoor residual spraying (**IRS**) with insecticides have contributed to substantial declines in the burden of malaria in the past 15 years [1,2]. However, the robustness and utility of current vector control faces two key biological challenges. First, the negative impacts of insecticide exposure on survival and reproduction impose strong selection for resistance [3]. This problem is exacerbated by the fact that there is a very limited selection of chemical insecticides approved for public health use; at present, pyrethroids are the only class of insecticides used on a wide scale on bed nets, and they account for two-thirds of the total product (by area) used in IRS for malaria control [4]. Accordingly, **physiological** (and, to a lesser extent, **behavioral**) **resistance** is now widespread across mosquito species and populations, threatening the effectiveness of the frontline insecticide-based interventions [1,5]. Second, the current core tools are most effective against *Anopheles* vectors that feed and rest indoors and exhibit a preference for feeding on human hosts during night-time [2]. Yet, in many locations, vectors exhibit more diverse behaviors, feeding on other hosts, feeding and resting outdoors, and/or feeding in the early evening [6–8]. A consequence of both of these challenges is that there are limits to how much LLINs and IRS alone can reduce transmission, even with further intensification and optimization [9]. This problem creates a pressing need for supplementary vector-control tools.

Exploration of vector-control tools is a rich area of research. A recent review commissioned by the United States President's Malaria Initiative highlighted examples of 12 broad technologies/approaches for new interventions, including new types of LLIN with resistance-breaking properties[i]. Another recent analysis evaluated the evidence for 21 existing and emerging

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vector-control tools excluding LLINs and IRS[ii]. Other reviews have focused on more specific strategies, such as biologically based or transgenic approaches [10,11]. Given these recent articles, our aim here is not to conduct an exhaustive review of prospective control tools. Rather, we outline two key criteria that we consider important in prioritizing the development of supplementary vector-control tools: a mode of action that is complementary to current tools, and a short timeline for implementation. Based on these criteria, we highlight a handful of tools/approaches that we feel have greatest immediate potential to add to the malaria vector-control tool kit.

Timeline to Impact

As described above, the WHO *Global Technical Strategy for Malaria 2016–2030* aims to reduce malaria transmission by at least 90% over the next 15 years. Similar ambitious targets are set out in the *Aspiration to Action* document prepared by the Bill and Melinda Gates Foundation[iii], which calls for a halving in transmission every 10 years, leading to ultimate eradication by 2040. Intercountry alliances, such as the Asia Pacific Malaria Elimination Network, aim for regional elimination by 2030[iv].

The Global Technical Strategy is informed by a modeling analysis which explores a range of future intervention scenarios that vary in terms of access to vector control (LLINs and IRS) and drug treatments (both seasonal malaria chemoprevention and first-line treatments with artemisinin combination therapy) [9]. The modeling analysis reveals a number of key insights (Figure 1). First, if vector control and drug use remain at current levels, malaria mortality is expected to increase in the next 10–15 years due to a changing immunity profile in the population, wherein people born after the current interventions were scaled up are exposed more slowly and acquire their first and subsequent cases at an older age. Second, if the effectiveness of existing tools falls (e.g., through evolution of resistance) the rebound in malaria burden will likely be more pronounced. Third, further intensification of existing core tools to 80 or 90% coverage can lead to reductions in malaria burden, and even elimination in some settings, but would fail to reach the anticipated targets in areas of intense transmission. Finally, only if supplementary tools are forthcoming within the near future is it predicted that the WHO targets can be achieved.

The requirement for supplementary tools to be implemented at scale within the next 5 or so years puts an emphasis on approaches that are close to field-ready, and limits the immediate utility of prospective tools that are still far from operational (Figure 1). For example, there is considerable interest in the potential of new gene-editing technologies for developing transgenic mosquitoes for use in population-replacement or population-suppression strategies [12–15]. Approaches to reduce **vector competence** by manipulating elements of the mosquito microbiome [16–18], or via transinfection with endosymbionts such as *Wolbachia* [19,20], are also being examined. However, given the current exploratory nature of this research (in most cases the research has yet to progress beyond laboratory-based proof-of-principle studies), together with the challenges and timelines of regulatory approval, it is questionable whether such technologies will achieve wide-scale operational use for malaria control within the next 8–10 years. This argument does not mean that these technologies cannot make valuable contributions somewhere down the line. Nonetheless, it is very difficult to see how they can play a substantial role in averting the present-day insecticide-resistance crisis, or in driving down malaria transmission in the next decade (Figure 1).

Complementing Existing Vector Control

Because transmission of malaria is so directly linked to the bite of the mosquito, a lot of research focuses on blood feeding behavior and factors affecting vector competence. Yet the life cycle of the adult mosquito involves much more than taking and digesting a blood meal.

Glossary

Anthropophilic: a preference for feeding on humans and resting in and around domestic dwellings.
Behavioral resistance: changes in vector feeding or resting behavior that reduce insecticide exposure.
Community-wide effect: a reduction in transmission risk at community level even though only a certain proportion of the community is protected directly by an intervention. It occurs, for example, when an intervention kills mosquitoes and so reduces the vector–human contact for the whole community.
Entomological inoculation rate (EIR): a measure of human exposure to infectious mosquitoes defined as the number of infectious bites received by a person over a given time period (usually a year).
Gonotrophic cycle: describes a life cycle of alternate feeding and laying eggs. The duration of the gonotrophic cycle is defined as the number of days that gravid mosquitoes take to lay their eggs after taking a blood meal.
Indoor residual sprays (IRS): spraying the walls and other surfaces of a house with a residual insecticide that is designed to kill mosquitoes as they rest after blood feeding.
Integrated vector management (IVM): the optimal use of diverse tools, tactics, and resources to reduce transmission of disease by vectors.
Long-lasting insecticide-treated net (LLIN): a bed net coated or impregnated with insecticide that is designed to remain effective for 3–5 years and 20 washes.
Physiological insecticide resistance: reduced susceptibility to an insecticide by changes in basic physiology, including target-site mutations that reduce neuronal sensitivity, and metabolic mechanisms that enhance detoxification.
Residual transmission: malaria transmission that persists after full operational coverage with effective LLIN and/or IRS interventions has been achieved.
Vector competence: physiological and behavioral characteristics that shape a vector's capability to transmit a pathogen (i.e., become infected following an infectious blood meal, successfully harbor the

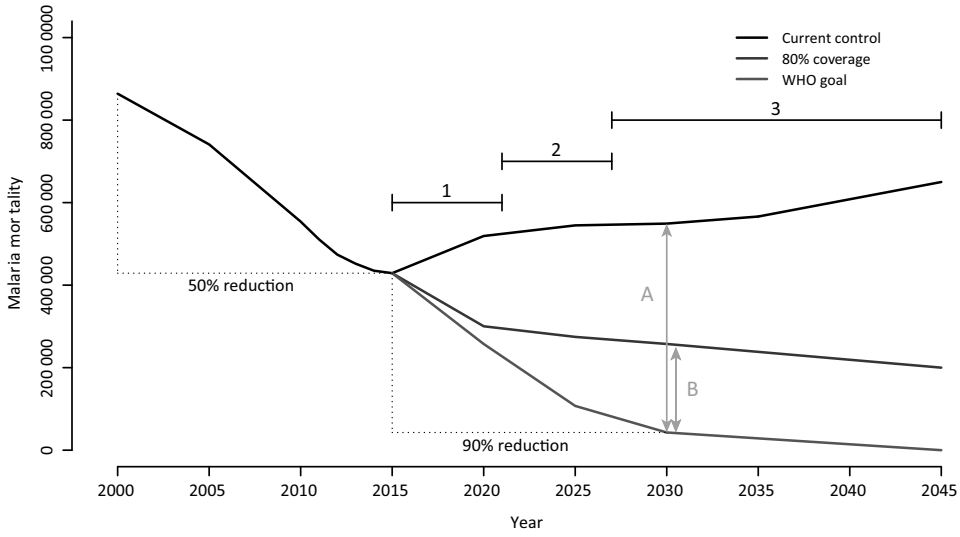

Figure 1. Estimate of Historic and Projected Global Deaths Due to Malaria Based on Different Control Scenarios. The figure shows estimates of global malaria deaths from 2000 to 2045. The 50% decline in malaria-related mortality recorded from 2000 to 2015 is largely attributable to the wide-scale implementation of vector-control tools [long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS)] [1,2]. The future projections are based on a model analysis that considers different scenarios of access to vector control, together with malaria drug treatments [9].
The graph was modified from Figure 1B of Griffin *et al*. [9] by using data from the 2016 *World Malaria Report* [89] to convert the original y-axis of 'deaths per 1000 people per year' into estimates of overall malaria mortality per year, and adding the target line for future decline in malaria deaths from the WHO Global Technical Strategy. The black line indicates resurgence in malaria deaths if control efforts remain at current levels. The blue line is the predicted decline in deaths assuming coverage of current control tools can be increased to reach 80% of the population at risk. The red line represents the target set out in the WHO Global Technical Strategy [1], which aims for a 90% decline in malaria deaths by 2030 and then ultimate elimination thereafter.
The arrows A and B illustrate the differences between the WHO target and the two control scenarios. Business as usual clearly represents a massive failure (A). Perhaps more notably, even substantial intensification of existing tools still yields a substantial shortfall (B). These gaps in control demonstrate the need for new interventions. The numbered horizontal lines refer to the estimated timelines for implementation of a range of prospective control tools where: (1) refers to tools that are close to field-ready (e.g., attractive toxic sugar baits, housing improvement, livestock targets, next-generation LLINs and IRS); (2) represents tools that require a few more years for product development (e.g., improved topical repellents, long-lasting endectocides for human use); and (3) represents tools that either for technical and/or regulatory reasons are still far from operational use (e.g., transinfection with *Wolbachia*, population replacement strategies using genetically modified mosquitoes and gene drive). The fact that the WHO target shows an immediate deviation from the two control scenarios highlights a critical role for tools that can be implemented in the short and medium term (1 and 2).

A young adult mosquito emerges from the aquatic larval habitat with a small reserve of energy [21]. Both male and female mosquitoes then consume sugars, mainly obtained from floral and extrafloral nectar, and honeydew [22]. Mating does not occur for a couple of days after the adults emerge. Males form mating swarms and virgin females enter these swarms, locate a male, and then exit as a couple to mate [23]. To complete the first **gonotrophic cycle**, most female mosquitoes must next take a blood meal. The host could be a human or, depending on the feeding behavior, an alternative vertebrate such as a cow [24]. Feeding can take place indoors or outdoors depending on the species and their populations [6]. To digest a blood meal safely, and before the onset of searching for an oviposition site, a female will rest for 2–4 days. Resting can take place indoors or outdoors, again depending on the species [25]. After blood digestion, a female has to find a suitable oviposition site, which, in some cases, can be distant and take several days to locate, during which time there is likely more demand for sugars [26]. Because human malaria parasites take around 8–12 days to complete the sporozoite cycle within the mosquito under optimum temperatures (and this can be considerably longer under

parasite as it develops, and pass the parasite on to a susceptible host in a later blood meal).
Zoophilic: a preference for feeding on nonhuman hosts (such as cattle) and potentially resting in and around livestock structures such as cattle sheds.

suboptimal conditions) [27,28], female mosquitoes must survive at least three such gonotrophic cycles before being able to transmit malaria [29] (Figure 2).

All of these mosquito activities, and the locations in which they take place, provide opportunities for disrupting the adult mosquito's life cycle, and hence, reducing transmission. LLINs and IRS work by lowering the contact rate between humans and vectors, either because the insecticide changes the normal feeding or host-searching behavior (repellency or deterrence) [30], and/or the insecticide causes mosquito death, affecting the age structure of the mosquito population and potentially the adult mosquito density [31]. Because of the importance of these core tools, and the potential for insecticide resistance to render them less effective, development of next-

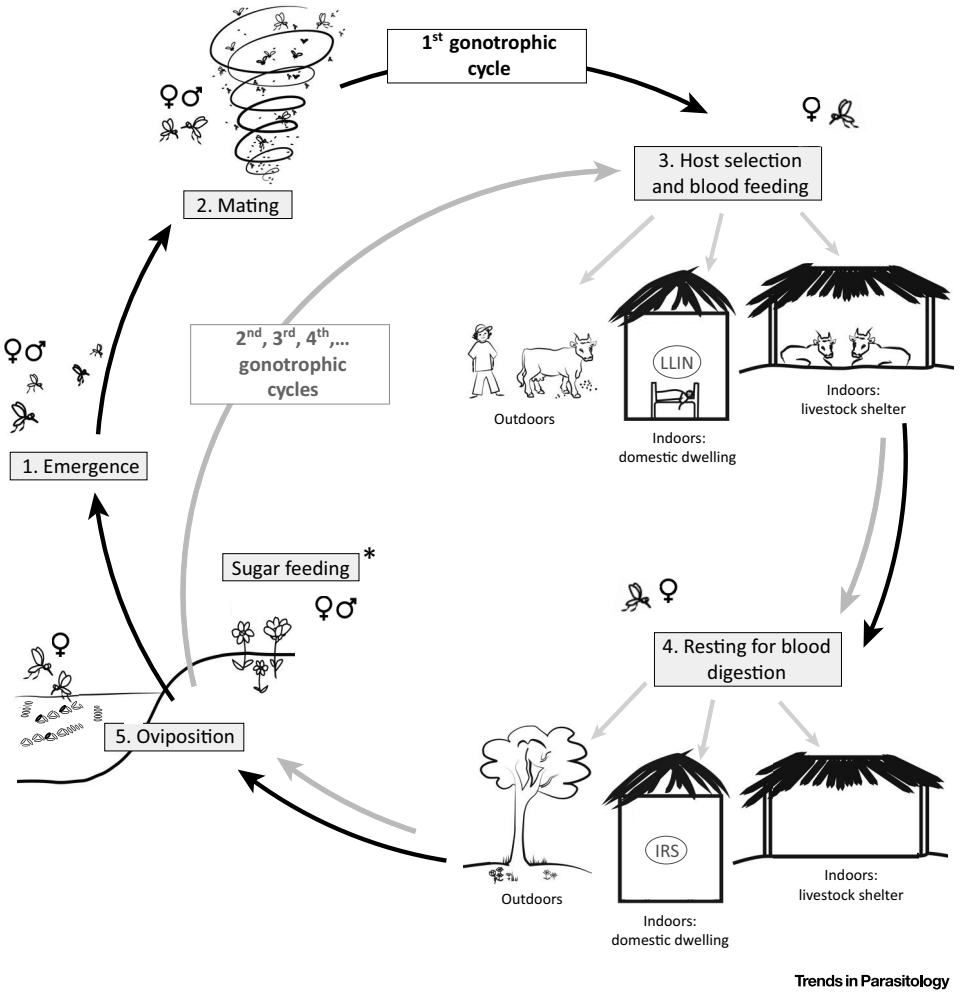

Figure 2. Diverse Behaviors and Activities of Adult Malaria Mosquitoes as They Progress From Emergence Through to Egg Laying Over One or More Gonotrophic Cycles. Adult mosquitoes emerge from aquatic habitats (1) and mate within a few days (2), potentially taking a sugar meal for energy (*). Male mosquitoes then tend to die quite quickly, while females go in search of a blood meal (3). Blood feeding could be on a diversity of hosts, either indoors or outdoors. After blood feeding, the mosquitoes will tend to rest for 2–4 days while they digest the blood to produce eggs (4). Resting can occur in a range of indoor or outdoor environments. Once the eggs are fully developed the mosquitoes then search for a suitable oviposition site (5), potentially taking another sugar meal (*) to boost energy reserves for flight. Once a suitable aquatic habitat is located and the eggs are laid, female mosquitoes can repeat the blood feeding and egg production process over subsequent days to complete multiple gonotrophic cycles.
Current core vector-control tools [long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS)] target female mosquitoes at just two points in the adult life cycle within domestic dwellings only.

generation LLIN and IRS products comprising novel active ingredients, that overcome resistance, is an important ongoing activity [32]. Nevertheless, LLINs and IRS target only mosquitoes inside the domestic dwelling, leaving activities such as sugar feeding, mating, outdoor biting, host searching and house entry, alternate host feeding, outdoor resting, etc. untouched (Figure 2). Also, LLINs and IRS generally impact only females. Supplementary tools that target adult mosquitoes more broadly, at multiple points across their life cycle, are needed to complement these established tools and, in so doing, address the challenge of **residual transmission** and create new opportunities for insecticide-resistance management.

Candidate Tools

In Table 1 we provide an illustrative (not exhaustive) list of adult vector-control tools that are currently being researched (i.e., have been published on in recent years) and assess them according to our criteria of 'field-ready' and 'complementary'. We also outline briefly some of the challenges we face in order to move forward to operational use. This assessment is somewhat subjective, but our aim is to highlight technologies that bring something new to the table (Figure 2) and identify a feasible timeline for implementation (Figure 1). Below, we discuss in more detail a number of tools/approaches that we feel have greatest immediate utility.

Sugar Feeding

Attractive toxic sugar baits (ATSBs), which utilize a mixture of an oral toxin, natural sugars, and floral attractants to lure mosquitoes [33,34], take advantage of the natural propensity of both male and female mosquitoes to sugar feed. ATSBs can be used in outdoor bait stations or indoor bait stations, or they can be sprayed directly onto nonflowering vegetation [35–37]. The products appear inexpensive and require minimal change in user behavior [38]. Moreover, the wide choice of candidate stomach toxins creates options for control of mosquitoes resistant to the currently used contact insecticides [39].

A small-scale field trial in Mali showed that ATSBs sprayed onto vegetation reduced the population of *Anopheles gambiae* s.l. by 90% [40]. A similar study in the Rift Valley showed a 95% reduction in female *Anopheles sergentii* populations, while completely eradicating males [41]. Even with indoor bait stations, both male and female mosquitoes were attracted to, and fed from, this source, with more than 90% reduction in populations [38]. Moreover, these studies report changes in population age structure towards younger mosquitoes; this is an important result as it is the old mosquitoes that are responsible for transmission. A recent modeling study showed that ATSBs could substantially reduce *An. gambiae* populations and associated **entomological inoculation rates** (EIRs) to near zero, in both sugar-resource-rich and sugar-resource-poor environments [42]. Evaluating this prediction empirically, and exploring the full range and potential usage of ATSBs in future integrated vector-control strategies more generally, are key next steps.

Swarm Sprays

Another underexploited target for vector control is swarming behavior [43]. The locations of mating swarms are stable over the seasons [44] and appear linked to swarm markers on the ground, such as wells, wood piles, or the limits between footpaths and grass [45,46]. These markers seem to provide visual cues for the males [43]. The proposed strategy for targeting these swarms is to use field observations and Geographic Information Systems (GIS) [43,47] to map swarm locations and then spray swarms with insecticide when they start forming, just after sunset. The swarms are generally accessible, as they are only 1–3 m above the ground, depending on the swarm markers [45,46].

A recent field trial conducted in Burkina Faso recruited a team of 20 volunteers from a village and targeted 300 swarm locations, spraying swarms with aerosols as they appeared over a

Table 1. Illustrative List of Prospective Tools/Approaches for the Control of Adult Malaria Vectors, Outlining Modes of Action, Whether the Tool is 'Field Ready', the Estimated Time to Operational Use, and Some of the Remaining Research and Development Challenges

Control tool[a]	Complementary mode of action	Field-ready technology	Esimated time to use	Challenges	Refs
Attractive toxic sugar bait	YES: Targets both sexes of diverse mosquito species; repeated exposure across life time; independent of blood feeding or resting behavior; resistance-breaking actives.	YES: Ongoing small-scale field trials; simple technology with available products.	0–5 years	Short lifespan when used as sprays on vegetation; further work needed to evaluate in different ecological contexts and to optimize within integrated vector management (IVM) strategies; nontarget evaluation.	[33–42]
Swarm sprays	YES: Targets males and also pregravid females; independent of blood feeding or resting behavior; resistance-breaking actives.	YES: Ongoing small-scale field trials; simple technology with available products.	0–5 years	Large number of swarm targets; demonstrate impact across diverse species and ecosystems; needs optimization within IVM; cost evaluations and implementation strategies required (who sprays and who pays?).	[43–49]
Housing improvement	YES: Prevents house entry and protects users without LLINs[b]; independent of insecticide resistance; potential for resistance-breaking actives in Eave Tubes.	YES: Numerous available approaches and new technologies (like Eave Tubes) under large-scale field evaluation; existing field trials and meta-analyses support impact; housing improvement is already happening across many disease-affected countries.	0–5 years	Further research required on appropriateness in different socioeconomic settings; need for cost-effectiveness evaluations and exploration of different implementation strategies.	[50–67]
Livestock targets	YES: Addresses problem of zoophilic vectors.	YES: IRS[c] of livestock structures can use existing technology; numerous topical insecticides and endectocides on the market.	0–5 years	Need for longer lasting endectocides to reduce treatment frequency; not all livestock are treatable or have defined housing structures; cost and effectiveness across different systems and socioeconomic contexts.	[68–82]
Spatial repellents	YES: Potentially protects users before they go indoors, and users without LLINs.	YES: Certain products already commercially available and used.	0–5 years	Need for improved long-lasting products; costs likely prohibitive in certain settings and require financial contribution from end-user (consumer products not covered by normal public health budgets); appropriate targeting and optimizing within IVM.	[83–87]
Next-generation LLINs	NO: Resistance-breaking, but same limitations as conventional LLINs.	YES: Certain nets are available now and more are under development.	0–5 years	Current next-generation nets cost 2–3 times as much as standard LLINs; not clear that they completely restore efficacy and improve control in all locations; resistance can still evolve.	[90–93]
Next-generation IRS	NO: Resistance-breaking, but same limitations as conventional IRS.	YES: Certain new products are available and more are under development.	0–5 years	New IRS products cost more than existing IRS so either more money needs to be made available or fewer houses are sprayed; resistance can still evolve; many countries don't use IRS.	[94–96]
Sterile insect technique via irradiation	YES: Targets all females and subsequent offspring; independent of blood feeding or resting behavior; independent of insecticide resistance.	YES/NO: Small-scale field trials with *Anopheles arabiensis* but not yet applicable to other species.	4–8 years	Fitness costs of irradiation; challenges of mass rearing and sorting of males; mating competition with wild types; dispersal constraints; mixed species complexes; public acceptance.	[10,11]

Table 1. (continued)

Control tool[a]	Complementary mode of action	Field-ready technology	Esimated time to use	Challenges	Refs
Topical repellents	YES: Independent of blood feeding or resting behavior.	YES/NO: Products do exist but little demonstrated protection against malaria infection (both field trials and meta-analysis).	4–8 years	Need clearer efficacy data; costs; short-duration products needing repeat application; user acceptance; potential for resistance.	[97–99]
Endectocide for humans	YES: Targets mosquitoes whenever they feed on humans, including outdoor biters.	YES/NO: Some products readily available and undergoing field-testing but persistence issues possibly limit current utility.	4–8 years	Need for longer lasting formulations; need better understanding of mode of action; need more efficacy data on lethal and sublethal effects; safety constraints; public acceptance; safety monitoring.	[78,81, 100–102]
Transinfection with *Wolbachia*	YES: Population replacement or suppression approaches work irrespective of blood feeding or resting behavior, and insecticide resistance.	NO: Laboratory proof-of-principle only.	8–10 years	Development of technology (stable transinfection in only one *Anopheles* species so far); works best with low-density populations; potential fitness costs affecting dispersal and mating behaviors; effectiveness across environments; mass rearing; species complexes; environmental and ethical safety; regulation; public acceptance.	[10,11, 19,20]
Population suppression strategies via genetic modification	YES: Targets all females and subsequent offspring; independent of blood feeding or resting behavior, and independent of insecticide resistance.	NO: Laboratory proof-of-principle only.	8–10 years	Development of technology (there is no clear product as yet for malaria vectors); potential fitness costs; effectiveness across environments; challenges of mass rearing; mating competition with wild types; dispersal constraints; mixed species complexes; environmental and ethical safety; regulation; public acceptance.	[10–18]
Population replacement strategies using genetically modified mosquitoes and gene drive	YES: Many possible modes of action independent of blood feeding or resting behavior, and independent of insecticide resistance.	NO: Laboratory proof-of-principle only.	8–10 years	Development of technology (there is no clear product as yet); potential fitness costs; effectiveness across environments; dispersal constraints; mixed species complexes; resistance evolution against transgene or gene drive; environmental and ethical safety; regulation; public acceptance.	[10–18]

[a]Tools highlighted in bold are those that have modes of action that complement current tools (i.e., they target different mosquito behaviors or different segments of the mosquito population compared to conventional LLINs and IRS) and are sufficiently advanced that they could be implemented in the near term (i.e., they either are, or are close to being, 'field ready').
[b]LLINs, long-lasting insecticide-treated nets.
[c]IRS, indoor residual sprays.

9-day period. These spray treatments reduced mosquito (*An. gambiae* s.l.) density by 80% over a period of 10 days compared with a control village, and also caused a significant reduction in the female insemination rate [48]. Other similar studies show equivalent results [43]. As with ATSBs, further work is required to fully evaluate and optimize the spraying pattern and frequency across a wider range of settings, and to determine cost effectiveness. However, swarm spraying requires little specialist equipment, and all the major African malaria vectors, as well as certain Asian and Latin American species [49], elicit swarming behavior, suggesting considerable potential for the approach. Importantly, swarm sprays target males and pre-blood-fed females, so any impact is independent of the blood feeding and resting behavior that can affect LLINs and IRS [43].

House Entry

Houses are not the only location where malaria transmission occurs, but they remain the most important transmission environment in many endemic areas [50–52]. Even with outdoor biting and transmission, there is evidence that a mosquito is likely to enter a house at some point during its life prior to delivering an infective bite [53]. Accordingly, one complementary vector-control intervention is to modify the house to limit mosquito entry.

Modern houses tend to be more protective against malaria than traditional houses made of natural materials that leave multiple gaps through which mosquitoes can enter [54], and in some settings they offer protection equivalent to LLINs [55]. What constitutes modern housing is context dependent, but generally includes a shift in the type of building materials from thatched roofs to metal, and from mud walls to brick or concrete. Houses might also include finished flooring, ceilings, improved doors, window screening, and closed eaves. All of these changes help to make a house more mosquito-proof and can reduce malaria in the inhabitants [56–59].

None of the standard house modifications require new technology *per se*, but there is a recent innovation that could add to the impact by combining house improvements with targeted insecticide treatment, effectively turning the house into a lethal lure. Open eaves are an important source of host attractant cues and a key entry point for *An. gambiae* s.l. in Africa [60,61]. Closing the eaves is, therefore, an important mosquito-prevention measure. Eave tubes are pieces of PVC pipe that can be fitted to partially reopen the eaves. The eave tubes contain an insert comprising insecticide-treated netting that kills mosquitoes as they attempt to enter the house through the tubes [62,63]. An electrostatic coating on the insert screening allows for the use of powder formulations of insecticides, a delivery method that is highly effective even against resistant mosquitoes [64]. One benefit of the lethal house lure approach is that it is a passive technology that protects everyone sleeping in the house (IRS is a household-level intervention but generally does not prevent house entry; LLINs provide personal protection but rarely does everyone in a house use a net). As coverage of eave tubes increases, a **community-wide** mass action **effect** is also predicted [65].

Eave tubes require only small quantities of insecticide per house, enabling the use of insecticide products that might be too expensive for use in IRS. Replacement of inserts is also very easy, potentially providing a method for delivering insecticides with rapid turnover that would not be appropriate for IRS or LLINs. Beyond diversifying the active ingredients available for vector control, the flexibility and potential for rapid turnover could provide a real opportunity to implement insecticide-resistance management strategies that use insecticide rotations, mosaics, or mixtures [66]. Other house modifications, such as insecticide-treated eave and window screening [67], or insecticide-treated eave baffles [68], could offer similar opportunities, and increase options for extending the 'lethal house lure' approach to a broader array of house types (note, however, that eave baffles are designed to allow mosquitoes to enter the house and so, like IRS, do not necessarily provide direct protection against biting). The cost effectiveness of any of these approaches requires further research, and will likely depend strongly on the nature of the local housing. However, leveraging private and public investment in housing improvement could provide a means to improve public health without adding burden to existing public health budgets.

Targeting Livestock

Certain key malaria vectors are strongly **anthropophilic**. However, there are many vector species or populations that exhibit more diverse behavior, feeding on livestock (**zoophilic** behavior) as well as on humans. While feeds on nonhuman hosts represent 'wasted bites' in terms of acquiring or passing on the malaria parasite, they allow the mosquito to escape the

effects of interventions, like IRS and LLINs, that center on the human host. Targeting these mosquitoes with livestock-based interventions could play an important role in reducing residual transmission [7,8,69].

Mosquitoes feeding on livestock could be targeted through treatment of livestock structures (e.g., IRS of cattle sheds). This approach is attractive as the technology exists, livestock structures tend to be less numerous than households (e.g., [70]), and many of the challenges that apply to conventional IRS (such as inconvenience of householders having to be available to grant access and remove furniture, concerns over odors or staining of walls, etc.) are less relevant [7]. In addition, it might well be possible to use different chemical products than those approved for use in domestic dwellings, providing opportunities for resistance management [7]. Where structures do not exist, livestock-baited tents [71,72] and the use of LLIN fences as livestock enclosures [73] have been shown to kill mosquitoes and reduce mosquito numbers indoors.

Direct treatment of cattle with insecticides by dipping, sponging, or spraying has also been shown to kill mosquitoes [74,75] and to reduce malaria in the human population [76]. One of the challenges in this approach is that many of the candidate insecticides are pyrethroid-based [72,77], and the pyrethroid resistance in *Anopheles* populations is particularly widespread in Africa. An alternative is the use of systemic veterinary insecticides (referred to as endectocides) that affect the mosquitoes upon blood feeding. Ivermectin has been successfully tested in cattle and demonstrated to both kill mosquitoes and shorten the lifespan of survivors [78,79]. Other candidate endectocides are also being explored [80,81], as well as slow-release formulations that could reduce the frequency of retreatment [82].

Spatial Repellents

Spatial repellents (i.e., airborne chemicals that reduce human–vector contact by eliciting one or more changes in insect behavior) have been researched for many years and shown to have potential to reduce transmission (see [83] for overview), including randomized controlled trials demonstrating epidemiological impact of commercially available products [84,85]. A feature of spatial repellents is that they can potentially provide protection in the evening before householders go to sleep and so could be complementary to LLINs [84]. They might also be utilized where LLIN or IRS use is minimal [86]. One of the operational challenges, and the subject of ongoing research, is the development of long-lasting formulations or delivery systems to increase user acceptance and cost-effectiveness [83,87]. However, the use of available consumer products (coils, vaporizers, impregnated mats, etc.) has been correlated with lower risk of malaria at the household level, depending on transmission environment and socioeconomic status [86]. As such, these tools already appear to be contributing, albeit with little strategic integration into control programs.

Concluding Remarks

Increasing the coverage and overall effectiveness of vector control is key to achieving the targets of the WHO Global Technical Strategy for malaria, and the broader goals of elimination and eradication. The current tools, LLINs and IRS, provide the foundation, and intensifying their use is a priority. To maintain the effectiveness of these core tools moving forward there is a need for novel chemical actives that circumvent insecticide resistance (but see Outstanding Questions). However, to supplement existing vector control, to target behavioral as well as physiological resistance, and to address the challenges of residual transmission, requires supplementary methods that target mosquitoes more broadly. Moreover, in order to avert an anticipated rebound in malaria due to waning natural immunity and potential impacts of insecticide resistance, it is essential that new tools enter into operational use within the next 5 or so years.

Outstanding Questions

How much will emerging pyrethroid resistance reduce the effectiveness of core vector-control tools? Better understanding of the effect size of resistance on malaria transmission would help to define the magnitude of the 'control gap' that needs to be filled by supplementary tools.

How good does a novel control tool need to be in order to justify implementation? Determining the value of a technology not only depends on local ecology and socioeconomic context but also becomes increasingly complex when multiple tools are deployed together. Integrated strategies might well deliver better overall control but it is almost inevitable that there will be some redundancy between tools.

How do we best combine tools to develop locally effective and sustainable integrated vector management strategies, and how should these integrated strategies be evaluated? Conventional randomized controlled trials are extremely challenging when there are multiple factorial combinations of treatments and when effect sizes become small.

Can we leverage mechanisms outside the traditional public health sector (such as consumer products and housing improvement) to promote technologies and help bridge funding shortfalls for malaria control?

Can regulatory and approval mechanisms be streamlined to facilitate adoption of new tools without compromising necessary data on safety and efficacy? For example, can measurements of entomological impact be used as alternatives to standard epidemiological impacts?

The tools we have highlighted here (ATSBs, swarm sprays, housing improvements, livestock treatments, spatial repellents) are among those that both complement existing control and have the potential to be implemented at scale in the near future. In order to make this a reality, a number of interrelated challenges remain (see Outstanding Questions). First, each of the candidate technologies needs further research to evaluate impact and achieve relevant regulatory approvals. Most crucially, there is a need to demonstrate epidemiological impact, as this is the current gold standard for evaluation. Large-scale epidemiological trials are underway for some tools, but further efforts (and hence funding) are required to build the evidence base. One uncertainty here is what constitutes a sufficient body of evidence given both the urgent need for supplementary tools, and the diversity of malaria transmission ecologies and socioeconomic settings. Once proof of principle has been demonstrated in a single epidemiological trial, it might be better to focus efforts on challenges of implementation, rather than conducting further trials in the hope of satisfying the notion of generality. Second, there is a need for economic evaluation and analysis of factors that influence the potential for scale-up, such as user acceptance, supply chains, and distribution networks, costs, and willingness to pay across different market sectors, etc. Third, there is a need to develop appropriate implementation strategies so that individual technologies can be tailored to local ecological and socioeconomic contexts, and combined into optimum **integrated vector management strategies** [88]. The emergence of supplementary technologies creates new challenges for operational control. For example, should a particular national malaria control program choose ATSBs, or endectocides, or eave tubes, or is there a benefit in combining all three? Answering such questions empirically through the classical approach of randomized controlled trials is extremely challenging. However, if this is the evidence that is required, such trials will need support. Progress to address these challenges over the next 5 years will maximize the chances that these tools can help to sustain the downward trajectory in malaria burden and provide the platform for the next generation of tools (e.g., transgenic mosquitoes) and approaches (e.g., combined vector, drug, and vaccine strategies) to deliver on the ultimate goals of elimination and eradication.

Author Contributions

All authors contributed to the manuscript.

Resources

[i]www.vector-works.org/wp-content/uploads/Vector-Control-Landscape-2015.pdf

[ii]www.rollbackmalaria.org/files/files/working-groups/VCWG/New challenges%2C new tools in vector control/2_Allison Tatarsky.pdf

[iii]www.mmv.org/newsroom/publications/aspiration-action-what-will-it-take-end-malaria

[iv]http://aplma.org/upload/resource/files/APLMA_Roadmap_final_EAS_2015.pdf

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Rethinking the extrinsic incubation period of malaria parasites

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Abstract

The time it takes for malaria parasites to develop within a mosquito, and become transmissible, is known as the extrinsic incubation period, or EIP. EIP is a key parameter influencing transmission intensity as it combines with mosquito mortality rate and competence to determine the number of mosquitoes that ultimately become infectious. In spite of its epidemiological significance, data on EIP are scant. Current approaches to estimate EIP are largely based on temperature-dependent models developed from data collected on parasite development within a single mosquito species in the 1930s. These models assume that the only factor affecting EIP is mean environmental temperature. Here, we review evidence to suggest that in addition to mean temperature, EIP is likely influenced by genetic diversity of the vector, diversity of the parasite, and variation in a range of biotic and abiotic factors that affect mosquito condition. We further demonstrate that the classic approach of measuring EIP as the time at which mosquitoes first become infectious likely misrepresents EIP for a mosquito population. We argue for a better understanding of EIP to improve models of transmission, refine predictions of the possible impacts of climate change, and determine the potential evolutionary responses of malaria parasites to current and future mosquito control tools.

Keywords: Malaria, Mosquito, Extrinsic incubation period, EIP, Temperature

Background

The extrinsic incubation period (EIP) of malaria, also called the period of sporogony, describes the time it takes for parasites to develop in the mosquito from point of ingestion *via* an infected blood meal, through to the point at which sporozoites enter the salivary glands and the mosquito becomes infectious. In the classic models of malaria transmission (e.g. [1–4]), the EIP is one of the most influential parameters because it interacts with adult mosquito survival rate as an exponential term, meaning that even very small changes in EIP can have a large effect on the number of mosquitoes living long enough to be able to transmit parasites. Changes in EIP potentially have much greater impact than equivalent changes in traits such as vector competence (i.e. the ability of vectors to become infectious) or vector density. Despite its epidemiological importance, EIP remains poorly characterized.

Our current understanding of EIP derives largely from research conducted in the early to mid-1900s, wherein the development times of the human malaria parasites *Plasmodium falciparum*, *P. vivax* and *P. malariae* were examined in the mosquito *Anopheles maculipennis* across a range of constant temperatures [5]. These data were used to construct degree-day models to predict the EIP of malaria parasites at a given environmental temperature [1, 2]. The models assume it takes a set number of accumulated degree days (DD) for malaria parasites to complete their development once mean daily temperature (T, in degrees Celsius) exceeds a lower temperature threshold for development (T_{min}). For *P. falciparum*, DD = 111 and T_{min} = 16 °C, giving EIP = 111/(T-16) [1]. For *P. vivax* the equivalent is EIP = 105/(T-14.5), and for *P. malariae* EIP = 144/(T-16) [1].

The Detinova degree-day models of *P. falciparum* and *P. vivax* described above have become lore [1]. Many contemporary studies that provide an estimate of EIP do so without acknowledging the source, let alone attempting any direct validation. However, in spite of their widespread use, the assumptions underpinning these models

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have received little attention. For instance, Nikolaev [5] defined EIP as the time at which sporozoites were first observed in the salivary glands of an individual infected mosquito, yet whether this is the most relevant measure in terms of overall transmission potential of a mosquito population is not clear. The degree-day models also assume that EIP can be estimated using mean temperatures alone. Whether other factors, such as parasite and vector genetics, or other sources of environmental variation, also play a role has been virtually ignored. Equally, whether there is a genetic basis for variation in EIP and potential for evolution in parasite development rate under different environmental conditions (e.g. in response to vector control interventions or climate change) is unknown. Our aim in the current paper is to examine these assumptions in order to improve our understanding of EIP, identify key knowledge gaps, and motivate further work to better characterize EIP moving forward.

What factors determine the EIP?

The influence of temperature

The original work of Nikolaev evaluated EIP of different malaria species across a range of constant temperatures (from 19–20 °C to 30 °C for *P. falciparum*, and 15–16 °C to 30 °C for *P. vivax*) [5]. In nature, however, mosquitoes do not live at a constant temperature but experience daily temperature fluctuations. There is now significant literature indicating that daily temperature variation can have a substantial effect on mosquito and parasite life history, beyond the effects of mean temperature [6–13]. In particular, theory and empirical evidence indicate that daily temperature fluctuations are likely to have the greatest influence toward the upper and lower thermal limits, with daily variation acting to increase parasite development rate under low mean temperatures, slowing development rate under high mean temperatures, and potentially having no net effect under intermediate conditions [6, 14, 15]. Thus, estimates of EIP derived under constant temperatures may not reflect the actual EIPs occurring in nature.

We are aware of no explicit empirical tests of how daily temperature variation impacts EIP of human malaria parasites. However, studies using a rodent malaria confirm the contrasting effects of daily temperature variation on parasite development rate under cool and warm conditions [6], so it seems likely that EIP of human malaria parasites could be similarly affected. Studies on dengue virus development within *Aedes aegypti* also show that temperature fluctuations shorten EIP under cool mean temperatures, but that fluctuations have no effect on virus development rate at higher temperatures, despite reduced vector competence [13].

Current degree-day models also define a lower temperature threshold below which development ceases. For *P. falciparum* T_{min} = 16 °C. However, the lowest temperature measured for *P. falciparum* in the studies of Nikolaev was 19–20 °C [5], and T_{min} = 16 °C was selected by Detinova [1] based on the earlier work of Moshkovsky [2] who fitted a linear regression to the parasite development rate data of Nikolaev and found the line crossed the x-axis at 16 °C. Other studies provide varied estimates of the lower thermal thresholds of human malaria, ranging from as low as 15 °C to as high as 24–26 °C for *P. falciparum*, and from 14.5 °C to 17.5 °C for *P. vivax* (see [16] and Table 1.3 in [17]). Few studies attempt to estimate EIP of *P. falciparum* at temperatures below 20 °C (Fig. 1). Thus, whether we can define 16 °C as the appropriate lower developmental threshold as used in the Detinova model [1] is currently unknown. This lack of knowledge is striking given that T_{min} is so integral to the degree-day model approach.

Additionally, the degree-day models assume the relationship between parasite development rate (the reciprocal of the EIP) and temperature is linear [1, 2]. In contrast, a number of recent theoretical studies describe malaria parasite development rate as a unimodal, nonlinear function [18–21]. Which of these approaches is most appropriate depends critically on whether there is an optimum temperature for development and whether the rate declines as temperatures increase above this optimum. Unfortunately, current evidence is again limited. The studies adopting unimodal functions include data from a very limited number of historic studies

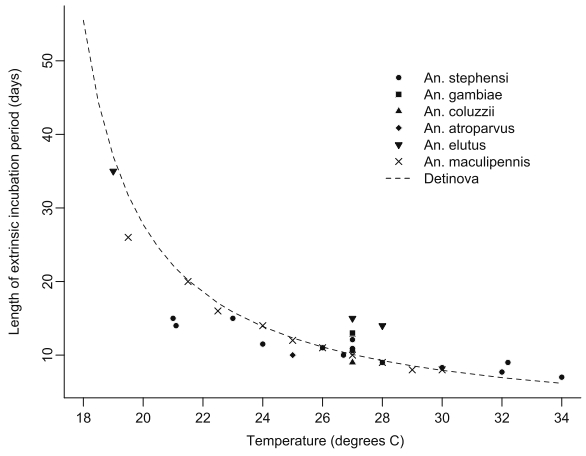

Fig. 1 Empirical estimates of EIP for *P. falciparum* across a range of studies. The dotted black line represents the standard degree-day model of Detinova [1] parameterized using the data for *An. maculipennis* [5]. Data points of the same shape indicate the same mosquito species but may derive from more than one study. The data are extracted from Mordecai et al. [21] (and references therein [26, 56]), together with Shapiro et al. [23], Nikolaev [5], Hien et al. [57] and Kligler & Mer [58]. Note that different studies vary in methods for estimating EIP. Though most report EIP as the time until first observation of sporozoites following an infectious feed, data points from [23] are derived from median EIP

where dissection of mosquitoes at high temperatures revealed no sporozoites in the salivary glands (e.g. [22]). However, the absence of sporozoites does not necessarily equate to zero growth rate. This distinction is not simply semantic; we ought to know whether high temperatures limit transmission because of a decline in vector competence (which could be direct parasite mortality or perhaps mediated *via* the mosquito), or because parasite growth slows and EIP becomes progressively longer. In the recent study of Shapiro et al. [23] there was no evidence of a non-linear decline in parasite development rate up to 34 °C, even though the proportion of mosquitoes becoming infectious declined at temperatures above 27 °C. More data are needed to resolve this fundamental issue.

Parasite genetic diversity

Nikolaev's study [5] identified differences in the EIP between three *Plasmodium* species. Other studies have further demonstrated interspecific variation in EIP [24–26]. The genetic basis for these differences in EIP is poorly understood. Additionally, whether there is intraspecific variation in EIP between parasite genotypes is unknown. The Detinova degree-day models assume no intraspecific variation but we are not aware of any empirical studies investigating this assumption.

Studies from other vector-borne pathogens provide some evidence of intraspecific variation in EIP. For example, the emergence of a new dominant genotype of West Nile virus in North America has been attributed to the new genotype having a shorter EIP in *Culex* mosquito vectors compared to the original strain [27]. Similarly, the Southeast Asian genotype of dengue serotype 2 virus has displaced the American genotype in several countries [28] which has been explained by its shorter EIP resulting in an estimated 2- to 65-fold increase in the vectorial capacity of the *Ae. aegypti* vector [29]. Additionally, differences in dissemination rate of three strains of dengue serotype 2 viruses within the same *Ae. aegypti* colony have also been observed [30], further suggesting that the pathogen's intraspecific variation in EIP is genetically influenced. However, intraspecific variation in EIP is not always observed among viruses. A single mutation between two isolates of chikungunya virus (CHIKV) favored transmissibility by *Aedes albopictus* [31] and has been associated with an outbreak that occurred in Indian Ocean territories, but no quantitative differences in EIPs between these strains were observed [32]. In addition, a statistical analysis aimed at estimating the relationship between temperature and EIP in three orbiviruses transmitted by *Culicoides* biting midges showed that the rate of virus replication was mostly consistent among the different pathogen genotypes [33].

Given the high levels of genetic variation within malaria parasite species [34–38], it seems likely that there could be genotypic variation for EIP [39]. Different *Plasmodium* genotypes have been shown to vary in their capacity to infect a specific mosquito species [40, 41], possibly due to different immune evasion mechanisms [42]. Additionally, parasite growth rates within the vertebrate host are under genetic control [39, 42]. Better characterizing intraspecific variation in sporogony could improve investigation of local transmission dynamics (e.g. [43]) and could help in understanding the spread of drug resistant genotypes (cf. [44, 45]).

Vector genetic diversity

There are approximately 70 species of mosquitoes in the genus *Anopheles* known to contribute to transmission of malaria parasites to humans [46]. The current degree-day models of EIP were derived from studies on one population of a single species, the Eurasian vector *Anopheles maculipennis* [5]. Few researchers would be happy to accept that all populations or species of *Anopheles* mosquitoes are equally permissive to malaria infection, and there has been substantial research investment to understand the genetic mechanisms underlying variation in susceptibility/refractoriness (e.g. [36, 47, 48]). Yet for EIP the prevailing assumption is that all vector species and populations are identical and the EIP is a property of the parasite response to mean temperature alone. Indeed, White & Rao [49] state "for lack of any evidence to the contrary, it must be assumed that differences in vector species does [*sic*] not affect the results [of EIP]".

In Fig. 1 we present all the available data we can find from studies that have explicitly measured EIP of *P. falciparum* (note that we followed the approach of Mordecai et al. [21] and excluded studies if they did not demonstrate adequate control of temperature, were unclear on parasite species, or had insufficient sample size such as reporting infections from dissection of single mosquitoes). The figure reveals that data are extremely sparse and that certain empirical estimates of EIP do not clearly match the standard degree-day model. Whether there are significant differences between vector species is impossible to say as there are insufficient data to generate species-specific EIP models for any of the key malaria vectors in Africa, Asia or Latin America.

In addition to the potential for interspecific differences in EIP between vectors (Fig. 1), there is the potential for intraspecific variation. In a recent study, Ye et al. [50] examined EIP of dengue across 40 genetically distinct families of *Aedes aegypti*. They showed significant differences in EIP (measured as time to detectable virus in the saliva) between families ranging from 4–14 days, and that variation in EIP was highly heritable (~40%).

Shorter EIPs were additionally correlated with shorter vector lifespans and higher virulence. This work demonstrates that EIP of dengue is largely controlled by variation in the mosquito genome. We are aware of no studies on malaria vectors examining intraspecific genetic variation in EIP. The data from Shapiro et al. [23] indicate differences between individual mosquitoes but the mechanisms are unclear. However, with evidence for genetic influence on other aspects of malaria parasite infection such as resistance/susceptibility [35, 47, 48, 51], interactions with insecticide resistance [52], and vector genotype × parasite genotype interactions [40, 53, 54], it would be surprising if there was no influence of mosquito genetics on EIP.

Other biotic and abiotic factors

The complex interplay between parasite and vector traits that determine overall transmission can be influenced by many factors [55–58]. Larval food limitation has been shown to decrease malaria parasite survival [59] and affect infection prevalence and intensity [60, 61]. The mechanisms behind these observations are not well understood but could be linked to altered immune response, resource allocation within the vector [59–61], or effects on adult body size that influence the blood meal volume and hence the number of infecting parasites (note that temperatures in the larval environment also impact ultimate adult size [62]). Importantly, quality of the larval habitat has been shown to affect EIP for both dengue [63] and *P. falciparum* [23] independent of temperature.

Food intake by adult mosquitoes can also affect parasite development. Relatively few studies have looked at the impact of sugar feeding on mosquito or parasite life history but there is evidence that nectar from different plants can potentially inhibit or enhance parasite load and rate of parasite development [57, 64]. Blood-feeding has also recently been shown to influence EIP of dengue virus in *Aedes* mosquitoes, with additional blood meals accelerating virus development [65].

Malaria parasites potentially compete with many organisms inside mosquitoes [66–69], including mixed infections with other malaria parasite genotypes [70, 71]. These interactions can impact parasite establishment and density *via* competition or immune-mediate mechanisms [72]. What effect they might have on EIP of malaria parasites is not known, but for dengue, the presence of an intracellular bacterial parasite (*Wolbachia*) has been shown to extend the EIP [73, 74]. There is further potential for parasite/pathogen-mediated effects *via* trans-generational immune priming, which can confer lasting protection within an individual [75] and in its offspring [76]. If parental exposure to parasites has consequences for malaria parasite resistance in the offspring [76], it is possible this could impact EIP, though this has not yet been explored.

How should EIP be measured?

The original work of Nikolaev [5] dissected mosquitoes at various time points following an infectious blood meal and defined EIP as the time at which sporozoites were first observed in mosquito salivary glands. Capturing the time of the first mosquitoes to become infectious might make sense: mosquitoes that allow rapid parasite migration through their bodies have more opportunities to infect humans at subsequent bites and so might be the most epidemiological relevant individuals. Equally, if parasite development is highly synchronized between individuals, then the time to first infection will likely be a reasonable approximation for the mosquito population as a whole. On the other hand, if development is widely distributed between mosquitoes, then a few early infectious mosquitoes might be unrepresentative of the total mosquito population, and be a poor predictor for force of infection.

A number of recent empirical studies (e.g. [23, 57]) have demonstrated that the time parasites take to reach the salivary glands is not the same for all mosquitoes in a population, even if they have received the same infectious blood meal and are maintained under identical conditions, again highlighting the need to understand sources of this variation [54]. Furthermore, variance in EIP and the median EIP value are affected by temperature [23]. Under warm conditions the median is shorter and there is less variation in duration of sporogony between mosquitoes, but as conditions cool, the median increases and the time between the first and last mosquitoes to become infectious can extend to several days, widening the distribution of EIP [23].

In Additional file 1, we present an outline of a model developed to examine whether different measures of EIP affect estimates of the probability that mosquitoes live long enough to become infectious. We based our analysis on the study of Shapiro et al. [23], which measured the EIP of *P. falciparum* in *An. stephensi* across six constant temperatures ranging from 21–34 °C. Briefly, the dynamics of sporogony were characterized by a logistic function (Fig. 2, Additional file 1), which enables us either to define individual measures of EIP (the 10-percentile, 50-percentile or 90-percentile), or to represent the full growth kinetics of parasites across the mosquito population.

In order to examine the proportion of infected mosquitoes that survive through the different measures of EIP we needed to estimate adult mosquito mortality rate. Many transmission models assume a constant daily mortality rate. In Fig. 3a we weight the proportion of mosquitoes that developed sporozoites at each temperature

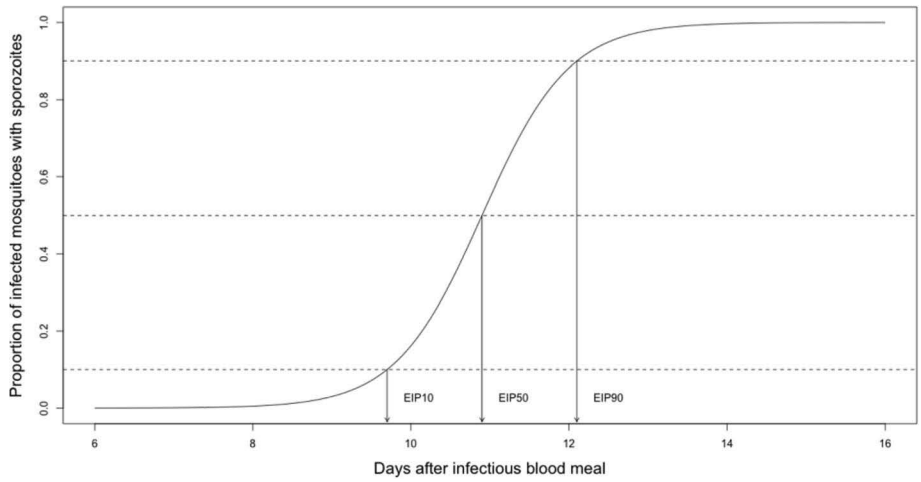

Fig. 2 Proportion of malaria-infected mosquitoes with sporozoites present in the salivary glands (i.e. becoming infectious) over time following an infectious blood meal. Here the dynamics of EIP are characterized using a logistic model following the approach of Paaijmans et al. [77] and Shapiro et al. [23, 60] (and see also data in Hien et al. [57]). The conventional way of estimating EIP is to measure the time at which sporozoites first appear in salivary glands of infected mosquitoes (approximating the EIP_{10}). However, given EIP is not perfectly synchronized between individual mosquitoes, the EIP could equally be characterized using alternative measures such as the median value for the mosquito population (EIP_{50}), or the time at which the maximum proportion of the population become infectious (approximating the EIP_{90}). In this illustrative example we assume all infected mosquitoes go on to become infectious. If conversion efficiency of oocysts to sporozoites is less than 100%, the asymptote will be reduced

by the proportion that survived through sporogony for a constant mortality rate of 10% per day, comparing the EIP_{10}, EIP_{50}, EIP_{90}, the standard degree-day model, and the full logistic model (Additional file 1: Table S1). In Fig. 3b we conduct a similar analysis but rather than assume a constant daily mortality rate we used the actual temperature-dependent mortality rates measured by Shapiro et al. [23] for each of the six temperatures (Additional file 1: Table S2).

Comparison of Fig. 3a with 3b shows that the pattern of adult mortality has a qualitative effect on the proportion of infected mosquitoes predicted to be alive and infectious for our different measures of EIP. With constant daily mortality rate there is a general trend for the proportion of infectious mosquitoes to increase as temperatures rise, since warmer temperatures shorten EIP whichever way it is characterized. With temperature-dependent mortality, however, the proportion of infectious mosquitoes tends to fall as temperature extends beyond 27 °C, since reductions in EIP are offset by increases in daily mosquito mortality rates at higher temperatures.

In addition, regardless of how mortality is estimated, at low temperatures the standard degree-day model tends to underestimate the probability of mosquitoes being alive and infectious compared to the estimates based on the empirical data of Shapiro et al. [23]. This difference largely derives from the fact that Shapiro et al. [23] reported more rapid sporogony than Nikolaev [5] at cooler temperatures. At temperatures above 27 °C the degree-day model increasingly approximates the EIP_{10}, which is to be expected as Nikolaev [5] estimated EIP from the first few mosquitoes to become infectious (which is close to the EIP_{10}) and the data of Shapiro et al. [23] and Nikolaev [5] are more similar at high temperatures. Perhaps most important is that the EIP_{50} yields almost identical values to approximations based on the full logistic model, while the EIP_{10} and EIP_{90} tend to over and under estimate the probability of a mosquito being alive and infectious, respectively. This result indicates that it is important to characterize the full dynamics of sporogony and that the distribution of EIP is better estimated using the median EIP (EIP_{50}), rather than beginning or end points of the distribution. This is not how EIP has been interpreted for almost a century.

Conclusions

Current understanding of EIP of malaria parasites is limited. There are very few empirical data and those that exist tend to report EIP inappropriately. Moreover, basic information regarding the genetic and environmental determinants of EIP is lacking. This is unfortunate as the potential environmental and genetic influences are numerous and likely to have profound evolutionary and epidemiological implications [77–83]. One obvious implication is that the intensity of malaria transmission will vary spatially and temporally depending on environmental fluctuations and specific vector-parasite combinations. It could be that effect sizes are small and that the established degree-day models capture the variation in

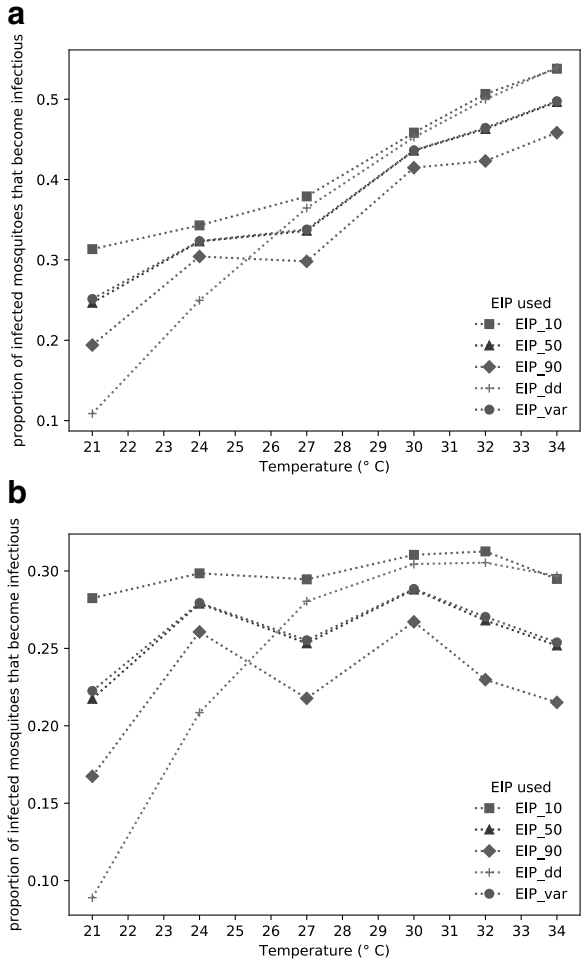

Fig. 3 The proportion of infected mosquitoes predicted to survive the duration of EIP and be able to transmit *P. falciparum* parasites at different temperatures. The EIP_{var} values refer to the full logistic models describing the dynamics of sporogony across six constant temperatures presented in Shapiro et al. [23]. The EIP_{10}, EIP_{50} and EIP_{90} values represent the 10-, 50- and 90-percentile points from the logistic curves. The EIP_{dd} values are from the classic Detinova degree-day model [1]. **a** Assumes a constant mortality rate of adult mosquitoes of 10% per day. **b** Assumes adult mortality rate to vary with temperature based on the data presented in Shapiro et al. [23]

EIP across time and space adequately. However, it could also be that mosquito species, mosquito condition, parasite strain, etc. have a substantial influence. This should not be an open question. There has been considerable speculation regarding possible impacts of climate warming on malaria transmission [19, 21, 81, 83], yet the effects could depend as much on the specifics of the local mosquito-parasite pairing as the absolute change in temperature itself. More empirical studies are required to rigorously examine EIP both as a stand-alone trait, and in the context of other essential components of vectorial capacity, such as mosquito density, adult longevity, and biting rate, which all contribute to overall transmission. Such studies would be facilitated greatly by the optimization of non-destructive methodologies allowing fine temporal resolution of EIP within individual mosquitoes, as is now possible for arboviruses [50, 84, 85]. In terms of transmission dynamics, it would also be valuable to determine the parasite's ability to adjust its development rate in response to environmental cues (adaptive phenotypic plasticity). For instance, can malaria parasites adaptively speed up their EIP when their transmission is compromised by the imminent death of their vectors (perhaps in old mosquitoes, those exposed to insecticides, or in the presence of competing parasites)? In a related way, given transmission is ultimately dependent on the bite of an infectious mosquito, it would be interesting to explore whether EIP could potentially be linked to biting rate and gonotrophic cycle. Like EIP, biting behavior is influenced by a suite of environmental factors [86, 87] and it is possible that the duration of EIP is rhythmically modulated to avoid the situation where the parasite is ready to be transmitted but the mosquito is not ready to feed, either because the mosquito is in the middle of a gonotrophic cycle [7] or because it is physiologically constrained [87, 88]. Such condition-dependent developmental strategies have been described in blood-stage malaria parasites [89, 90] and deserve considerations in infected mosquitoes. Finally, understanding the extent to which EIP is genetically variable is also crucial to understanding the capacity of EIP to evolve in response to malaria interventions or mosquito life history, as genetic variation fuels evolution. Current core vector control tools (long-lasting insecticide-treated bed nets (LLINs) and indoor residual insecticide sprays (IRS)) act, in part, by changing mosquito population age structure [91, 92]. These tools exploit the fact that the EIP is long relative to the lifespan of most mosquitoes, and that mosquitoes take multiple blood meals throughout their lifetime. By increasing the probability of mortality per blood feeding event, LLINs and IRS reduce the number of mosquitoes that live long enough for the parasite to complete EIP. Other prospective control tools also target the 'old infectious' mosquitoes [91, 92]. There is now a substantial industry built around understanding and managing the evolutionary responses of mosquitoes to insecticides and other vector control tools (e.g. see [93]). Whether vector control tools can drive evolutionary changes in EIP and select for parasite clones with shorter EIPs is unknown but should, perhaps, become part of an extended insecticide-resistance monitoring process. The fitness of parasites should increase with shorter EIP, unless faster developing parasites inflict higher mortality costs on mosquitoes or come with fitness trade-offs to the parasite such as reduced infectivity (as discussed in [54]). Whether mosquito fitness is affected by EIP length of malaria

parasites is unknown. The implications for transmission could depend on relationships with relevant transmission traits such as mosquito longevity or parasite load [54]. For example, are fast developing clones also those that are the most virulent and reduce mosquito longevity? Are fast developing parasites also those that produce the fewest transmissible stages? These potential trade-offs and constraints may have important implications for understanding the evolutionary potential of EIP. More broadly, the effects of parasite drug resistance and mosquito insecticide resistance - two important sources of genetic variation - on EIP deserve attention.

Additional file

Additional file 1: Text. Numerical approximation of proportion of vectors surviving to become infectious assuming logistic EIP. **Table S1.** Comparison of results for approximation of probability of surviving from infection to infectiousness using logistic model. Results used for plots in main text are highlighted. Chosen D_{max} and δ give results consistent to 6dp with results from ten times smaller δ and D_{max} of 100 *vs* 30, indicating that for the intended purpose, no material benefit would be gained from using smaller δ or larger D_{max}. **Table S2.** The temperature-related values used for *k, tM*, and μ , taken from Shapiro et al [23]. (DOC 68 kb)

Abbreviations

EIP: Extrinsic incubation period; EIP_{10}: Extrinsic incubation period measured as the time until 10 percent of infected mosquitoes become infectious; EIP_{50}: Extrinsic incubation period measured as the time until 50 percent of infected mosquitoes become infectious which equates to the median time of sporozoite development; EIP_{90}: Extrinsic incubation period measured as the time until 90 percent of infected mosquitoes become infectious

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Availability of data and materials

All data analyzed and statistical methods used are included in this review or cited in the references.

Authors' contributions

MBT, JRO and TL originally conceived of the idea for a manuscript to reevaluate how EIP is defined through the VectorBiTE EIP working group. PL constructed figures and models to demonstrate quantitative examples for how EIP estimates vary. SAW, ES, FB and PB developed ideas with MBT, JRO and TL at the 2017 VectorBiTE EIP working group meeting and assisted in writing and editing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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