Impact of glutathione-s transferase and cytochrome P450 metabolic resistance on the effectiveness of various bed nets against *Anopheles funes*tus, a major malaria vector in Africa

Thesis submitted in accordance with the requirements of the Liverpool School of Tropical Medicine for the degree of Doctor in Philosophy by MENZE Djantio Benjamin

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Abstract

The scale-up of insecticide-based interventions, including Indoor Residual Spraying (IRS) and Long Lasting Insecticidal Nets (LLINs), has contributed to the significant reduction of malaria burden in the past decade. Unfortunately, growing insecticide resistance in malaria vectors is threatening these successes. However, the impact of resistance, especially metabolic resistance, on the effectiveness of vector control tools against pyrethroid-resistant mosquito populations, remains a topic of debate. One of the key challenges of assessing the impact of metabolic resistance on the effectiveness of these insecticide-based interventions has been the lack of molecular markers for insecticide resistance, notably metabolic resistance. The recent detection of key genetic markers conferring metabolic-mediated resistance (*GSTe2* marker and *CYP6P9a*/b markers) is providing the opportunity to now address this question. In this work, we used an experimental hut trial, and the markers recently made available to investigate the direct impact of metabolic resistance on the effectiveness of insecticide-based interventions.

The characterization of the area selected for experimental hut trial revealed that *Anopheles funestus* sensu stricto (s.s.) (80%) and *Anopheles gambiae* s.s. (20%) are the two main species. High levels of resistance to pyrethroids and organochlorines were noticed. Moderate resistance was observed against bendiocarb (carbamate) in both species, but relatively higher in *Anopheles gambiae s.s*. In contrast, full susceptibility was observed for the organophosphate malathion. The PBO synergist assays with pyrethroid type I and II revealed a significant recovery of the susceptibility in *Anopheles funestus s.s.* population (48.8 to 98.1% mortality and 38.3 to 96.5% mortality, respectively). The DDT/pyrethroid 119F-*GSTe2* resistant allele (28.1%) and the dieldrin 296S-*RDL* resistance (9.7%) were detected in *Anopheles funestus s.s.* The high pyrethroid/DDT resistance in *Anopheles gambiae* correlated with the high frequency of the 1014F knockdown resistance allele (63.9%). The 1014S-*kdr* allele was detected at low frequency (1.97%). The *Plasmodium* infection rate was 20% in *Anopheles gambiae,* whereas *Anopheles funestus* exhibited an oocyst infection rate of 15 and 5% for the sporozoite infection rate. Using experimental hut trials and genotyping of a glutathione S-transferase resistance marker (L119F-*GSTe2*), we noticed that PBO-based nets

induced a significantly higher mortality rate than pyrethroid-only nets. Blood feeding rate and deterrence were significantly higher in all LLINs than control, although greater for PermaNet 3.0 than PermaNet 2.0 but not between Olyset nets. Genotyping the the L119F-*GSTe2* mutation revealed that, for permethrin-based nets, 119F-*GSTe2* resistant mosquitoes have a greater ability to blood feed than susceptible [Olyset Plus (OR= 3.1; P=0.00007) and Olyset (OR=3.0; P=0.00007)] while the opposite effect is observed for deltamethrin-based nets. For Olyset Plus, a significant association with exophily was observed in resistant mosquitoes (OR=11.7; P=0.003). Furthermore, *GSTe2*-resistant mosquitoes (cone assays) significantly survived with PermaNet 2.0 (OR=2.1; P=0.002) and even more with PermaNet 3.0 (OR=30.1; P<0.0001) whereas no association was observed for Olyset Plus. L119F-*GSTe2* was confirmed to confer resistance to permethrin, Deltamethrin, and DDT. Also, genotyping of the CYP6P9a/b markers revealed that homozygous resistant (RR) were better able to survive exposure to Olyset nets than homozygous susceptible (SS) : (OR=7.03; P< 0.01) for *CYP6P9a* and (OR=5.1; P<0.01) for *CYP6P9b.* In addition, homozygous resistant (RR) show a greater ability to blood feed when compared to homozygous susceptible (SS) (OR= 3.1; p<0.001) for *CYP6P9a* and (OR= 8.01; p<0.001) for *CYP6P9b*. Further analysis demonstrates that mosquitoes double homozygous resistant (RR/RR) at both genes had by far a significant ability to reduce LLINs efficacy (Blood feeding: RR/RR v SS/SS; OR= 9.3; p<0.001) compared to single homozygous (Blood feeding RR v SS; OR= 4.5; p<0.001).

This study shows the greater efficacy of PBO-based nets against pyrethroid-resistant malaria vectors. Reduced efficiency of LLINs and the increased ability to survive and blood feed of *CYP6P9a*/b and *GSTe2* resistant mosquitoes highlight the impact of metabolic resistance.

Dedication

To my lovely wife Njapdounke Moundou Khadij Epse MENZE, I know that my long absence, for field works in Bankim or my training abroad, took me away from you. I am so grateful to you.

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List of abbreviation

ace-1*: acetylcholinesterase*

Ae*: Aedes*

AChE: acetylcholinesterase

An: Anopheles

BAC: Bacterial artificial chromosome

Bti: *Bacillus thuringiensis*

CRID: Centre for Research in Infectious diseases

DDT: Dichlorodiphenyltrichloroethane

DNA: Deoxyribonucleic acid

FANG: *An. funestus* from Angola

FUMOZ-R*: An. funestus* from Mozambique-Resistant

GPIRM: global plan for insecticide resistance management in malaria vectors

GLMM: Generalised Linear Mixed Model

GSTs: glutathione-S-transferases

ITNs: Insecticide treated nets

IRM: Insecticide Resistance Management

IRS: Indoor Residual insecticide Spraying

IVCC: Innovative Vector Control Consortium

LLINs: Long-Lasting Insecticidal Nets

LSTM: Liverpool School of Tropical Medicine

LSM: Larval source Managment

IRS: Indoor Residual Spraying

IRM: Insecticide resistance managment

Kdr: Knockdown resistance

s.s: sensu stricto (in the strict sense)

OVM: ovale malariae vivax

s.l: sensu lato

P: Plasmodium

PBO: Piperonyl butoxide

PCR: Polymerase Chain Reaction

RFLP: Restriction fragment length polymorphism

qPCR : Quantitative polymerase chain reaction

VGSC: Voltage Gated Sodium Channel

WP: Wettable Powder

WHO: World Health Organization

WHOPES: World Health Organization Pesticide Evaluation Scheme

Chapter 1: Literature review

1.1 Background

Despite all the progress made in malaria control, the disease remains a major public health concern. In 2018, it was noticed 405 000 deaths from malaria worldwide and 228 million cases (WHO, 2019b). Children under the age of 5 accounted for two thirds (67%) of global malaria deaths in 2018 (WHO, 2019b). In 2018, 93% of malaria cases were found in the WHO African Region. More than half of all cases were in six countries: Nigeria (25% of cases); the Democratic Republic of the Congo (12%); Uganda (5%); Côte d'Ivoire, Mozambique and Niger (4% each) (WHO, 2019b). All of Cameroon's population of 25 million inhabitants live in malaria endemic areas (71% living in high transmission areas and 29% living in low transmission settings). In 2017 in Cameroon, a total of 2,093,009 cases of malaria were confirmed by diagnostic test, 49% of these cases were classified as severe malaria. Malaria cases represent 24% of all medical consultations in health facilities and 45% of all hospitalizations. Malaria accounts for the greatest proportion of hospitalizations in the Adamawa, Far North, and North regions accounting for 60%, 56%, and 56% of all hospitalizations in these regions, respectively. In 2017, 3195 malaria deaths were reported in health facilities, which represented 13% of all-cause deaths. The proportion of deaths due to malaria is highest in the Far North (26%) and North (27%) regions (PNLP, 2018; WHO, 2019b)

Malaria is transmitted by pathogens, which is an intracellular protozoan belonging to the genus *Plasmodium* discovered in 1880 by Laveran (Mouchet et al., 2004). In sub-Saharan Africa, *An. gambiae, An. Arabiensis* and *An. funestus* are the major malaria vectors (Ranson et al., 2011). The introduction of new tools, such as new diagnostics, more effective antimalarial medicines, and introduction of the RTS, S malaria vaccine in Ghana, Kenya, and Malawi as a WHO pilot program has given hope regarding malaria control (WHO, 2019b). Despite all these innovations, malaria control relies heavily on Insecticide-based interventions (Bhatt et al., 2015).

Insecticide-based interventions are reducing the malaria burden. Malaria prevalence and malaria incidence have been reduced by 40 % between 2000 and 2015 (Bhatt et al., 2015). During that same period, 663 malaria cases were averted, and insecticide-treated bed nets were found to be the most significant contributor to this decrease by contributing up to 68 % of the reduction observed (Bhatt et al., 2015). Bed nets remain the mainstay of insecticides

treated materials for vector control with 582 million bed nets delivered between 2014 and 2016, out of which 505 million were delivered to sub-Saharan Africa (WHO, 2017). An estimated 61% of children under five living in sub-Saharan Africa slept under an ITN compared to 26% in 2010 (WHO, 2019b). In Cameroon, strategies against malaria rely heavily on long lasting insecticidal nets (LLINs) distribution. In 2011, 8 657 075 LLINs were distributed by the government all over the country. Between 2013 and 2015, 12 356 059 LLINs were distributed. The last distribution took place in 2019 and 14 697 104 LLINs were distributed. In 2017 the bed nets coverage in Cameroon was 76.6% (Antonio-Nkondjio et al., 2019).

Unfortunately, growing insecticide resistance against the four insecticide classes available (organochlorine, organophosphate, carbamate, and especially pyrethroids the only class fully approved by WHO for LLINs and ITNs)- in one of the major malaria vector *An. funestus* (Menze et al., 2016; Menze et al., 2018b; Riveron et al., 2014a; Riveron et al., 2016a) is threatening these successes. Within the Funestus complex, *An. funestus s.s.* is the most widely distributed across Africa (Dia et al., 2013). In malaria vectors, the insecticide resistance is conferred mainly through target resistance and metabolic resistance (Hemingway and Ranson, 2000). The knockdown resistance (*kdr*) in the voltage-gated sodium channel is not yet selected in *An. funestus* s.l., (Irving and Wondji, 2017) Resistance in *An.funestus* is mainly metabolic and mediated fully or partially by P450 monooxygenases (Coetzee and Koekemoer, 2013; Wondji et al., 2012). *GSTs* are, besides cytochrome P450s and esterases, one of the main enzyme families conferring metabolic resistance to insecticides (Hemingway, 1999) either through a direct metabolism or by catalyzing the secondary metabolism of substrates oxidized by cytochrome P450s (Hemingway and Ranson, 2000). Over-expression of GST epsilon 2 (*GSTe2*) has been associated with DDT and pyrethroids resistance in several mosquito species, including *An. gambiae* (Mitchell et al., 2014), *An. funestus* (Riveron et al., 2014c) and *Aedes aegypti* (Lumjuan et al., 2011). In *An. funestus*, the structural analysis revealed that a leucine to phenylalanine amino acid change (L119F) in *GSTe2*, enlarging the substrate-binding pocket of the enzyme, was conferring DDT/pyrethroid resistance in West and Central African populations (Riveron et al., 2014c). In *An. funestus s.l*. The insecticide resistance is displaying different patterns across Africa. In Eastern and Southern Africa, metabolic resistance in *Anopheles funestus* s.l. is mediated by P450 monooxygenases (Riveron et al., 2014a; Riveron et al., 2013). In the same malaria vector geographically located in western and Central Africa,

metabolic resistance is mainly mediated by Glutathione-S-transferases epsilon 2 (*GSTe2*) (Djouaka et al., 2016b; Menze et al., 2016; Riveron et al., 2014b).

The WHO Global Plan for Insecticide Resistance Management (GPIRM) is highlighting the need to assess the impact of insecticide resistance to implement proper insecticide resistance management (IRM) strategies to preserve the efficacy of current and future insecticides. However, the extent of the impact of this resistance, notably, metabolic resistance on the effectiveness of insecticide-based interventions such as Long lasting Insecticidal Nets (LLINs) remains unclear.

Epidemiological and entomological approaches are usually considered as useful methods to investigate the impact of insecticide resistance (WHO, 2012a).

However, the epidemiological approach, based on malaria prevalence and malaria incidence can be jeopardized by the fact that malaria transmission is affected by many confounding factors such as the local habitation favorable to a mosquito bite, the weather and the fickleness of health care system (Kleinschmidt et al., 2015; WHO, 2012a, b). Recognising the limitations of the epidemiological approach, the alternative is to measure entomological outcomes, such as relative mortality and feeding success of resistant and susceptible vectors in controlled conditions using experimental huts (N'Guessan et al., 2009; N'Guessan et al., 2007b; WHO, 2012b). The limitations of this second approach are: firstly, the trial is performed at a small scale and secondly, the difficulty in measuring the level of resistance in individual mosquito due to lack of genetic markers (Strode et al., 2014; WHO, 2012b). It is, therefore, challenging to assess the impact of insecticide resistance, especially the metabolic resistance for which no DNA-based markers previously existed. This gap is a significant obstacle to the implementation of IRM in areas where insecticide resistance to the malaria vector is driven by metabolic resistance since its impact could not be assessed. However, recent efforts have detected major DNA-based markers for metabolic resistance for *GST* in both *An. funestus* (Riveron et al., 2014b), but also P450s in *An. funestus* (Mugenzi et al., 2019; Weedall et al., 2019). These include a key genetic marker in the glutathione S-transferase epsilon two gene (*GSTe2*) conferring a metabolic-mediated resistance to pyrethroids and DDT in the major malaria vector *An. funestus* in West and Central Africa (Riveron et al., 2014b). Concerning the P450 family genes, further genomic analyses revealed a complex evolution of resistance, including copy number variation, transposon insertion, and gene conversion. Detection of causative cis-regulatory mutations allowed designing the first DNA-based P450 assays in

malaria vectors for the *CYP6P9a* (Weedall et al., 2019) and *CYP6P9b* genes (Mugenzi et al., 2019). These markers now allow addressing questions regarding the direct impact of *GST*- and P450-mediated metabolic resistance on insecticide-based interventions such as LLINs.

Taking advantage of the recent detection of these DNA-based molecular markers for metabolic resistance in the P450 and *GST*, we used experimental huts in Cameroon to assess the performance of several bed nets against pyrethroid resistant *An. funestus, and* to evaluate the impact of this metabolic resistance on the effectiveness of long lasting insecticidal nets.

1.2 Malaria History

Malaria comes from the Italian word mal-aria or "bad air" as, for a long time, malaria was associated with marshes. This disease occurred in southern Europe and around the Mediterranean until the beginning of the twentieth century and was eradicated by the disappearance of vectors following the efforts of mosquito control and the raising of the standard of living. As early as the 4th century, Hippocrates had already made the first descriptions of the clinical manifestations of the disease characterized by chills, fever, and sweats. These symptoms occur according to specific daily sequences hence the names of third and fourth fevers. However, it is at the end of the 19th century that Alphonse Laveran noticed the presence of the parasite in the blood of a sick subject and that Ronald Ross discovers its transmission by mosquitoes (Mouchet, 2004). Nevertheless, it is to Giovanni BATTISTA GRASSI that we owe the knowledge of the Plasmodium development cycle in the mosquito (Wery, 1995).

1.3 Pathogenic agents of malaria

The pathogens responsible for malaria are intracellular protozoa discovered in 1880 by Laveran (Mouchet, 2004). They are transmitted to humans following a bite of a female infected mosquito of the genus Anopheles. These parasites are also transmissible during blood transfusions or congenital (Wery, 1995). Five species of *Plasmodium* are at the origin of malaria in humans. They differ from one to another by several specific epidemiological, biological, and clinical characteristics:

***Plasmodium falciparum* Welch, 1897** is the most widespread species. This species is strongly established in tropical Africa. Its incubation period is 7 to 15 days, and its longevity is less than one year. It is at the origin of the third malignant fever, the most lethal. It is a parasite of red blood cells of all ages, with erythrocytic schizogony occurring in deep organs. In case of complications, the parasite can pass into the central nervous system; we then speak of cerebral malaria (Mouchet, 2004).

***Plasmodium malariae* Laveran, 1881** occurs throughout the Afro-tropical region at very variable frequencies of 2-45%. It is generally more common in forest areas. Its longevity is

about 21 days, and it mainly attacks old red blood cells. Its recrudescence can go up to 10 or 20 years, by reactivation of latent erythrocyte forms despite the absence of hypnozoites (Baudon et al., 1987).

***Plasmodium vivax* Grassi and Feletti, 1890** has a wide distribution but are absent in populations carrying Duffy antigen. This species is responsible for the most common benign tertian fever. Its incubation lasts about 15 days and can last up to 7 months. It is a parasite of young red blood cells that causes remittent fevers for a period of up to two years due to the presence of hypnozoites (Baudon et al., 1999).

***Plasmodium ovale* Stephens, 1922** is mainly present in Africa. It causes mild benign fever. Its longevity is 15 days, and relapses are lasting up to 5 years due to the presence of hepatic hypnozoites (Danis & Mouchet, 1991, Mouchet et al., 2004).

***Plasmodium knowlesi* Knowles, 1932** is genetically close to *Plasmodium vivax* and morphologically *Plasmodium malariae*. It was previously known as a parasite of monkeys but has recently been found in humans in several South Asian countries (Singh et al., 2004; Sermwittayawong et al., 2012).

1.4 Malaria vectors

The total number of *Anopheles species* worldwide is around 484 species (Harbach, 2004). In Sub-Saharan Africa, about 140 *Anopheles species* are present. Most of them are members of species complex or group. The two important complex or group containing the majority of these species are *An. gambiae* complex and *An. funestus* group (Gillies and Coetzee, 1987). The major vectors in Africa are *An. gambiae s.s., An. coluzzii, An. funestus s.s* and *An. arabiensis* (Coetzee et al., 2013; Ranson et al., 2011; White, 1974).

1.4.1. *An. gambiae* complex

Among *An. gambiae* complex we can noticed: *An. gambiae* s.s*, An. arabiensis, An. melas, An. quadriannulatus, An. quadriannulatus B* and *An. bwambae* (Carnevale et al., 1992).

These species are morphologically identical but can be quickly and simultaneously identified using a specific PCR based on the polymorphism of ribosomal DNA (Scott et al., 1993). Cytogenetic studies have shown that species of *An. gambiae* complex is genetically distinct and characterized by chromosomal inversions, which correspond to adaptations to specific

climatic conditions (aridity, temperature). Some of these species have strict ecological requirements that define their geographic distribution (Coluzzi et al., 1979). This is the case of *An bwambae* larvae, which prefer the saltwater of the Semliki forest in Uganda (White, 1985), larvae of *An. melas* and *An. merus* which evolve in brackish waters, respectively, on the western and eastern facades of Africa (Manguin et al., 1996), *An. gambiae* s.s. found in clear, shallow, sunny waters. *An. quadriannulatus* is widespread in southern Africa (form A) and Ethiopia (form B), but its zoophilic nature limits its ability to transmit malaria (Gillies and Coetzee, 1987; Hunt et al., 1998). The species of this complex are present in regions of degraded forest or humid savannah and thus occupy the entire African equatorial belt. Other subdivisions have been described within the species *An. gambiae* s.s., in particular, the molecular forms M and S between which the gene flows are minimal, which suggests a phenomenon of speciation in progress (Della Torre et al., 2002; Torre et al., 2001).

An. gambiae was recently split into two species, one remaining as *An. gambiae* s.s (formally called S form) while a new species called *An. coluzzii* (formally M molecular form) (Coetzee et al., 2013). In Cameroon beside *An. funestus, An. gambiae.,* we can notice some vectors of local importance such as *An. nili* and *An. moucheti,* which are also significantly involved in malaria transmission (Awono-Ambene et al., 2004) (Carnevale et al., 1999).

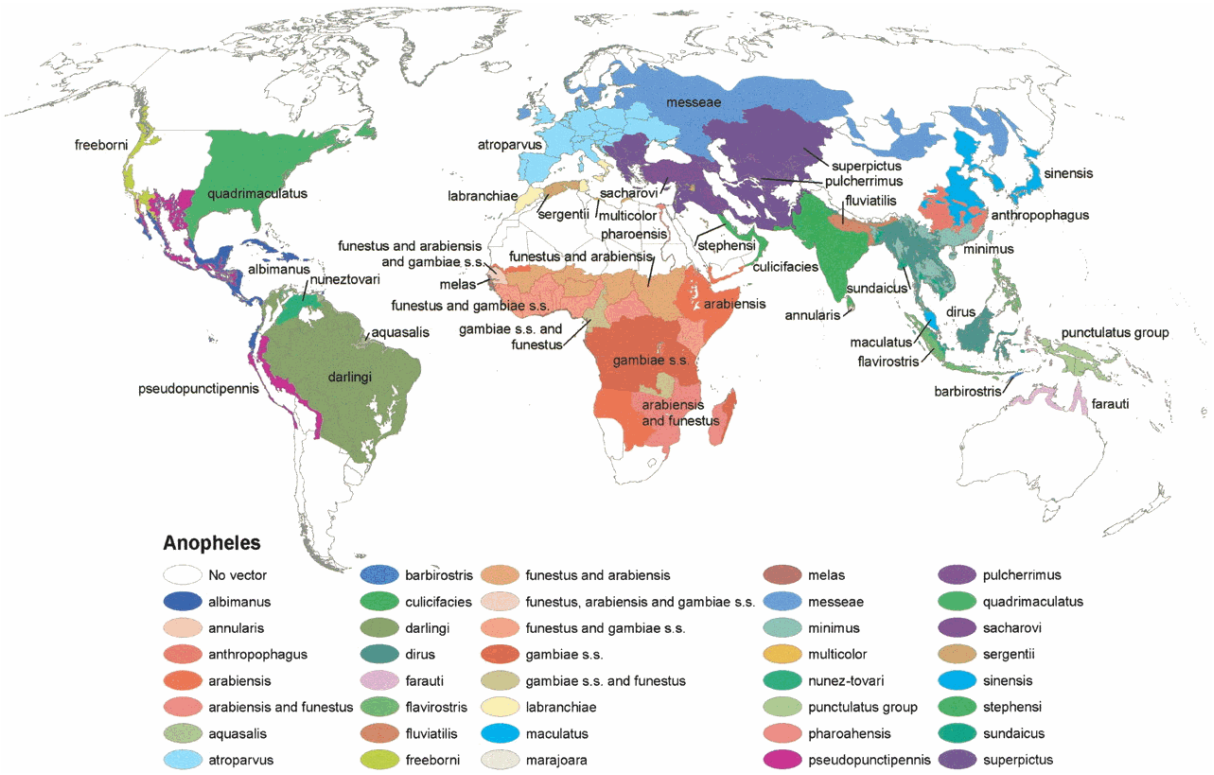

Figure 1.1: Map showing dominant malaria vector species across the world. Source: http://www.ajtmh.org/docserver/fulltext/14761645/70/5/486f1_C4TT.gif

1.4.2 *An. funestus* group

An. funestus is a major malaria vector in Africa. It has a wide geographic distribution covering the entire Afro-tropical region (Figure 1.2) (Coetzee and Koekemoer, 2013; Dia et al., 2013). The larval stages evolve in large bodies of clear water, permanent or semi-permanent, with emergent vegetation high enough to shade the surface of the water (Dia et al., 2013). (Figure 1.3).

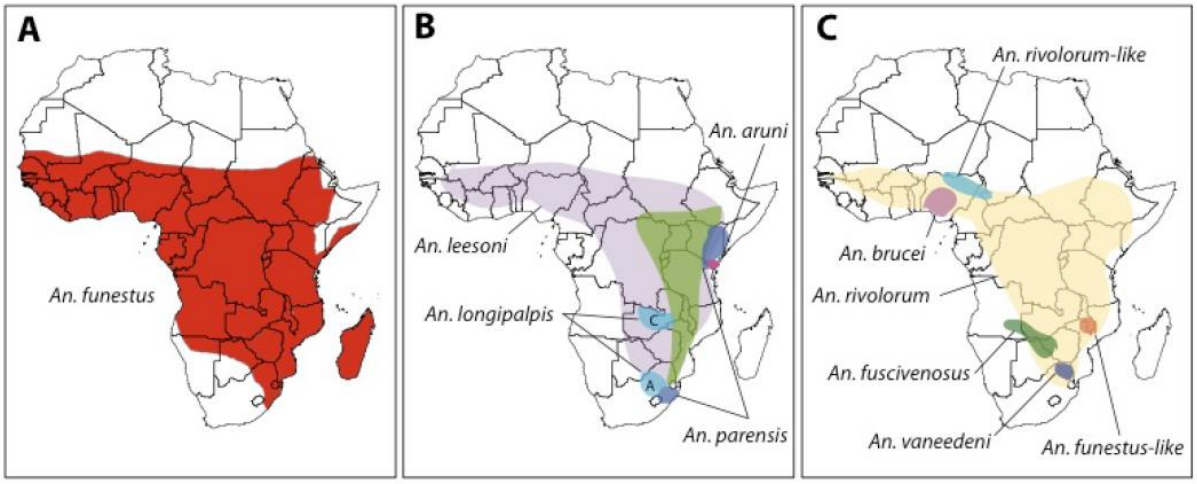

Figure 1.2: Distribution of the 13 species of the Funestus Group in Africa, A: *Anopheles funestus*, B: *An. leesoni, An. longipalpis* (type A and C), *An. aruni* and *An. parensis* (Courtesy of Dr. S. Manguin), C: *An*. *rivolorum, An. rivolorum-like, An. funestus-like, An. vaneedeni, An. fuscivenosus* and *An. brucei* (Courtesy of Dr. S. Manguin).Adapted from (Dia et al., 2013).

Figure 1.3: Breeding sites of *An. funestus.* Mibellon in the Adamawa region, the republic of Cameroon. *An. funestus* breeds all over the year in the lake, which provides shaded areas thanks to the lake vegetation.

The Funestus group includes 13 species grouped into three subgroups (Table 1.1) (Dia et al., 2013). These are: *An. funestus, An. vaneedeni, An. parensis, An. Aaruni, An. funestus-like, An. confuses, An. longipalpis type C, An. leesoni, An. longipalpis type A, An. rivulorum, An. rivulorum-like, An. brucei* and *An. fuscivenosus*(Dia et al., 2013).

Table 1.1: African distribution of the Funestus group (Dia et al., 2013)

African species of the Funestus Group				
Subgroup	**species**	**Geographical distribution**	**Host preference**	**Vector role**
Funestus	*An. funestus*	continental	anthrophilic	major
	An. funestus-like	local	unknown	unknown
	An. Aruni	local	unknown	unknown
	An. Confusus	regional	Zoophilic	unknown
	An. parensis	regional	unknown	minor
	An. Vaneedeni	local	unknown	unknown
	An. Longipalpis type C	local	Zoophilic	unknown
Minimus	*An. Leesoni*	continental	Zoophilic	minor
	An. Longipalis type A	local	Zoophilic	unknown
Rivulorum	*An. Rivulorum*	continental	Zoophilic	minor
	An. Rivulorum-like	local	unknown	unknown
	An. Brucei	local	unknown	unknown
	An.fuscivenosus	local	unknown	unknown

Several molecular biology techniques have been developed for the precise identification of species from the Funestus group (Cohuet et al., 2003; Koekemoer et al., 2002; Spillings et al., 2009). The most recent is based on the polymorphism of the ITS2 region of ribosomal DNA; it identifies *An.funestus s.s., An. vaneedeni, An. rivulorum, An. rivulorum-like, An. leesoni, An. parensis* and *An. funestus-like* (Spillings et al., 2009).

Cytogenetic studies within the species *An. funestus* have made it possible to characterize two chromosomal forms called Kiribina and Folonzo, with a contrasting polymorphism, probably reflecting a phenomenon of ongoing speciation (Costantini et al., 1999).

The biology and vectorial capacity of the funestus group species are very different. Only *An. funestus* is anthropophilic and endophilic and plays an essential role in the transmission of malaria (Antonio-Nkondjio et al., 2002; Fontenille et al., 1997).

Other species of the funestus group, due to their zoophilia and exophilia, are of less importance in medical entomology. Only *An. rivulorum* has been mentioned as a secondary vector of malaria in Tanzania (Wilkes et al., 1996).

Despite being a major vector of malaria, *An. funestus* has been neglected for several decades. Most of the efforts are focused on the members of the gambiae complex (Coetzee and

Fontenille, 2004). This inattention was attributed to the difficulty of colonizing *An. funestus* in the laboratory.

1.5 Malaria control

Controlling malaria is appearing to be a very complicated matter, and it will be dangerous to consider the situation from one angle (Shiff, 2002). To control malaria efficiently, it is recommended to consider an integrated approach, including vector control, which can be based on mosquitoes biology, parasite epidemiology, and human behavior patterns (Mouchet and Carnevale, 1998). Malaria control relies heavly on: (i) Chemotherapy and Chemoprophylaxis to treat the infection caused by *Plasmodium*, (ii) Vector control. In addition to chemotherapy and vector control, rapid diagnostic tests (RDTs) play a key role in malaria elimination. It gives the opportunity of trained members of the community to carry out malaria diagnosis accurately and advise suitable treatment, reducing any delay between the start of fever and treatment.

1.5.1 Treatment of Infection: Chemotherapy and Chemoprophylaxis

Malaria control through the treatment relies on molecules belonging to several classes:

(i) 4-aminoquinolines (chloroquine, quinine, mefloquine, amodiaquine and halofantrine) or 8-aminoquinolines (primaquine).

(ii) The antifolate compounds (pyrimethamine, proguanil, chlorcycloguanil, dapsone, and sulfadoxine).

(iii) The artemisinin and derivatives (artemisinin, artesunate, artemether, arteether, dihydroartemisinin).

(iv) The hydroxynapthoquinone atovaquone (Schlitzer, 2008).

The treatments from all the classes mentioned above are effective and can be advised depending on the severity of the disease. Chloroquine which is a member of 4-aminoquinolines group has been the most successful single drug for the treatment and prophylaxis of malaria. It is a safe and affordable drug, and was effective before resistant strains began to emerge in the 1960. Treatments are advised according to the intensity of

malaria. For the treatment of acute uncomplicated malaria, artesunate-amodiaquine is recommended. For severe malaria cases injectable artesunate is recommended. Sulfadoxine-pyremethamine +amodiaquine is recommended as seasonal preventive treatment of malaria. Chemotherapy to control malaria is threatened by the emergence of resistance to antimalarials that usually appear after a few years (Ashley et al., 2014; White, 2014).

1.5.2 Malaria Vaccines

To prevent malaria, about 38 *Plasmodium falciparum*, and 2 *Plasmodium vivax* candidate malaria vaccine are in the pipeline to be processed and approved (Karunamoorthi, 2014). The recent introduction of the RTS,S (The '**R**' stands for the central repeat region of Plasmodium (P.) falciparum circumsporozoite protein (CSP); the '**T**' for the T-cell epitopes of the CSP; and the '**S**' for hepatitis B surface antigen) malaria vaccine in Ghana, Kenya and Malawi as a WHO pilot program has given hope regarding malaria control (WHO, 2019b).

1.5.3 Vector control

The absence of a 100 % effective vaccine introduced for clinical used to make malaria prevention to rely on chemotherapy and vector control programs at the moment. Vector control holds chemical methods and biological methods.

1.5.3.1 Biological method

To maintain the efforts put in place to fight against malaria, we need to explore other methods to circumvent insecticide resistance. The biological approach to control malaria transmission relies on method like the use of fungus. A recent study showed improved efficacy of an arthropod toxin expressed by fungus against resistant mosquitoes (Bilgo et al., 2017; Lovett et al., 2019). Other important fungal considered in vector control belong to the genera of Coelomomyces, Culicinomyces, Beauveria, Metarhizium, Lagenidium, and Entomophthora (Yassine et al., 2012).

Microbial agent residue like *B. thuringiensis var. israelensis*, serotype H14 (Bti) is widely used as a microbial insecticide to control vector species (Rowe et al., 2003). A large community

scale based program where Bti was applied weekly in Dar Es Salaam has contributed to the reduction of malaria infection prevalence (Geissbühler et al., 2009). *Bti* has been shown to be non-toxic for humans mammals, birds, fish and plant and very efficient to control Culex pipiens (Altalhi, 2005).

In addition, biological methods using the intracellular bacterium Wolbachia can transform the popultion. This method is particularly attractive since this maternally-inherited agent offers a potent mechanism to invade natural populations through cytoplasmic incompatibility (Moreira et al., 2009). When *Wolbachia* are introduced into mosquitoes, they interfere with pathogen transmission and influence life traits such as lifespan (McMeniman et al., 2009). *Aedes aegypti* infected with *Wolbachia* from *Drosophila melanogaster* has successfully invaded two natural *Aedes aegypti* populations from Austria a few months after their release (Hoffmann et al., 2011). This investigation has demonstrated that *Wolbachia*-infected mosquitoes have contributed to the reduction of dengue virus transmission. The results of this study indicate that *Wolbachia*-based strategies can be considered as a good approach to control Dengue

1.5.3.2 Physical approaches

The physical methods to control malaria consist of the cleaning of the environment. These include drainage of stagnant body water, regular cleaning of the wet patch, verification of windows, and other openings. Research conducted in the Tanzanian city of Dar es Salaam by the Liverpool School of Tropical Medicine and the Ifakara Health Institute demonstrates that covering the window with untreated net can suppress the population and intensely reduce malaria prevalence. The study also provided evidence that increasing coverage of complete window screening was linked with reduced biting densities of *Anopheles gambiae* complex and *Anopheles funestus* group (Killeen et al., 2019). One of the most important approach to reduce malaria transmission is Larval source management (LSM) (Fillinger and Lindsay, 2011). Larviciding and environmental management were the only tools available to contain malaria in the early twentieth century.

1.5.3.3 Chemical method

For chemical methods, four insecticides classes are available: organochlorine (DDT), pyrethroid such as permethrin (type I), Deltamethrin (type II), organophosphate (malathion) and carbamates (Bendiocard) (WHO, 2017). The Organochlorine and Pyrethroid are targeting the voltage-gate sodium channel in insect neurons (Brogdon and McAllister, 1998) The Organophosphate and carbamates are targeting the acetylcholine-esterase in the synapse of the insect system nervous. The chemical method is implemented through insecticide-treated materials such as Long-lasting insecticidal nets (LLINS) and Indoor residual spray (IRS).

LLINs: At the moment, vector control relies heavily on LLINS. LLINS is one of the most effective tools that we have at the moment for vector control, it acts as a physical barrier, and the sub-lethal effect of pyrethroid contributes to reducing mosquito's density (Bhatt et al., 2015; Maia et al., 2018; Richardson et al., 2018). Several LLNs brands have been requalified by WHO (WHO, 2019a).

LLINS including Piperonyl butoxide (PBO): LLINs treated with two different active ingredients (each with a different mode of action) are on the table as options to ensure the continued effectiveness of LLINs. Beside the nets with two different active ingredients, we already have nets treated with a pyrethroid combined with a synergist to contribute to the maintenance of the effectiveness of pyrethroid-only nets. Piperonyl butoxide (PBO) is a 'synergist' that can boost the effectiveness of certain insecticides against resistant mosquito populations. PBO inhibits the inner defense mechanisms of insects, the most important are the cytochromes P450, a superfamily of enzymes containing monooxygenases P450 which is the primary route of detoxification in insects, causing the oxidative breakdown of insecticides like pyrethroid (Gleave et al., 2018).

Table 1.2: List of WHO Prequalified LLINs for vector Control (adapted from (WHO, 2019a)

LLINs	Manufacturer	Active ingredient
Olyset Net	Sumitomo Chemical Co., Ltd	Permethrin (1000 mg/m2)
Olyset Plus	Sumitomo Chemical Co., Ltd	Permethrin (1000 mg/m2) + Piperonyl Butoxide (1%)
Interceptor	BASF SE	Alpha-cypermethrin (200 mg/m2)

Interceptor G2	BASF SE	Alpha-cypermethrin (100 mg/m2) + Chlorfenaypr (200 mg/m2)
Royal Sentry	Disease Control Technology, LLC	Alpha-cypermethrin (261 mg/m2)
Royal Sentry 2.0	Disease Control Technology, LLC	Alpha-cypermethrin (203 mg/m2)
Royal Guard (120 denier)	Disease Control Technology, LLC	Alpha-cypermethrin (5.5 g/kg) + Pyriproxyfen (5.5 g/kg
PermaNet 2.0 (75 denier)	Vestergaard S.A.	Deltamethrin (1.8 g/kg)
PermaNet 2.0 (100 denier)	Vestergaard S.A.	Deltamethrin (1.4 g/kg)
PermaNet 3.0 (roof)	Vestergaard S.A.	Deltamethrin (4 g/kg) + Piperonyl Butoxide (25 g/kg)
PermaNet 3.0 (side/75 denier)	Vestergaard S.A.	Deltamethrin (2.8 g/kg)
PermaNet 3.0 (side/100 denier)	Vestergaard S.A.	Deltamethrin (2.1 g/kg)
Duranet LLIN	Shobikaa Impex Private Limited	Alpha-cypermethrin (261 mg/m2)
MiraNet	A to Z Textile Mills Ltd	Alpha-cypermethrin (180 mg/m2)
MAGNet	V.K.A. Polymers Pvt Ltd	Alpha-cypermethrin (261 mg/m2)
VEERALIN	V.K.A. Polymers Pvt Ltd	Alpha-cypermethrin (216 mg/m2) + Piperonyl Butoxide (79 mg/m2)
Yahe LN (75 denier)	Fujian Yamei Industry & Trade Co Ltd	Deltamethrin (1.85 g/kg)

Yahe LN (100 denier)	Fujian Yamei Industry & Trade Co Ltd	Deltamethrin (1.4 g/kg)
SafeNet	Mainpol GmbH	Alpha-cypermethrin (200 mg/m2)
Yorkool LN (75 denier)	Tianjin Yorkool International Trading Co., Ltd	Deltamethrin (1.8 g/kg)
Yorkool LN (100 denier)	Tianjin Yorkool International Trading Co., Ltd	Deltamethrin (1.4 g/kg)
Panda Net 2.0	LIFE IDEAS Biological Technology Co., Ltd.	Deltamethrin (76 mg/m2)
Tsara Boost	NRS Moon netting FZE	Deltamethrin (440 mg/m2)+ Piperonyl Butoxide (120 mg/m2)
Tsara Soft	NRS Moon netting FZE	Deltamethrin (80 mg/m2)
Tsara Plus (Roof)	NRS Moon netting FZE	Deltamethrin (3 g/kg) +Piperonyl Butoxide (11 g/kg)
Tsara Plus (side)	NRS Moon netting FZE	Deltamethrin (2.5 g/kg)

IRS: At the moment, vector control relies heavily on tools like IRS. IRS should be considered in comparison to LLINs, and the most appropriate should be chosen according to the environment. IRS is not easy to implement because it is costly so the coverage cost can be a stopping factor. We also have the logistic aspect, because of logistic concern IRS can be challenging to apply. However, many manufacturers are releasing new formulations showing great results. Some of them are listed below (WHO, 2019a).

Table 1.3: List of some WHO Prequalified IRS forVector Control (adapted from (WHO, 2019a)

IRS	Manufacturer	Active ingredient (AI)	AI concentration	Formulation type
SumiShield 50 WG	Sumitomo Chemical Co., Ltd.	Clothianidin, WG	50%	WG

Fludora Fusion	Bayer S.A.S.	Clothianidin, Deltamethrin	Clothianidin: 50% Deltamethrin: 6.25%	WP-SB
Actellic 300CS	Syngenta Crop Protection AG	Pirimiphos-methyl	300g/L	CS
ICON CS - ITN Kit	Syngenta Crop Protection AG	Lambda-Cyhalothrin 25g/L CS	25g/L	CS
Fendona 10 SC	BASF SE	Alpha-cypermethrin	9.6%	SC
K-Othrine WG250	Bayer S.A.S.	Deltamethrin	25% (250 g/kg)	WG; WG-SB

Larviciding: The purpose of this method is to reduce the larva density to reduce the vectorial capacity of the vector. Temephos, Malathion, and pirimiphos-methyl are used for this achievement (WHOPES, 2013), but the non-persistence of these insecticides in the breeding site makes this method to require constant spray in water. Because of this reason, this method appears to be expensive. However, in the context of the rapid increase in insecticide resistance, outdoor malaria transmission, and the changing behaviour of malaria vectors, larviciding when it is well could add value to malaria control (Antonio-Nkondjio et al., 2018).

1.6 Insecticide resistance

Insecticide resistance is defined as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species" (IRAC, 2018). Insecticide resistance is conferred mainly through target resistance and metabolic resistance, which are grouped as physiological resistance. In addition reduced penetration resistance and behavioral resistance are also observed (Hemingway and Ranson, 2000; IRAC, 2010).

1.6.1 Target site resistance

Most of the insecticide classes target a specific action site in the nervous system. Target-site resistance takes place when this action site is modified. As a result of this modification, the insecticide effect on the mosquito is reduced. Three types of target resistance are observed: The voltage-gate sodium channel modification (knock-down resistance: Kdr) responsible of

organochlorine and pyrethroid resistance (Hemingway and Ranson, 2000; Martinez-Torres et al., 1998), acetylcholine-esterase modification responsible of organophosphate and carbamate resistance (Weill et al., 2004) and the gamma aminobutyric acid (GABA) modification responsible of dieldrin resistance (RDL) (Wondji et al., 2011).

Knockdown resistance (Kdr): *Kdr* resistance has been detected in several insect species, including many mosquitoes such as *An. gambiae, Culex pipiens,* and *Aedes aegypti* (Hemingway and Ranson, 2000). The most common mutation is observed at the 1014 codon position with leucine to phenylalanine replacement (L1014F), as also found in *An*. *gambiae* (Martinez-Torres et al., 1998). However, another mutation is observed in the same codon in both and *An gambiae* and *An. arabiensis* with a Leucine to Serine replacement (L1014S), which was originally observed in East Africa (Hemingway and Ranson, 2000) while the L1014F is predominant in West Africa. The studies carried out until now revealed no *Kdr* presence in *An. funestus* (Irving and Wondji, 2017; Wondji et al., 2012). However recent analysis of the polymorphism of a portion of the voltage-gated sodium channel gene (VGSC) of *An. funestus* from Cameroon (Gounougou) confirm the absence of the L1014F/S Kdr mutation but revealed 3 novel amino acid changes I877L, V881L, and A1007S. However, no association was established between VGSC polymorphism and pyrethroid/DDT resistance (Menze et al., 2016).

Acetylcholinesterase: Acetylcholinesterase mutation is due to glycine to serine substitution (G119S) in many mosquito species. G119S has been reported in West Africa in *An. gambiae* (Weill et al., 2004). The same mutation has also been found in *An arabiensis* (Casimiro et al., 2006). But never found in and *An. funestus* (Cuamba et al., 2010). Recently, a study by (Ibrahim et al., 2016) detected in *An. funestus* population from Southern Africa a novel mutation (N485I) associated with carbamate resistance (Ibrahim et al., 2016).

GABA receptor: Several mutations conferring insecticide resistance have been detected in insects (Corbel and N'Guessan, 2013). For the malaria vector, *Anopheles gambiae*, alanine to glycine substitution in the Rdl locus is conferring resistance to dieldrin (Du et al., 2005). The A296S mutation in the Gaba receptor has also been described and associated with Dieldrin resistance in *Anopheles funestus* population from Cameroon and Burkina Faso (Wondji et al., 2011). However, this mutation was not observed in *An. funestus* population from the Eastern and Southern parts of Africa (Malawi, Uganda and Mozambique).

Although dieldrin is not used any more, this mutation is still present in the *Anopheles funestus* population from the Northern part of Cameroon even if decreasing over time. In 2006 the RR

genotype frequency of RDL in Northen Cameroon was around 80% , then 30% in 2012 and less than 5% in 2015 (Menze et al., 2016). This is due to a recovery of susceptibility since the selection pressure has been removed (Menze et al., 2016).

1.6.2 Metabolic resistance

All insects possess enzymatic systems that detect toxin and initiate the metabolism of these compounds to detoxify their body. Metabolic resistance takes place when the insecticide cannot reach the target site because of the detoxification action of specific enzyme families. This type of resistance occurs through different potential mechanisms, including Gene amplification, gene up-regulation, and allelic variation. Three enzyme families are primarily involved in detoxification: monooxygenases, glutathione-Transferases, and esterase (IRAC, 2011), although other families are also associated (Hemingway and Ranson, 2000).

Cytochrome P450 monooxygenases: Cytochrome monooxygenases are an important enzyme group present in almost all animals, and they play a major role in detoxification (Feyereisen, 1999; Scott, 1999). Monooxygenases have been involved in resistance to several insecticide classes in many mosquito species across Africa. Monooxygenases have been associated with a pyrethroid, organophosphate, and carbamate resistance (Hemingway and Ranson, 2000). In *An. funestus* two duplicated cytochrome P450 genes *CYP6P9a* and *CYP6P9b* have been associated with pyrethroid resistance in the southern part of Africa (Mozambique and Malawi) (Amenya et al., 2008; Riveron et al., 2013; Wondji et al., 2009) as well as the *CYP6M7* gene (Riveron et al., 2017b). *CYP6P3* and *CYP6M2* have also been associated with pyrethroid resistance in *An. gambiae* in Benin and Nigeria (Djouaka et al., 2011; Mitchell et al., 2012; Müller et al., 2008). A recent study by (Riveron et al., 2017b) revealed that *CYP6P9a*, *CYP6P9b*, and *CYP6M7* genes, involved in pyrethroid resistance in *An. funestus* were found upregulated in *Anopheles funestus* population from Congo after qRT-PCR using non-exposed mosquitoes (Riveron et al., 2017b).

Glutathione-S-transferases: Glutathione-S-transferases (*GSTs*) are mainly involved in DDT resistance (Hemingway and Ranson, 2000). Using genome-wide transcriptional and functional analyses combined with structural and population genetics studies, we conclusively

demonstrated that a single amino acid change in the binding pocket of the glutathione-s-transferase epsilon 2 (*GSTe2*) gene, coupled with increased transcription, confers a high level of DDT resistance and cross-resistance to pyrethroids in the major African malaria vector *An. funestus* (Figure 1.4) (Riveron et al., 2014b). The same study detected, for the first time, a molecular resistance marker for metabolic resistance and designed a DNA-based diagnostic assay currently use to identify and map the distribution of resistance conferred by *Gste2* across Africa. *GSTs* have been associated with DDT resistance in *An. funestus* population across several countries in Africa including Benin in West Africa (Djouaka et al., 2016a) but also Cameroon in Central Africa (Menze et al., 2016) through a mutation in the *GSTe2* gene (L119F)(Figure 1.5) (Riveron et al., 2014).

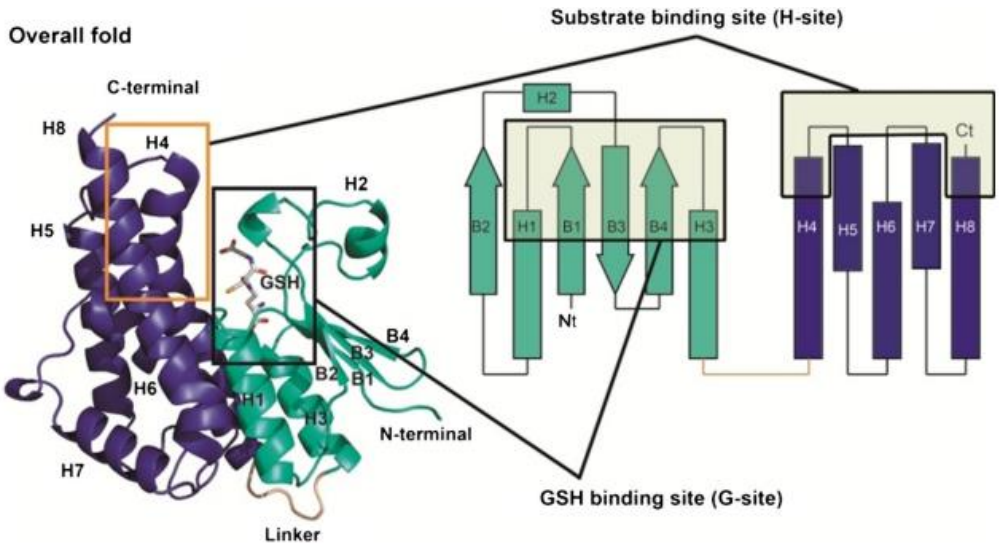

Figure 1.4: Topology of *GSTe2* showing the C- and N-terminals, the GSH binding pocket (G-site) and the substrate-binding pocket (H-site). AU, arbitrary units; BN, Benin; GSH, glutathione; MAL, Malawi; MW, molecular weight; UG, Uganda; vol, volume. Adapted from (Riveron et al., 2014b).

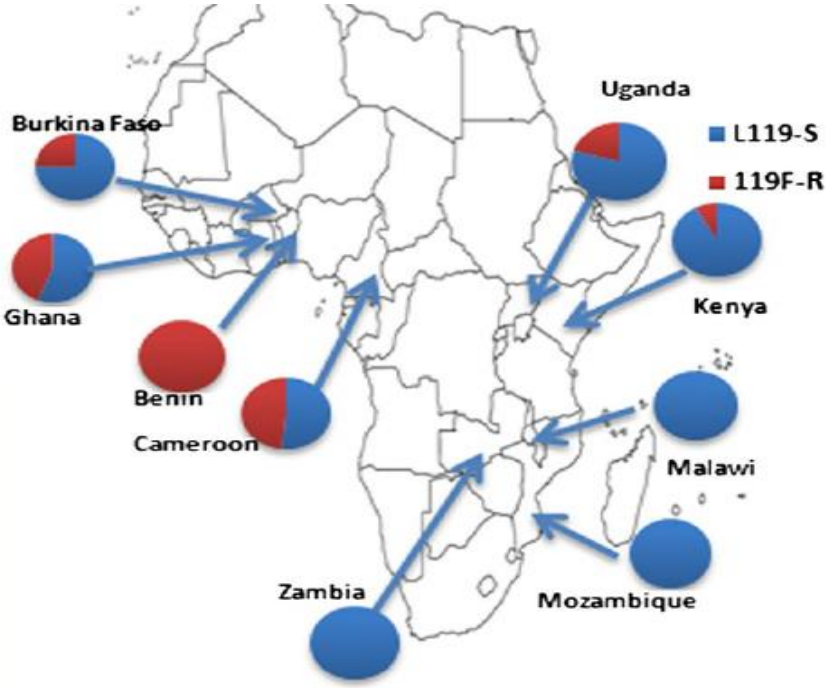

Figure 1.5: The geographical distribution of L119F allele across Africa in *Anopheles funestus*. sl population (Riveron et al., 2014b).

Esterase: The selection pressure due to the use of organophosphate and carbamate has led to the overexpression of non-specific carboxylesterases(CCE) in many species including mosquitoes, aphids, and cockroaches (Hemingway et al., 2004). Several studies have associated CCE in the hydrolysis or sequestration of ester bonds in insecticides (Corbel and N'Guessan, 2013). Carboxylesterases have been associated with temephos resistance in *Ae aegypti* (Poupardin et al., 2014). Bioassays on *Ae. aegypti* larvae from Thailand detected a high level of temephos resistance with ratios ranging from 3.5 fold in Chiang Mai to nearly ten fold in Nakhon Sawan (NS) province. Synergist assays and microarray results suggested and increased activities of carboxylesterase. Gene profilindg have shown that carboxylesterase genes (CCEae3a) were up to 60 time upregulated when comparing sample resistant to temephos to susceptible (Poupardin et al., 2014).

In *Culex quinquefasciatus* population known as a major vector of human disease and an important target of the vector control program, insecticide resistance has been related to esterase (Gordon and Ottea, 2012).

In *Anopheles sinensis* a major malaria vector in eastern Asia, resistance to pyrethroid has been associated with Carboxyl esterases, RNA-seq spotted five overexpressed CCE genes, and qRT-PCR identified 12 overexpressed CCE genes. The main CCE genes linked with pyrethroid resistance were the α-esterase ten and acetylcholinesterase 1 (Wu et al., 2018).

Gene profiling analysis in *An. funestus* population has detected some candidate carboxylesterases genes overexpressed (Riveron et al., 2014a). Current studies are going on for their validation.

1.6.3 Penetration resistance

Massive use of insecticide have contributed to the selection of a more sophisticated resistance mechanism. Penetration resistance refers to some changes in the cuticle that will reduce the diffusion of insecticide in the insect body (Balabanidou et al., 2018). A review by Balabanidou et al has identified two main mechanisms by which penetration resistance is occurring: (i) The cuticle thickening and (ii) The altering of cuticle composition. These cuticular modifications are happening as a result of the over-expression of several genes, cuticular protein, and ABC transporters. The upregulation of *CYP4G16* and laccase two have also been identified as responsible of cuticular modifications (Balabanidou et al., 2018).

In *An. gambiae* population, the contribution to cuticle changes in insecticide resistance has been assessed. Microscopy studies and characterization of lipid extracts have demonstrated that resistant mosquitoes had a thicker cuticle and a significant increase in cuticular hydrocarbon content. The overexpression of *CYP4G16* and *CYP4G17* has been associated with the increase in cuticular hydrocarbon content (Balabanidou et al., 2016).

In *An. funestus* population from South Africa, pyrethroid resistance has been associated with changes in the thickness of the cuticle. A positive correlation between mean cuticle thickness and time knockdown during exposure to permethrin was observed. In addition the mean cuticle thickness was significantly high in samples resistant to permethrin compared to susceptible (Wood et al., 2010). Besides metabolic based resistance with is the main insecticide resistance mechanisn in *An funestus* population, penetration resistance is rising as an auxiliary mode of resistance.

1.6.4 Behavioural resistance

Insects can respond to insecticide exposure by changing their behavior to avoid the lethal effect of the insecticide. In some cases, metabolic resistance and target resistance are supported by behavioral resistance (IRAC, 2010). A change of biting behavior was observed in *An funestus* population from 2 villages in Benin after massive LLINs coverage (Moiroux et al., 2012). Human-landing catch was conducted indoor and outdoor in Lokohouè and Tokoli, two localities in Benin. The study was carried out one year before the LLIN coverage and three years later. During the study, the median catching time and the proportion of outdoor biting were compared. In Tokoli the percentage of outdoor biting moved from 45 to 681 %. Just one year after the LLINs coverage. In the next village, we notice 26% of the *An. funestus* population biting after 6 am. This evaluation was proof of change in biting behavior (Moiroux et al., 2012). Studies are going on to evaluate and give more evidence of behavioral resistance as a mechanism of resistance with genetics and a heritable basis (Zalucki and Furlong, 2017).

1.7 Evolution of Insecticide resistance

Insecticide resistance including all the four classes of insecticides is now noticed all over Africa (Antonio-Nkondjio et al., 2017; Djouaka et al., 2016c; Menze et al., 2016; Menze et al., 2018b; Mulamba et al., 2014b; Riveron et al., 2016b). Resistance to pyrethroids, the insecticide class, commonly used in LLINs, is increasing rapidly. Pyrethroid resistance has been noticed in many *An. gambiae* populations across Africa. In *An. gambiae* s.s. population from Côte d'Ivoire, resistance to cyfluthrin, lambda-cyhalothrin, alpha-cypermethrin,deltamethrin, and permethrin was observed. Using the same population, resistance was also observed with deltamethrin-impregnated nets (Chandre et al., 1999). Further studies in Côte d'Ivoire in 2012 in the locality of Tsassodji have confirmed the resistance to permethrin (mortality: 42.0%), to deltamethrin (mortality: 58.8%) and lambda-cyhalothrin (mortality:67.7%) in *An gambiae* population (Alou et al., 2012). *An gambiae* from Mibellon in Cameroon was also found highly resistant to pyrethroid with mortality ranging from 0% to 4.44% (Menze et al., 2018b).

Beside pyrethroid resistance, organophosphate and carbamate resistance were also found. *An. gambiae* s.l. from Bouake in Côte d'Ivoire was found to be resistant to carbamates (bendiocarb and carbosulfan) with mortalities ranging from 13.9% to 67% (Zoh et al., 2018). Organophosphate resistance was observed in *An. gambiae* population from Benin. After 30 minutes of exposure to CDC bottles treated with fenitrothion. *An. gambiae* from Tanguieta and Malanville, two localities in Benin were found resistant with mortalities ranging from 57.14% (Malanville) to 76.72% (Tanguieta) (Aïzoun et al., 2013).

In *An. funestus* population, insecticide resistance to diverse classes of insecticides such as pyrethroids, carbamates, organochlorines, and organophosphate are increasingly reported across Africa although with contrasting patterns (Cuamba et al., 2010; Djouaka et al., 2016a; Menze et al., 2016; Menze et al., 2018b; Mulamba et al., 2014b; Riveron et al., 2016b). In southern Africa, *An. funestus* populations are highly resistant to pyrethroids and carbamates (Cuamba et al., 2010; Morgan et al., 2010; Riveron et al., 2016b; Wondji et al., 2012) whereas populations from East Africa remain fully susceptible to carbamates but are resistant to pyrethroids and DDT (Kawada et al., 2011; Mulamba et al., 2014a; Mulamba et al., 2014b). In West Africa, *An funestus* populations have been reported to be resistant to pyrethroids, DDT, and moderately resistant to carbamates (Djouaka et al., 2016a; Menze et al., 2016; Menze et al., 2018b; Riveron et al., 2016b).

1.8 Impact of the resistance to insecticides on the effectiveness of vector control tools

The extent of the impact of insecticide resistance, notably, metabolic resistance on the effectiveness of insecticide-based interventions such as Long-lasting Insecticidal Nets (LLINs) remains unclear. There is currently an intense debate with sometimes opposite results as published recently (Kleinschmidt et al., 2018a; Protopopoff et al., 2018a) on the impact of insecticide resistance on the effectiveness of insecticide-based interventions. This is illustrated with two recent publications, one highlighting the limited impact of resistance from a multicentre study (Kleinschmidt et al., 2018a) whereas a randomized control trial comparing PBO-net vs pyrethroid only revealed a significant epidemiological benefit with PBO nets highlighting a negative impact of resistance (Protopopoff et al., 2018a). This contrasting result

shows the challenge of addressing this fundamental question. However, the lack of resistance markers for metabolic resistance has been one of the limiting factors.

Two approaches (epidemiological and entomological) are usually considered as useful methods to investigate the impact of insecticide resistance (WHO, 2012b). However, the epidemiological approach relaying on malaria cases investigation is not suitable since malaria transmission is affected by many confounding factors (Kleinschmidt et al., 2015; WHO, 2012b). These confounding factors, such as the local habitation favorable to the mosquito bite, the weather, and the fickleness of the health care system, can blind the real link between Insecticide resistance and malaria transmission. Recognising the limitations of the epidemiological approach, the alternative is to measure entomological outcomes, such as relative mortality and feeding success of resistant and susceptible vectors in controlled conditions using experimental huts (N'Guessan et al., 2009; N'Guessan et al., 2007b; WHO, 2012b).

1.8.1 Case of the impact of insecticide resistance on the effectiveness of vector control tools in Benin.

Experimental hut trial was used in Benin to check the efficacy of ITN (Lambdacyalothrin) in two localities of Benin: Ladji an area where mosquitoes are highly resistant with a high level of Kdr and Malanville an area where mosquitoes are susceptible. In Ladji, reduction of the efficacy of the insecticide was noticed with a low mortality rate (29.8%) and a high level of blood-feeding (82%). In Malanville where mosquitoes were susceptible, the mortality was around 98.5%, and the blood-feeding 3% (N'Guessan et al., 2007b). These results highlight the fact that Insecticide resistance is changing the efficacy of nets. Unfortunately, in this case, the direct impact of kdr could not be assessed as both populations had different genotype patterns. Metabolic resistance could not be determined since there are no molecular markers available.

1.8.2 Case in South Africa where pyrethroid resistance led to the increased of malaria cases

In 1996 a national decision was made to switch from DDT to Pyrethroid using IRS. A few years later, a strident increase of malaria cases was observed. The bioassays performed revealed that *An. funestus* was resistant to Pyrethroid and susceptible to DDT. These mosquitoes also showed a sporozoite rate of 5.4 %, which is high compare to what usually observed in South Africa. When they reverted to DDT, they noticed a reduction of malaria transmission (Hargreaves et al., 2000). This observation further highlights the fact that insecticide resistance reducing the efficacy of IRS negatively impact malaria transmission.

1.8.3 Other studies on the impact of insecticide resistance on the effectiveness of vector control tools

A study by (Strode et al., 2014) presented a systematic review and Meta-analysis, including cone assays, tunnel assays in the laboratory and field studies using experimental hut from many authors. Cone assays showed reduced knockdown and mortality associated with a high level of resistance. For tunnel assay, because of a high level of resistance, reduced efficacy of ITNs was also observed when looking at the mortality and passage of mosquitoes through the net (Strode et al., 2014). For experimental hut, independently of the resistance profile, a clear difference in the entomological outcomes was observed when compared to the treated hut to the untreated. However, a large amount of heterogeneity between the different studies creates a situation where it was difficult to conclude. A strong recommendation to people involved in the area to standardize the protocol was raised. For example, the characterization of the resistance pattern before the study, the season, the rotation of the sleepers, the washing procedure, decontamination of the hut, and exclusion of males from the analysis were suggested for standardization (Strode et al., 2014).

Another study by (Churcher et al., 2016b), used a Meta-analyses of bioassay studies and experimental hut trials (figure 1.8)to demonstrate how emerging insecticide resistance is changing the number of malaria cases. Transmission dynamic models reveal that even very

low level of resistance can increase malaria incidence because of a reduction of mosquito's mortality For example only 30% of resistance in a population with 10% malaria prevalence could cause an additional 245 malaria cases per 1000 per year (Churcher et al., 2016b).

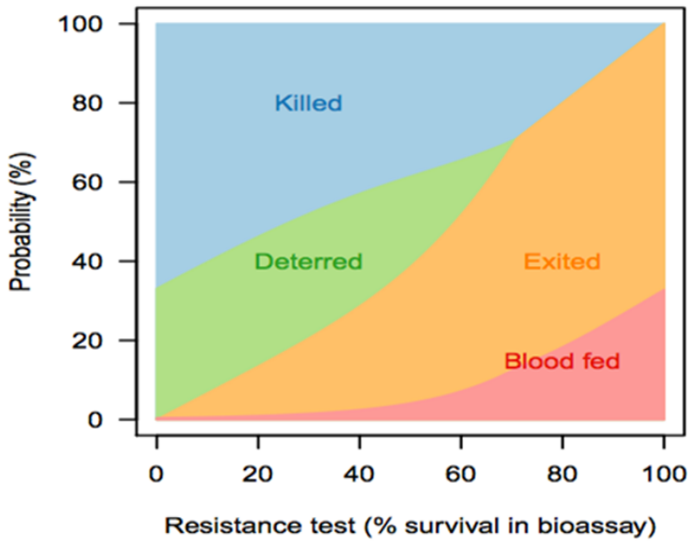

Figure 1.6: Average probability that blood-feeding mosquitoes will be killed, deterred from entering, exit without feeding or successfully feed and survive during a single feeding attempt and how this changes with the population prevalence of pyrethroid resistance. From (Churcher et al., 2016b).

1.9 Evaluation of the impact of metabolic resistance

1.9.1 DNA-based marker for GST-based metabolic resistance

The first DNA-based diagnostic for *target-site resistance* was established two decades ago (Martinez-Torres et al. 1998). Detecting DNA-based markers for metabolic resistance has been more challenging. The lack of markers for metabolic resistance has made it difficult to monitor its spread in the field, and assess its real impact on ongoing control interventions and malaria transmission. However, recent significant progress has been made. The first success was the case of the *GST*-based metabolic resistance with the detection of a Leucine-to-Phenylalanine amino acid change at position 119 in the GST epsilon 2 (*GSTe2*) in the major malaria vector *An. funestus* (Riveron et al 2014). Over-expression of GST epsilon 2 (*GSTe2*) has been associated with DDT and/or pyrethroids resistance in several mosquito species, including *An.*

gambiae (Mitchell et al., 2014), *An. funestus* (Riveron et al., 2014c) and *Aedes aegypti* (Lumjuan et al., 2011).

In *An. funestus*, the role of the L119F mutation, with the resistant allele being more efficient at metabolizing DDT, was confirmed by functional characterization of recombinant *GSTe2*. It has also been demonstrated that *GSTe2* directly metabolizes the pyrethroid permethrin (Riveron et al., 2014b). Structural analysis revealed that a leucine to phenylalanine amino acid change (L119F) in *GSTe2*, enlarging the substrate-binding pocket of the enzyme, was conferring DDT/pyrethroid resistance in West and Central African populations (Riveron et al., 2014c) (Figure 1.9). Based on this Riveron et al 2014 also designed a TaqMan assay to detect the three genotypes of the mutation. The TaqMan assay was later used for genotyping alive and dead mosquitoes after exposure to DDT and pyrethroid and to demonstrate using the odd ratio that *GSTe2*-L119F correlates with DDT and Pyrethroid resistance (Riveron et al., 2014b). Further studies confirm the role of allelic variation of the gene as GAL4-UAS transgenic expression in Drosophila flies demonstrates that the resistant 119F allele is conferring a high level of resistance to DDT and permethrin when compared to the L119 (Riveron et al., 2017a). In addition, this single amino acid change was used to design a simple field applicable DNA-based diagnostic tool (Tchouakui et al., 2019c) (Figure 1.10).

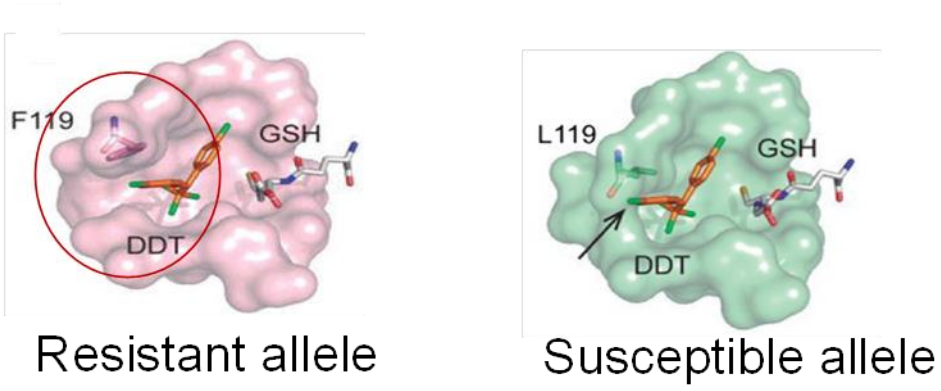

Figure 1.7: Image representing the substrate biding pocket from Benin (purple) and Uganda (green). The H site's representation demonstrates how the DDT docks well into the *GSTe2* in mosquitoes from Benin and does not with the *GSTe2* in mosquitoes from Uganda. Adapted from (Riveron et al., 2014b).

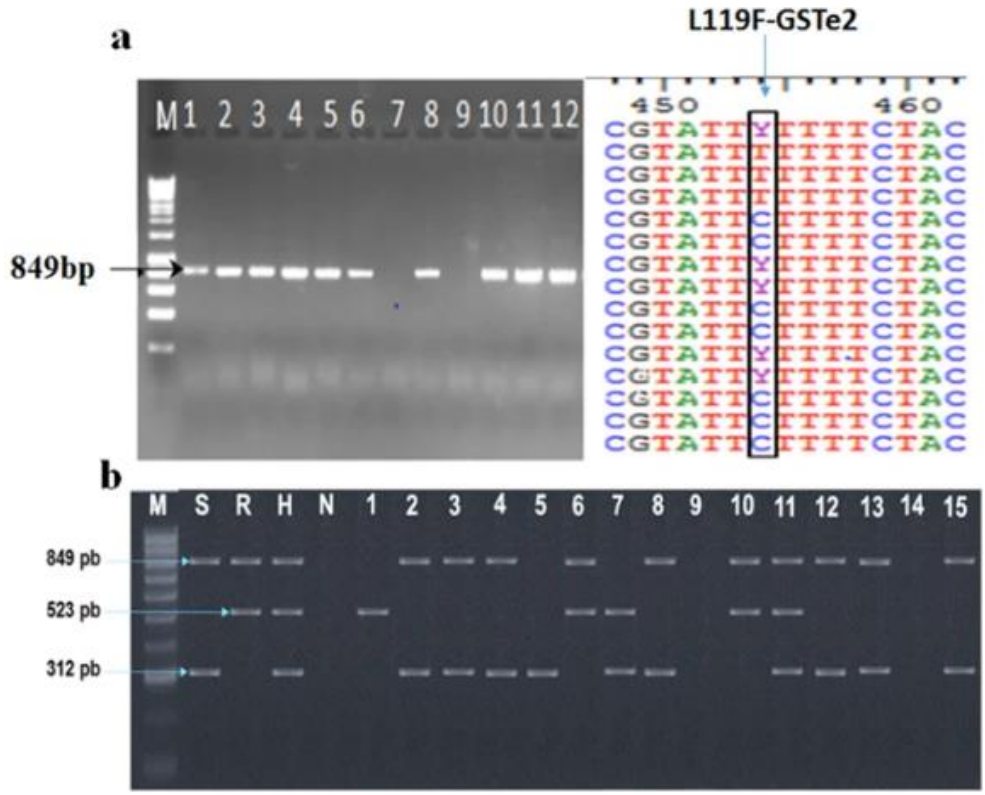

Figure 1.8: Allele-Specific -PCR for genotyping of the L119F-*GSTe2* mutation. (a) Amplification by PCR of the *GSTe2* gene in *An. funestus* s.s. (b) Agarose gel of AS-PCR showing the L119F *GSTe2* mutation in *An. funestus* s.s. at 849 bp. Resistant at 523 bp, susceptible at 312 bp, and heterozygote mosquitoes at both 523 bp and 312 bp. Adapted from (Tchouakui et al., 2019a).

1.9.2 DNA-based marker for Cytochrome P450-based metabolic resistance

1.9.2.1 DNA-based marker for *CYP6P9a*

Regarding P450 family genes, further genomic analyses revealed a complex evolution of resistance, including copy number variation, transposon insertion, and gene conversion. From *An. funestus* samples across Africa, 800-bp sequence immediately upstream of *CYP6P9a* were compared to detect cis-regulatory mutations controlling *CYP6P9a/b*-based pyrethroid resistance. Following the comparison, very low diversity was observed in the genomes of mosquitoes from different regions. However, mosquitoes from southern Africa consistently showed different polymorphisms when compared to other Africa regions. This polymorphism was including the presence of an AA insertion eight bp upstream of a putative CCAAT box through an A/C substitution (Figure 1.11) (Weedall et al., 2019).

The detection of causative cis-regulatory mutations allowed designing the first DNA-based P450 assay in malaria vectors (Weedall et al., 2019). An image of the gel showing how the 3 *CYP6P9a* genotypes detected is present in chapter 2 (section 2.4.4; Figure 2.12).

These molecular markers recently made available are providing a unique opportunity to now address questions regarding the direct impact of metabolic resistance on insecticide-based interventions such as LLINs.

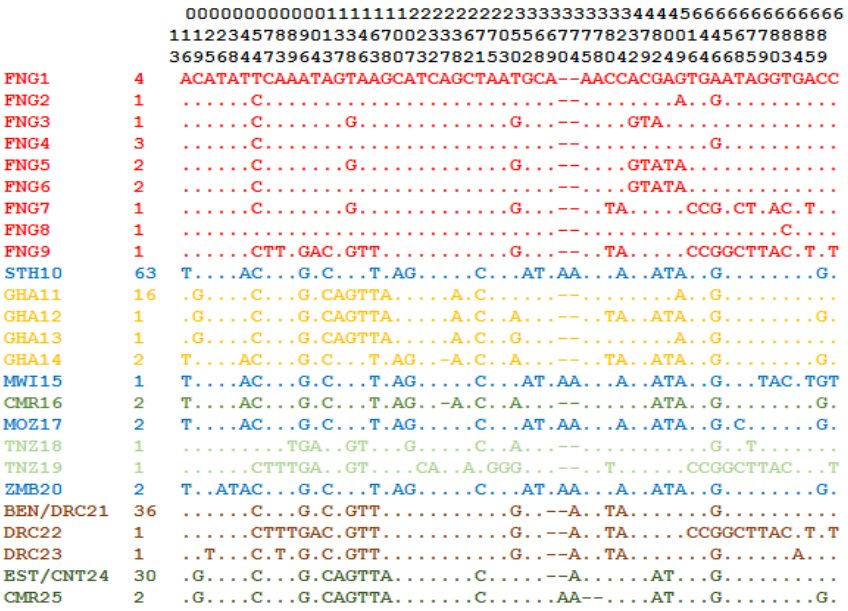

Figure 1.9: Polymorphic sites and haplotypes across Africa showing AA insertion eight bp upstream of a putative CCAAT box through an A/C substitution and genetic diversity patterns of an 800bp cis-regulatory genomic fragment of *CYP6P9a* Africa-wide. Haplotypes are labeled with prefixes from the country. CMR is Cameroon, GHA is Ghana, BEN is Benin, TNZ is Tanzania, DRC is the Democratic Republic of Congo, MOZ is Mozambique, STH is Southern Africa, MWI is Malawi, ZMB is Zambia, EST/CNT is East/Central Africa, FANG is FNG. Adapted from (Weedall et al., 2019).

1.9.2.2 DNA-based marker for *CYP6P9b*

Transcriptional and genomic analysis of *An. funestus* mosquitoes across Africa showed complex and diverse molecular changes underlying insecticide resistance trough differential gene expression patterns and genomic signatures like cis-acting regulatory elements regulating the expression of detoxification genes. To understand the potential regulatory elements controlling the over-expression of *CYP6P9b*, the full 1 kb intergenic region between

CYP6P9b and *CYP6P5* in individual resistant (FUMOZ) and susceptible (FANG) mosquitoes was amplified and sequenced. An indel of 3 bp (AAC) was consistently observed with a deletion in the resistant FUMOZ and presence in susceptible FANG. Based on this, a DNA-based diagnostic assay to detect *CYP6P9b*-mediated resistance was designed (Figure 1.12) (Mugenzi et al., 2019). An image of the gel showing how the 3 *CYP6P9b* genotypes detected is present in chapter 2 (section 2.4.5; Figure 2.13).

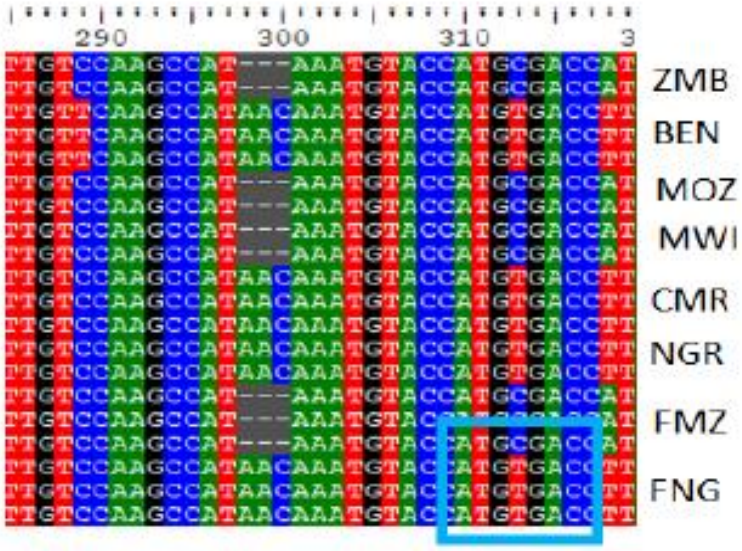

Figure 1.10: Sequences showing fixed variations linked with *CYP6P9b*-mediated pyrethroid resistance, including the AAC insert only found in resistant mosquitoes and the C/T variant tightly linked to the resistant haplotype. Adapted from (Mugenzi et al., 2019).

1.10 Development of new insecticide products for vector control

The high level of resistance observed among the Anopheles population (Menze et al., 2016; Menze et al., 2018b; Riveron et al., 2017b) is a concern for the vector control program in Cameroon and Africa. In addition, the reduced efficacy of bed nets currently used in public health associated with a high level of resistance is also reported (Allossogbe et al., 2017; Churcher et al., 2016a; N'Guessan et al., 2007b). This reduced efficacy of the product available for vector control at the moment due to the high level of resistance in Anopheles field population is an urgent call for the need to develop new products (Zaim and Guillet, 2002). To raise this issue, the Innovative Vector Control Consortium (IVCC) is working hard in collaboration with Bill et Melinda Gates, chemical industry, and academia to find new products for vector control (Hemingway et al., 2006). This collaboration aims to provide by 2023 new

chemical and technological tools to reduce the burden of malaria, Dengue, and other vector-borne diseases (Hemingway, 2014).

Some products with different mode of action previously used in the agricultural sector are now implemented in public health for vector control. Chlorfenapyr, for example, a pyrrole insecticide was found to be very efficient against *Ace1* and *Kdr* resistant mosquitoes after a simulation of experimental hut trial (N'guessan et al., 2007a). After experimental hut using ITNs and IRS Chorfenapyr was also found to be successful in killing pyrethroid-resistant *Anopheles gambiae* and *Culex quinquefasciatus* (N'Guessan et al., 2009). A duo nets made by the combination chlorfenapyr mixed with alpha-cypermethrin was found to be effective against resistant mosquitoes in West Africa (N'Guessan et al., 2016). Another study comparing chlorfenapyr IRS + Interceptor LN (alpha-cypermethrin) vs interceptor G2 LN (duo nets combining chlorfenapyr + alpha-cypermethrin) vs Interceptor LN alone in Cove in Benin was also done. The combination of pyrethroid + Chlorfenarpyr was found to be more effective when compared to pyrethroid alone (Ngufor et al., 2017a). This efficiency of the mixture is due to the fact that the chlorfenapyr with a different mode of action increased the mass killing while the pyrethroid with the repellent effect provided the personal protection (Ngufor et al., 2017a).

Fludora® Fusion is a new product for vector control developed by Bayer. This product is the first IRS application combining two insecticides with a different mode of actions (the neonicotinoid insecticide clothianidin and the pyrethroid insecticide deltamethrin). Fludora shows great results against resistant mosquitoes using experimental hut in Benin with 80% mortality and up to 6 months lasting efficacy (Figure1.13) (Agossa et al., 2018).

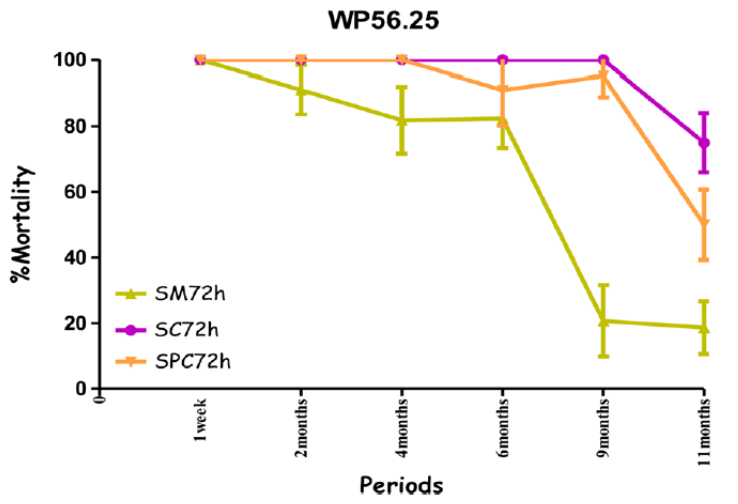

Figure 1.11: Mortality obtained with wild *An. gambiae* s.s. in operational conditions in Benin using mixture clothianidin 200 mg/m2+deltamethrin 25 mg/m2 (WP 56.25). Adapted from (Agossa et al., 2018).

Another study in Cove in Benin also demonstrated good performance of fludora at different concentrations on different surfaces (Figure 1.14) (Ngufor et al., 2017b).

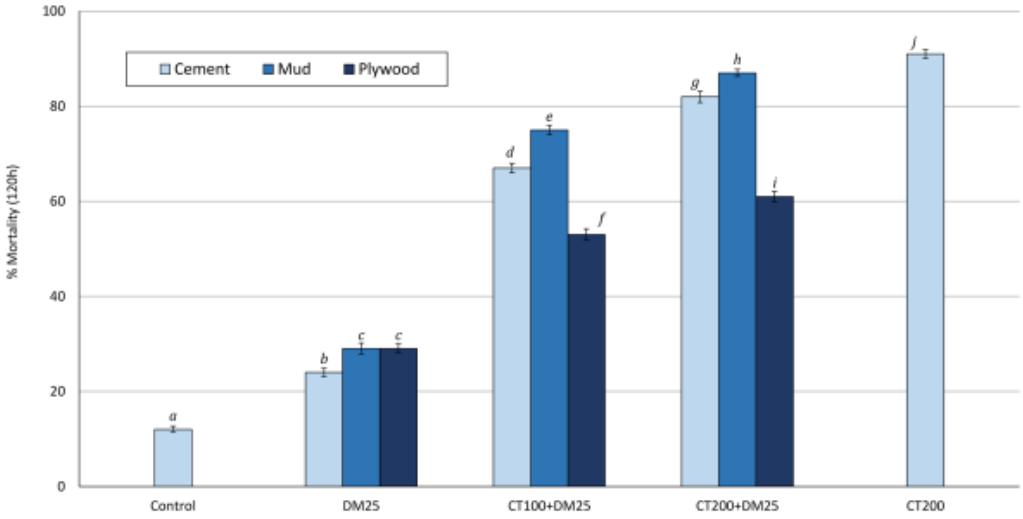

Figure 1.12: Mortality against wild pyrethroid-resistant *An gambiae* in operational conditions on different wall surfaces. Bars with a similar letter are not significantly different at 5%. DM = deltamethrin, CT = clothianidin. Adapted from (Ngufor et al., 2017b).

SumiShield™ 50WG is an Indoor Residual Spray (IRS) containing a new Mode of Action active ingredient. The active ingredient is made of clothianidin 50% w/w a water-dispersible granule (WG). SumiShield 50WG is odorless, has low toxicity, readily dilutes in water, and is easy to transport. Between 2016 and 2017, a trial to determine the efficacy of SumiShield 50WG, against wild Anopheles arabiensis was carried out in the in Moshi village in the Republic of Tanzania. The biological efficiency of Sumishield was assessed in Moshi in Tanzania. An optimal efficacy was observed for up to six months post spray and 100% mortality of mosquitoes by 144 to 168 hours post-exposure to treated surfaces (Kweka et al., 2018).

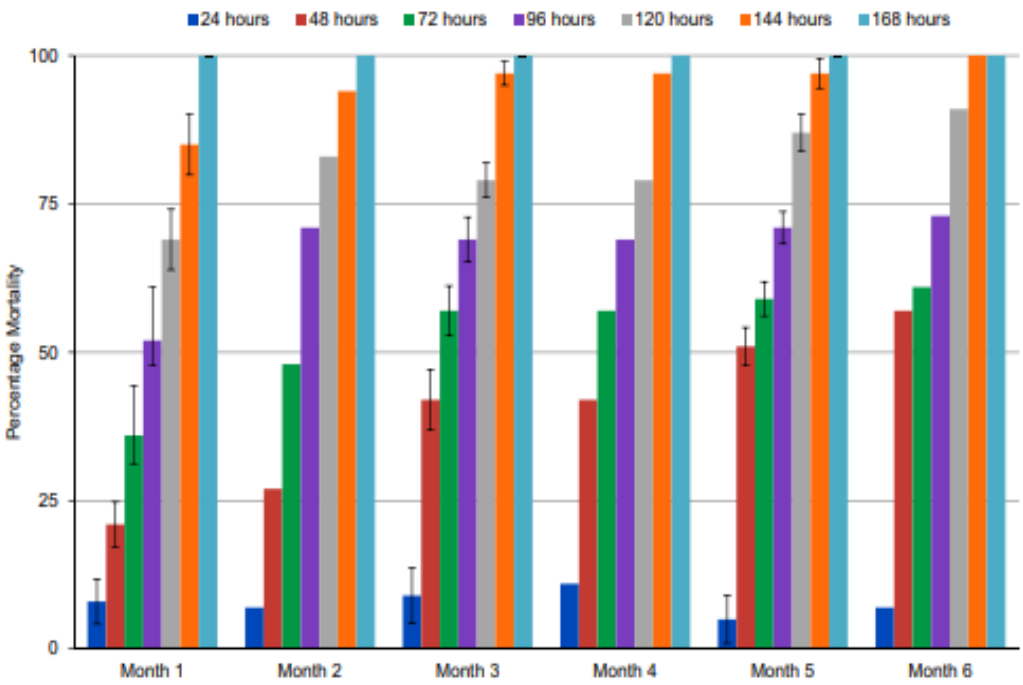

Figure 1.13: Residual efficacy observed after 30 minutes of exposure to SumiShield 50WG treated surfaces in houses in Moshi village in Tanzania. It was adapted from (Kweka et al., 2018).

Besides new insecticide formulation, a new combination called Olyset duo made of permethrin + an insect growth regulator that disrupts the maturation of oocytes in mosquitoes was also tested (Ngufor et al., 2016b). The offspring of blood-fed *Anopheles gambiae* exposed to Olyset duo after the experimental hut trial was reduced by 71% with unwashed net and by 43% with washed (Ngufor et al., 2016b). A cluster-randomized controlled trial in Burkina Faso also demonstrates the reduction of malaria incidence after the introduction of Olyset Duo. The prevalence of malaria went from 2·0 episodes per child-year when using standard bed nets to 1·5 episodes per child-year in the group using Olyset Duo (incidence rate ratio 0·88 [95% CI 0·77-0·99; p=0·04]) (Tiono et al., 2018).

New technology like Microencapsulation used with an existing insecticide like chlorpyrifos methyl can increase their residual activity (N'Guessan et al., 2010b). The creation of new technology to deliver insecticide is also an option to improved vector control. Insecticide-treated wall linings (ITWL) and Long-lasting pyrethroid treated plastic sheeting, also known as

a durable lining (DL) were developed to improve the delivery of insecticide and improve vector control (Messenger et al., 2012; Sharma et al., 2009).

1.11 Insecticide Resistance management

The idea behind insecticide resistance management is to preserve and extend the continued effectiveness of actual and future insecticides. As a response to the WHO assembly and the board Roll Back Malaria, the global plan for insecticide resistance management (GPIRM) was designed in 2012. This plan now acknowledges that if strong measures are not taken to monitor insecticide resistance to preserve the continued effectiveness of current and future insecticide or to prevent the spread of resistance, consequences could be calamitous for vector control programs (WHO, 2012b).

To tackle the problem, several methods such a: i) Rotation, ii) Mosaic, iii) Combination, and iv) Mixtures have been suggested. All these techniques are based on two principles: 1) a fitness cost principle based on the recovery of the mosquitoes' susceptibility when the selection pressure is removed (rotation and mosaics). 2) Overcoming and challenging the resistance by putting in place strategy to kill resistant mosquitoes (mixtures and combination) (Denholm and Rowland, 1992; WHO, 2012b).

1.11.1 Mixtures

Mixtures are when two or more compounds of different insecticide classes are mixed to make a single product or formulation so that the mosquito is guaranteed to come into contact with the two classes at the same time (Ngufor et al., 2016b; WHO, 2012b).

1.11.2 Combination

In a combination of interventions, two control interventions are used in a house (e.g., pyrethroids on nets and an insecticide of a different class on the walls (Kleinschmidt et al., 2009; Okumu et al., 2012; WHO, 2012b).

1.11.3 Rotation

In this method, two or preferably more, insecticides with different modes of action are rotated from one year to the next (WHO, 2012b) (Figure 1.16). There is no recent case of the use of this method in vector control, and it was introduced once a long time ago in West Africa to control black flies (KURTAK et al., 1987).

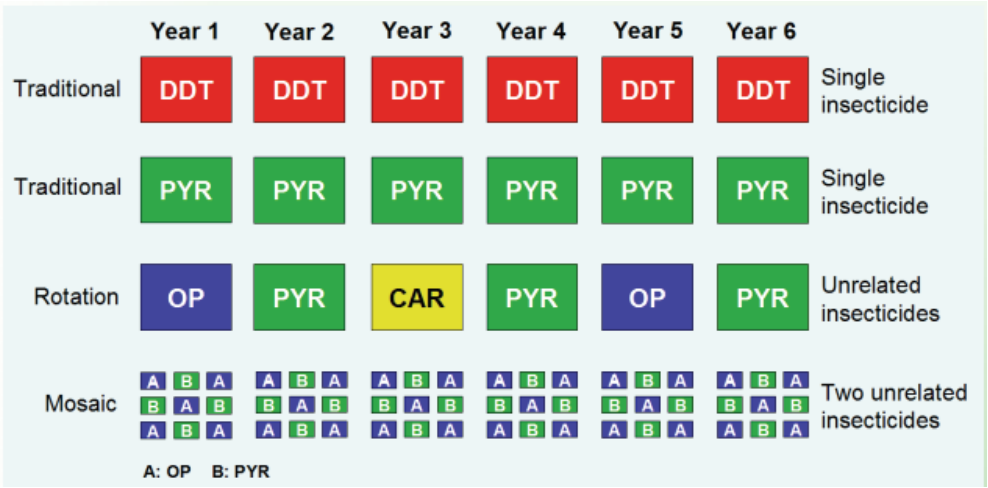

Figure 1.14: Study design describing some model of rotation and mosaic. Adapted from (IRAC, 2014b).

1.11.4 Mosaic

Mosaic spraying: one compound is used in one geographic area and a different compound in neighboring regions, the two being in different insecticide classes (GPIRM, 2012). The aim of this is to create a dilute effect that will slow down the selection of the resistance (figure 1.16). The mosaic method was tested in Mexico to control *Anopheles albimanus.* Despite the observation of enzymes activity patterns variation, the probability of high-level resistance development when using mosaic regime was significantly lower when compared to the rate obtained when using pyrethroid alone (Fgure 1.16). (IRAC, 2014a). Mosaic is also used in LLINs fabrication to produce the *two-in-one bed nets*. These nets are usually treated with two compounds, one on the site and another one on the roof (Guillet et al., 2001; Hougard et al., 2003).

1.12 Study objectives

1.12.1 General objectives

The WHO Global Plan for Insecticide Resistance Management (GPIRM) highlighted the need to investigate how insecticide resistance is changing the efficiency of bed nets to implement suitable insecticide resistance management strategies (IRM) in order to preserve the efficacy of current and future insecticides. However, the extent of the Impact of this resistance, notably, metabolic resistance on the effectiveness of insecticide-based interventions such as Long-lasting Insecticidal Nets (LLINs) remains unclear. Taking advantages of the markers recently made available, this study aims to investigate the impact of insecticide resistance on the effectiveness of vector control tools against the major African malaria vector *An.funestus*
The rise of insecticide resistance may jeopardize the efficiency of vector control tools since resistance can reduce their efficacy. The hypothesis is that resistant mosquitoes (RR or RS) will be more able to survive exposure to insecticide and will have a greater ability to take the blood meal. If this happens, we will be able to conclude that insecticide resitance is reducing the effectiveness of bed nets.
We will try to answer the following question: Can metabolic resistance to pyrethroids reduce the effectiveness of bed nets by increasing the ability of the mosquitoes to survive and to bloodfed when exposed to bed nets?

1.12.2 Specific objectives

The specific aims are:

1. To determine the bionomics and insecticides resistance profiling of malaria vectors at a selected site for experimental hut trials in central Cameroon (Chapter 3).

2. To evaluate using experimental hut, the performance of PBO-based and pyrethroid-only nets against the malaria vector *An. funestus* and to investigate how *GSTe2* metabolic mediated resistance is impacting that performance (Chapter 4).

3. To evaluate the performance of PBO-based and pyrethroid-only nets against the hybrid lab strain Fumoz-Fang and to investigate how duplicated *CYP6P9a* and *CYP6P9b* cytochrome p450 genes are impacting that performance (Chapter 5).

Chapter 2: Basic methodology

Several experiments were carried out during this thesis. To assess the performance of bed nets against free-flying mosquitoes and released mosquitoes, experimental huts were used following the WHO guidelines (WHO 2013). WHO susceptibility tests were carried out to assess the resistance profile of the local population of mosquitoes. Cone test was carried out to control the quality of bed nets using a susceptible lab strain. Cone tests were also used to assess the performance of bed nets against resistant mosquitoes in lab conditions. To characterize the local population in the site where the experimental station is located, TaqMan was used for genotyping of the resistance molecular markers, including *Kdr* and *Ace* 1, and evaluate the *Plasmodium* infection rate. Standard PCR was used for molecular identification of species and to establish the species diversity. PCR-RFLP was carried out to genotype the P450 markers, and AS-PCR was used for the *GSTe2* marker.

2.1 Experimental hut trial

2.1.1 Study site and Experimental hut design

The experimental hut station is located in Mibellon (6°4'60''N, 11°30'0''E), a village in Cameroon located in the Adamawa Region; Mayo Banyo Division and Bankim Sub-division. At the experimental station, 12 huts built following the World Health Organisation (WHO) standard, are available for a wide range of experimental hut trials. *An. funestus* s.s. is the main malaria vector in the area (Menze et al., 2018b). *Mansonia* sp*,* and *Culex sp* are also present in the area*.* Mosquitoes in the area are highly resistant to pyrethroids and DDT, as described in chapter 3. The trial was carried out for 12 weeks during the rainy season from 10th July to 16th October 2016.

The huts were built following the prototype recommended by WHO for the West African region (WHO, 2013d). The hut was constructed on a concrete base of cement surrounded by a drain channel to trap ants. The walls were made from concrete bricks and plastered inside and outside with a plaster made from a mixture of cement and sand. The roof was made from corrugated iron, and the ceiling was made from plywood. The four windows located on three sides of the hut were designed to create an angle with a 2 cm gap, which will facilitate the entry of mosquitoes flying upward and prevent the mosquitoes from escaping once they have

entered the hut. A veranda trap was built at the back of the hut according to WHO protocol (WHO, 2013b) (Figure 2.1). A curtain was used as a separation between the veranda and the rest of the hut. Before bedtime, each sleeper was required to raise the curtain to allow mosquitoes to take refuge in the veranda. In the morning, it was recommended that the sleeper lowers the curtain before starting the collection, to allow a separate collection of mosquitoes in the veranda and the hut. In this study the experimental hut design was used in several occasions. To assess the performance of Olyset, Olyset Plus, PermaNet 2.0 and PermaNet 3.0 against *Anopheles funestus* from Mibellon and to see how GSTe2 impact that performance,the mosquitoes were collected during 12 weeks (72 nights) between July and October 2016. The latin square design (Chapter 4; Section 4.2.5) was applied first for six weeks, but the number of mosquitoes collected was not enough, so we added another 6 weeks. To assess the performance of Olyset, Olyset Plus, PermaNet 2.0 and PermaNet 3.0 against the crossing Fumoz-Fang to see how CYP6P9a and b genes affect that performance, 50 to 100 mosquitoes from the lab strain were realseased during 6 nights in June 2018.

Figure 2.1: Mibellon experimental station in the republic of Cameroon **A)** Design of the experimental huts used at the experimental station in Mibellon, Cameroon. **B)** Volunteer sleeping in the huts during the study.

2.1.2 Mosquito collection

Mosquitoes were collected every morning, using hemolysis tubes from: (i) inside the nets, (ii) in the room: floor, walls and roof, and (iii) in the veranda exit trap. Mosquitoes collected from each compartment were kept separately in a bag to avoid any mixing between samples from different compartments. (Figure 2.2). Samples were transferred to the laboratory for identification. After identification, samples were then classified as dead, alive, blood-fed, or unfed (Appendix 1). The 'alive' samples were kept in the paper cup and provided with sugar solution for 24 hours and mortalities monitored. Samples were then kept adequately in labelled Eppendorf tubes with Silicagel for dead and in RNA later for Alive (Figure 2.3).

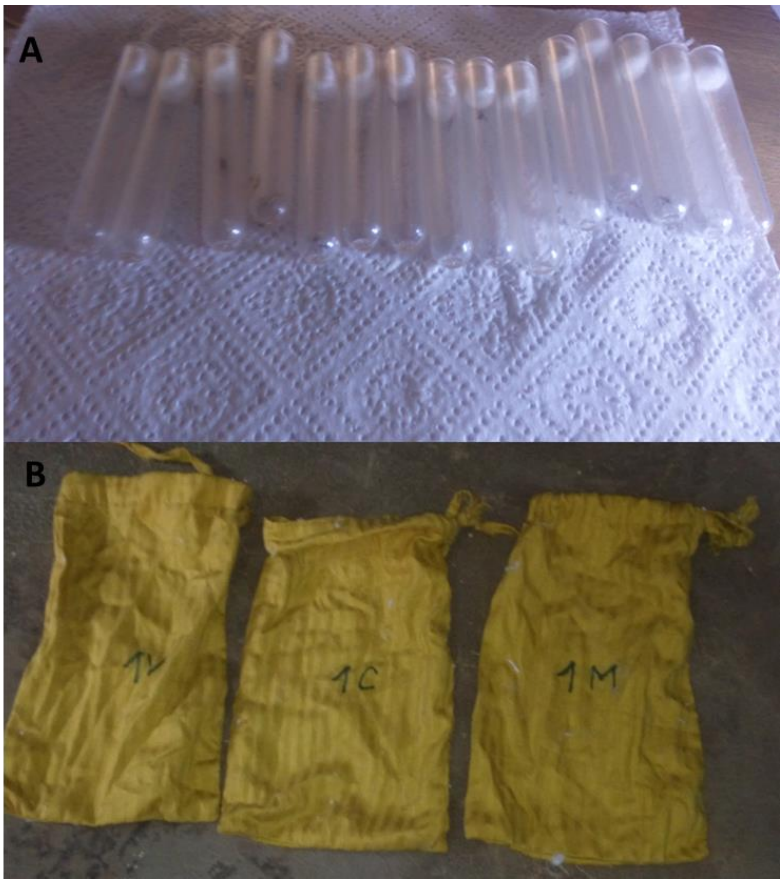

Figure 2.2: Sampling of mosquitoes from the huts: **A)** Hemolysis tubes used to collect mosquitoes in the huts at the Mibellon experimental station in Cameroon. **B)** Bags used to kept samples collected per compartment (V=veranda, C=case, M=mosquito nets).

Figure 2.3: Sample storage after collection: Mosquitoes are kept in labeled Eppendorf tube and transferred at -20°C for further analysis.

2.1.3 Bed nets performance assessment

The performance of the bed nets was expressed relative to control (untreated nets) (Appendix 1) in term of:

i-Deterrence/entry rate: the reduction in hut entry relative to control. Deterrence (%) = 100 x (Du – Dt) / Du, where Du is the total number of mosquitoes found in the untreated hut (control), and Dt is the total number of mosquitoes in the treated hut.

ii-Entry rate (%) = 100 x (Ht/Hn) where Ht is the total number of mosquitoes found in the hut, and Hn is the total number of mosquitoes collected in all the six huts.

iii-Exophily: the proportion of mosquitoes found exited in the veranda trap. Exophily (%) = 100 x (Ev/Et) where Ev is the total number of mosquitoes found in veranda, and Et is the total number of both inside the hut and veranda.

iv-Blood feeding rate (BFR). This rate was calculated as follows: Blood feeding rate = (N mosquitoes fed) x 100 / total N mosquitoes. Where "N mosquitoes fed" was the number of mosquitoes fed, and "total N mosquitoes" was the total number of mosquitoes collected.

v-Blood-feeding inhibition (BFI): the reduction in blood-feeding in comparison with the control hut. Blood feeding inhibition is an indicator of personal protection (PP). More precisely, the personal protection effect of each bed net is the reduction of blood-feeding percentage induced by the net when compared to control. The protective effect of each bed net can be calculated as follows: *Personal protection (%) = 100 x (Bu – Bt) / Bu, where Bu* is the total number of blood-fed mosquitoes in the huts with untreated nets and *Bt* is the total number of blood-fed mosquitoes in the huts with treated nets (WHO, 2013b).

vi-Immediate and delayed mortality: the proportion of mosquitoes entering the hut that is found dead in the morning (immediate death) or after being caught alive and held for 24 h with access to sugar solution (delay mortality) (WHO, 2013b). In this study, we focused on the overall mortality calculated as follows: Mortality (%) = 100 x (Mt/MT) where Mt is the total number of mosquitoes found dead in the hut and MT is the total number of mosquitoes collected in the hut.

2.2 Susceptibility tests

To carry out the susceptibility test, blood-fed *Anopheles* mosquitoes were collected every morning between 06:00 and 11:00 in the households from Mibellon. Mosquitoes were obtained using this method since *An. funestus,* which is the principal vector in the area, has breeding site difficult to access. The household owners gave verbal consent before starting

mosquito collection. Mosquitoes were collected in January 2017 using the Prokopack electrical aspirator (John W Hook, Gainesville, FL, USA) and kept in netted paper cups that were stored in a cool box. Samples were later transported to the insectary of the LSTM Research Unit at OCEAC in Yaoundé (Cameroon) with standard insectary condition (temperature was 25%±2, and the humidity 80%±10). As previously described, the field-caught females were morphologically identified according to morphological keys (Gillies and Coetzee, 1987). Mosquitoes were then kept 4 to 5 days until they became fully gravid and induced to lay eggs using the forced eggs-laying method (Morgan et al., 2010). Eggs obtained from individual mosquitoes were pooled in cups for hatching and then transferred in trays where Tetramin® baby fish food was used for feeding. (Cuamba et al., 2010; Morgan et al., 2010). The F1 obtained were tested in WHO tube to assess their susceptibility against deltamethrin 0.05% and permethrin 0.75%. (Figure 2.4). For each test, four replicates of 25 mosquitoes were used. Knockdown was recorded after 1 hour and mortality after 24h following WHO guidelines (WHO, 2016). Mortality between 98–100% indicates the susceptibility of the mosquitoes. Mortality of less than 98% suggests suspected resistance whereas mortality of less than 90%, means the resistance of mosquitoes The test was validated if the mosquito mortality in control was less than 5%. But with a mortality more than 20%, the test was invalid and had to be repeated. On the other hand, if the mortality was between 5 and 20%, the overall mortality of the exposed mosquitoes is corrected by applying the formula of Abbott (Abbott, 1925).

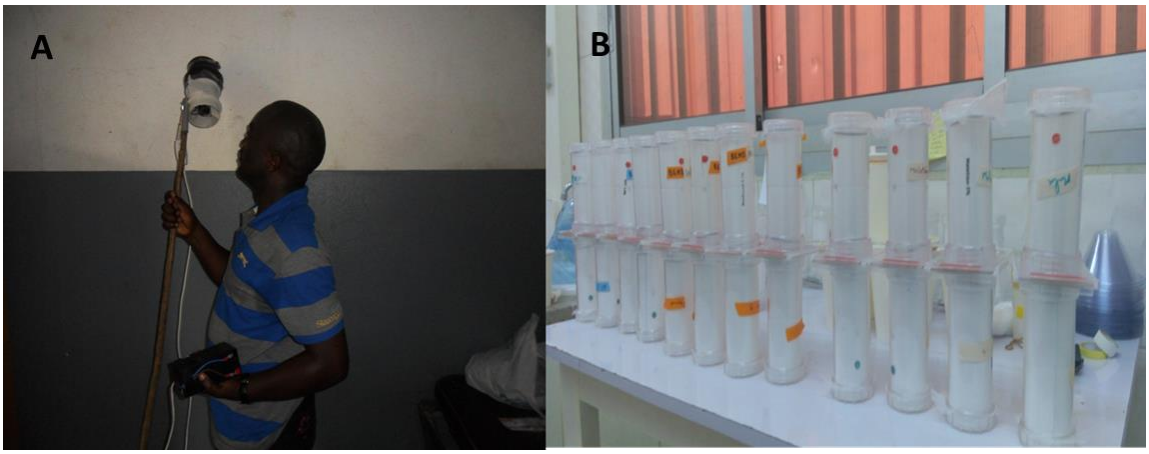

Figure 2.4: Mosquitoes collections and bioassays. **A)** Blood fed indoor resting mosquitoes were collected using an electrical aspirator in Cameroon during this study **B)** Susceptibility test following WHO protocol using F1 samples performed in the insectary at the LSTM Research Unit, CRID in Cameroon.

2.3 Cone assays

Cone assays were carried out on several bed nets to assess their performance in lab conditions. These tests were carried out using fragments of LLINs (30 cm × 30 cm). Ten unfed 2-5 day-old females mosquitoes were introduced into each cone placed on the LLIN for 3 min. After exposure, the mosquitoes were removed from the cones using a mouth aspirator and then transferred into paper cups and provided a 10% sugar solution. Mosquito knock-down was recorded after 60 min. A negative control (untreated net) was included in each series of cone tests. Mortality post-exposure was recorded after 24 h of observation. The temperature was around 25° C ± 2° C and humidity 80% ± 10 % during the assays (Figure 2.5).

Figure 2.5: Cone bioassay of long-lasting insecticidal mosquito nets performed in the insectary of the LSTM research Unit in Cameroon during this study.

2.4 Molecular characterization in terms of species diversity and molecular base of the resistance

Beside field and insectary work, a range of molecular analysis was conducted in the laboratory including PCR and TaqMan assay (Figure 2.6).

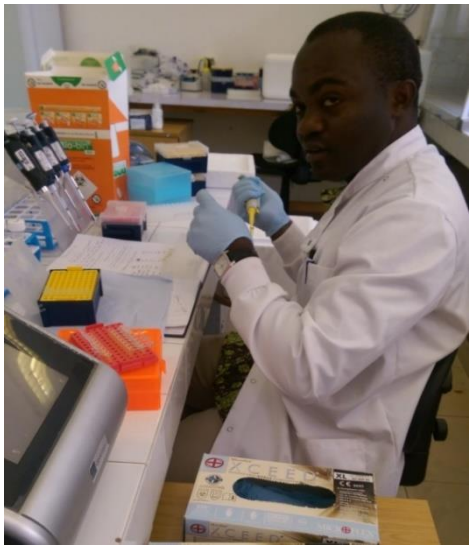

Figure 2.6: Performing molecular assays at the LSTM Research Unit in Cameroon during this study.

2.4.1 PCR species identification for *An.s funestus* group.

For the species identification by cocktail PCR, the mixture was made of one (1) µl DNA (see DNA extraction protocol in appendix 2), in a total volume of 25 µl. 0.85 µl (10µM) of the universal primer forward and reverse were used. Six other primers (Table 2.1) for the most common member of *An. funestus* group was also used. The DNA was amplified using a thermocycler (Gene Amp PCR System 2700, Singapore). The PCR machine was set according to the following condition: 94°C-2min (denaturation), 35 cycles of 94°C -30 seconds (denaturation), 45°C 30 seconds (annealing), 72°C -40 seconds (extension), and a final extension at 72°C -5 (Appendix 3). A mixture of 2 µl of loading buffer and three µl of PCR product was run on a 1.5% gel containing 5ul of Ethidium bromide. The gel was run at 120 volts for 45 min in 1xTAE buffer(Figure 2.7) and the bands visualized using the Trans-illuminator G box (SynGene) connected to a PC with the software gene snap (SynGene, UK). (Koekemoer et al., 2002).

Table 2.1: *S*pecies-specific primer sequences and temperatures included in the PCR species identification of Funestus group. Adapted from (Koekemoer et al., 2002)

Primer name	Sequence (5′ to 3′)	Tm (°C)
UV	TGT GAA CTG CAG GAC ACA T	55.34
FUN	GCA TCG ATG GGT TAA TCA TG	52.4
VAN	TGT CGA CTT GGT AGC CGA AC	58
RIV	CAA GCC GTT CGA CCC TGA TT	58.8
PAR	TGC GGT CCC AAG CTA GGT TC	60.5
LEES	TAC ACG GGC GCC ATG TAG TT	60.2

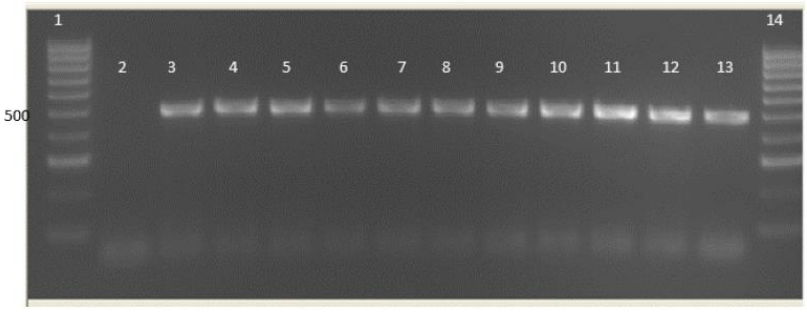

Figure 2.7: PCR species identification performed using some samples from Mfou (locality in the centre of Cameroon). Lane 1 and 14 DNA marker, lane 3-positive control, lane 2-negative control, and lane 4 to 13 *An. funestus* s.s.

2.4.2 TaqMan for Plasmodium infection rate evaluation

Collected *An. funestus* females were dissected in two parts: the head and thorax together and the abdomen separately. Genomic DNA was extracted separately from the head/thorax and abdomen and the oocyst and the sporozoite rate evaluated using the TaqMan assay (Bass et al., 2008) on MX 3005 machine (Agilent, Santa Clara, CA, USA). For *An. gambiae*, due to the low number of samples, the whole mosquito was used, and only the overall *Plasmodium* infection rate estimated. One µl of DNA sample was used as a template in a 3-step PCR program with denaturation at 95̊C for 10 min followed by 40 cycles of 15 sec at 95̊C and 1 min at 60̊C. The primers (Falcip+: TCT-GAA-TAC-GAA-TGT-C, OVM+: CTG-AAT-ACA-AAT-GCC, Plas-F: GCT-TAG-TTA-CGA-TTA-ATA-GGA-GTA-GCT-TG, Plas r: GAA-AAT-CTA-AGA-ATT-TCA-CCT-CTG-ACA) were used together with two probes labeled with fluorophores: FAM to detect *Plasmodium falciparum*, and HEX to identify *Plasmodium ovale*, *Plasmodium vivax* and *Plasmodium malariae. Plasmodium falciparum* samples and a mix of *Plasmodium ovale*, *Plasmodium vivax* and *Plasmodium malariae* were used as positive controls (Figure 2.8).

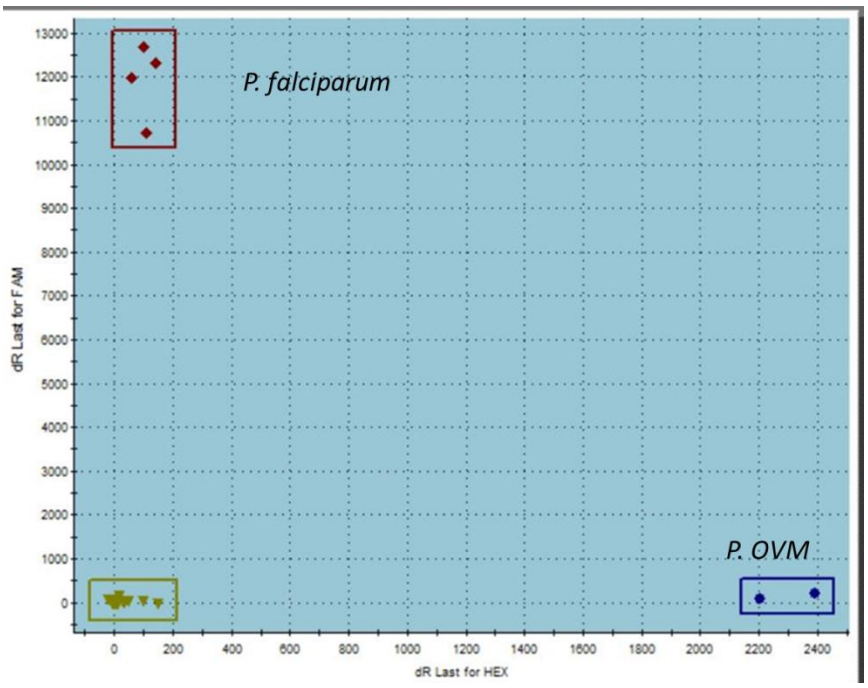

Figure 2.8: Taqman results (scatter plot) showing the distribution of *P. falciparum* and *P. OVM*. Two probes labelled with fluorophores: FAM to detect *Plasmodium falciparum*, and HEX to detect *Plasmodium ovale*, *Plasmodium vivax* and *Plasmodium malariae.*

2.4.3 TaqMan for L1014F, L1014S *kdr* and A296S-*RDL* genotyping

TaqMan genotyping assays were performed in 10 µl volume containing 1× Sensimix (Bioline), 80× primer/probe mix, and one µl template DNA. Probes were labeled with two specific fluorophores FAM and HEX, FAM, to detect the resistant allele and HEX to detect the susceptible allele. The assay was performed on an Agilent MX3005 real-time PCR machine with cycling conditions of 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min as described previously (Bass et al., 2007; Djouaka et al., 2016b) (Figure 2.8 and 2.9).

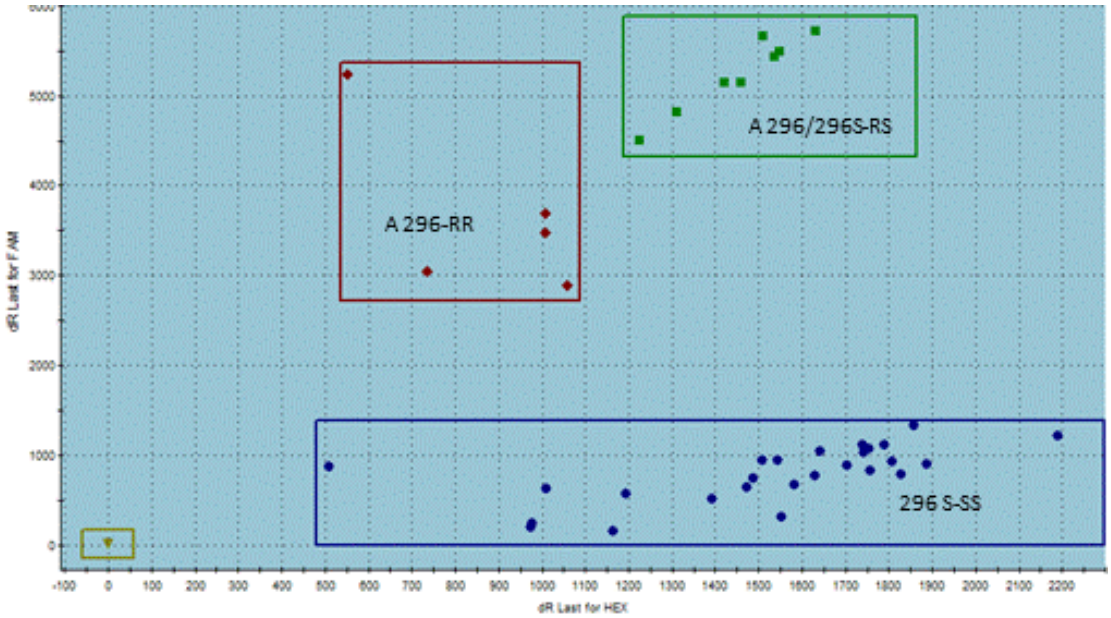

Figure 2.9: Genotyping Taqman results (scatter plot) showing the three genotypes detected. Image describing the 3 genotypes of the A296S *RDL* mutation in Mibellom.

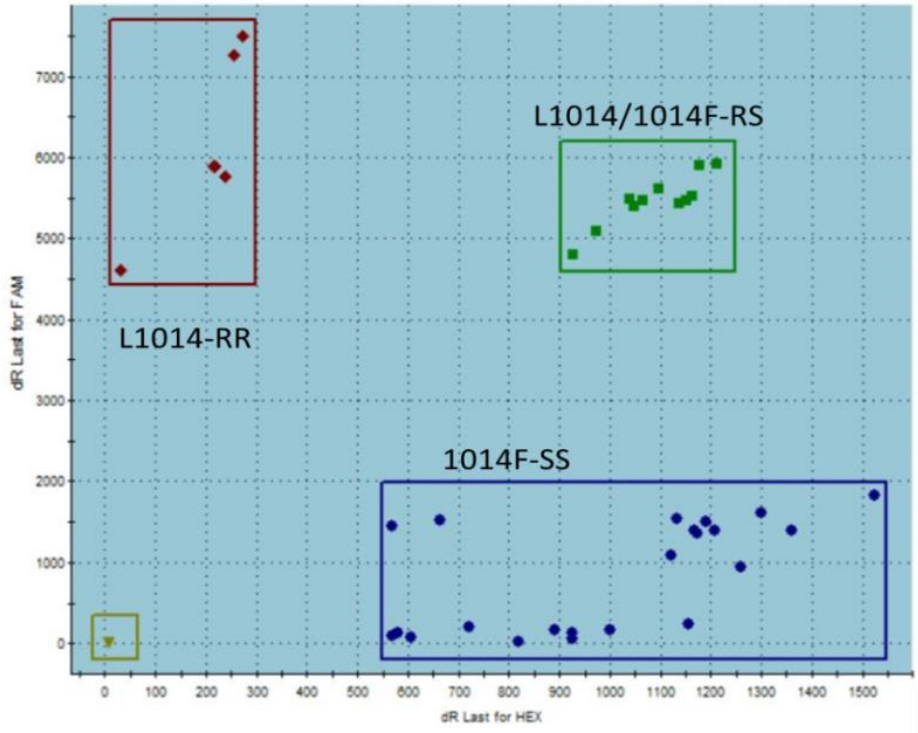

Figure 2.10: Genotyping Taqman results (scatter plot) showing the three genotypes detected. Image describing the 3 genotypes of the *Kdr* West L1014F mutation.

2.4.3 Genotyping of the L119F-*GSTe2* marker using the allele-specific PCR

Samples classified as dead, alive, blood-fed, unfed, room, and veranda were used for DNA extraction using the Livak protocol (Appendix 1) (Livak, 1984). The L119F-*GSTe2* mutation was genotyped to assess how the glutathione S-transferase gene, *GSTe2*, impacts the performance of the bed nets. An allele-specific PCR (Tchouakui et al., 2019d; Tchouakui et al., 2018) was used to detect the three genotypes of the L119F-*GSTe2* mutation (RR, RS, and SS). The PCR was carried out using 10 mM of each primer and 1 µL of gDNA as the template in 15 µL reaction containing 10X Kapa Taq buffer A, 0.2 mM dNTPs, 1.5 mM MgCl2, 1 U Kapa Taq (Kapa Biosystems, Wilmington, MA, USA). Amplification was carried out using thermocycler parameters: 95° C for 5 min; 30 cycles of 94° C for 30 sec, 58° C for 30 sec, 72° C for 45 sec, final extension at 72° C for 10min. The following primers were used: L119F-Fwd: ATG ACC AAG CTA GTT CTG TAC ACG CT; L119F-Rev: TTC CTC CTT TTT ACG ATT TCG AAC T; L119F-Res1: CGG GAA TGT CCG ATT TTC CGT AGA AtAA; L119-F-Sus1: CAT TTC TTA TTC TCA TTT ACA GGA GCG TAaTC. PCR products were separated on 2% agarose gel by electrophoresis. The bands

corresponding to different genotypes were interpreted as described by (Tchouakui et al., 2019a; Tchouakui et al., 2019d) (Figure 2.11).

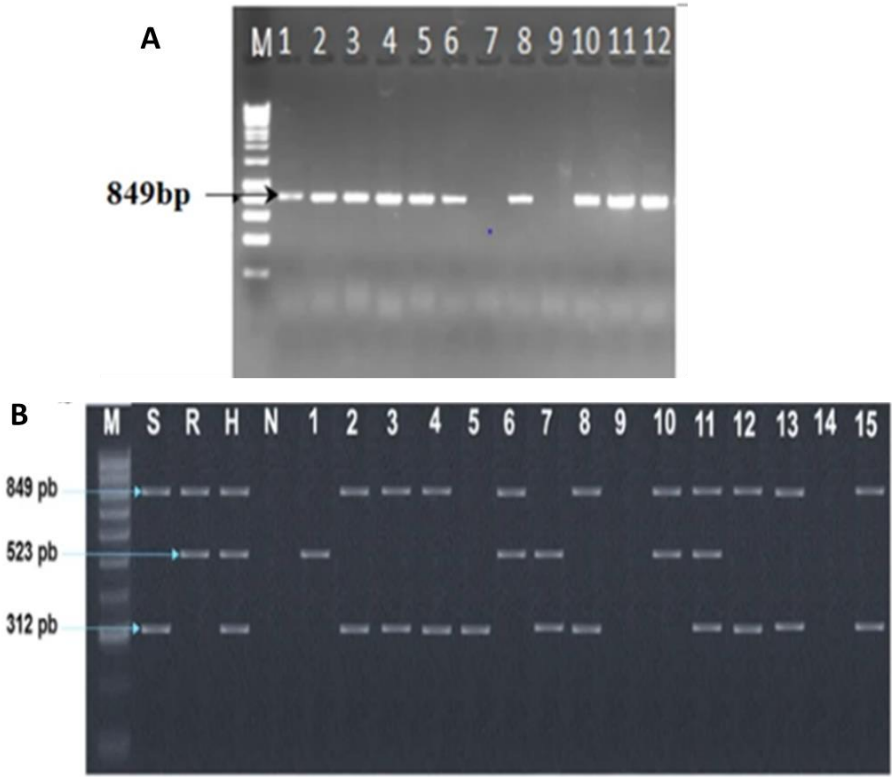

Figure 2.11: Agarose gel describing the genotyping of L119F-*GSTe2* mutation. **A)** Gel showing the amplification of *GSTe2* gene in *An*. *funestus* s.s. **B)** AS-PCR is showing the L119F gste2 mutation in *An*. *funestus* s.s. 849 bp (Resistant), 523 bp (susceptible), 523 bp, and 312 bp (heterozygote).

2.4.4 Genotyping of the *CYP6P9a*-R maker using PCR-RFLP

The PCR was carried out using 10 mM of each primer and 1 µL of gDNA as the template in 15 µL reaction containing 10X Kapa Taq buffer A, 25 mM dNTPs, 25 mM MgCl2, 1 U Kapa Taq (Kapa Biosystems, Boston, MA, USA). Amplification was carried out using thermocycler parameters: 95° C for 5 min; 35 cycles of 94° C for 30 sec, 58° C for 30 sec, 72° C for 45 sec, and a final extension at 72° C for 10min. The following primers were used: **RFLP6P9aF forward primer** 5'-TCCCGAAATACAGCCTTTCAG-3; **RFLP6P9aR reverse primer** 5'-ATTGGTGCCATCGCTAGAAG-3'. Three µL of the PCR product were migrated on 1.5% agarose gel. The expected PCR product is **450bp.** The digestion with Taq 1α followed all this. (Taq 1α

0.2 µL, PCR product 5 µL, 10x NEBuffer 1 µL, distilled water 3.8 µL, total reaction volume). This mix was incubated at 65°C for 2 hours. Three µL of the digestion product was migrated on 2% gel. Amplicons from resistant mosquitoes were cut into two bands with sizes of 350bp and 100 bp, and the susceptible remains undigested with the band at 450bp (Figure 2.12).

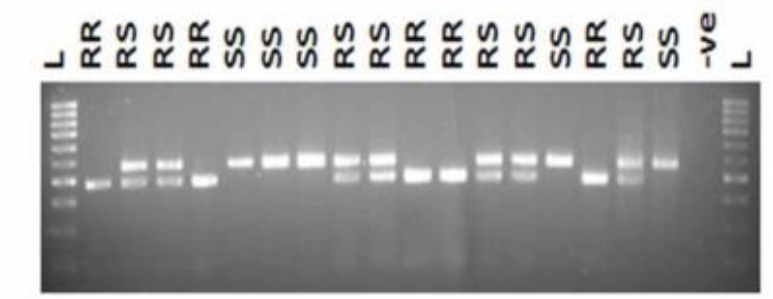

Figure 2.12: Agarose gel showing the results of the PCR-RFLP for *CYP6P9a* genotyping: Amplicons from resistant mosquitoes are cut into two bands with sizes of 350bp and 100 bp and the susceptible remains undigested with the band at 450bp.

2.4.5 Genotyping of the *CYP6P9b*-R maker using PCR-RFLP

The PCR was carried out using 10 mM of each primer and 1 µL of gDNA as template in 15 µL reaction containing 10X Kapa Taq buffer A, 25 mM dNTPs, 25 mM MgCl2, 1 U Kapa Taq (Kapa Biosystems, Boston, MA, USA). Amplification was carried out using thermocycler parameters: 95° C for 5 min; 35 cycles of 94° C for 30 sec, 58° C for 30 sec, 72° C for 45 sec, and a final extension at 72° C for 10min. The following primers were used: **6p9brflp_0.5F** 5'-CCCCCACAGGTGGTAACTATCTGAA-3'; **6p9brflp_0.5R** 5'-TTATCCGTAACTCAATAGCGATG-3'**.** Three µL of the PCR product were migrated on 1.5% agarose gel. The expected PCR product is **550bp.** The digestion with the Tsp451 restriction enzyme followed all this. (***Tsp*451**0.2 µL, PCR product 5 µL, 10x NEBuffer 1 µL, distilled water 3.8 µL, total reaction volume). This mix was incubated at 65°C for 2 hours. Three µL of the digestion product was migrated on 2% gel. Amplicons from susceptible mosquitoes were cut into two bands with sizes 400bp and 150 bp, and the resistant mosquito remains undigested with the band at 550bp (Figure 2.13).

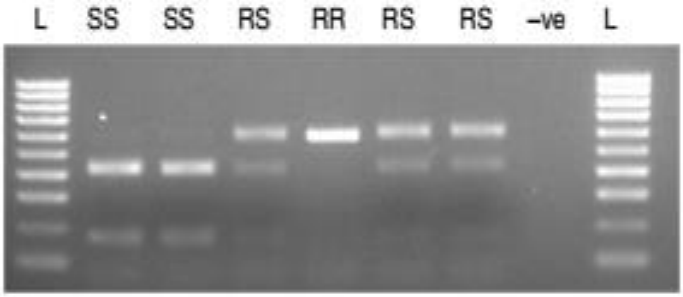

Figure 2.13: Agarose gel showing the results of the PCR-RFLP for CYP6P9b genotyping Susceptibles cut in two bands with sizes 400bp and 150 bp and the resistant remains undigested with the band at 550bp.

2.4.6 Data analysis

Experimental hut trial: To calculate the proportion of each entomological outcomes and the level of significance between the treatments and between the control for each entomological outcomes, the XLSTAT software (Addinsoft, Berkeley, CA, USA) was used. To conteract the problem of multiple comparison, the bonferroni correction was applied using an alpha of 0.01 as the cut off for significance. The numbers of mosquitoes collected in the huts with different treatments were analyzed by negative binomial regression. The effects of the treatments on each of the main proportional entomological outcomes (exophily, blood-feeding, and mortality) were assessed using binomial generalized linear mixed models (GLMMs) with a logit link function, fitted using the 'lme4' package for R 3.6. (R Development Core Team, 2019) (Ngufor et al., 2016a; Toe et al., 2018a). A separate model was fitted for each outcome and each mosquito species. In addition to the fixed effect of each treatment, each model included random effects to account for the following sources of variation: between the huts used in the studies; between the sleepers who slept in the huts; between the ten weeks of the trial (Appendix 4).

Test of association between the genes (*GSTe2* and *CYP6P9a/b*) and the entomological outcomes: To investigate the association between the genes and the ability of the mosquitoes to survive, blood feed or escape, Vassar stats were used to estimate the Odds ratio based on a fisher exact probability test with a 2x2 contingency table.

Hut effect analysis: The one-way analysis of variance (ANOVA) using Prism 7.0 (GraphPad, San Diego, CA, USA) was used to determine whether there were any statistically significant differences between the means of the mosquitoes collected from the six huts (Appendix 5). For all the analyses in this study, an alpha of 0.05 was used as the cut off for significance.

Construction of graph: Most of the graphs in this documents was constructed using the software Prism 7.0 (GraphPad, San Diego, CA, USA).

Chapter 3: Bionomics and insecticides resistance profiling of malaria vectors at a selected site for experimental hut trials in central Cameroon

3.1 Context of the study

Despite the recent progress made in reducing the malaria burden in sub-Sahara Africa through the deployment of insecticide-based interventions such as long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS), malaria remains an important health issue (Bhatt et al., 2015). Malaria control in Cameroon has recently witnessed a significant scale-up of vector control interventions through the distribution of LLINs, pushing up the bed net coverage to 70.9% of the population (MICs, 2014; PNLP, 2016, 2018). However, the effectiveness of these insecticide-based control tools is threatened by the emergence of insecticide resistance in major malaria vectors across African countries, including Cameroon (Menze et al., 2016; WHO, 2012a, b; WHO/GPIRM, 2012). It is imperative to design and implement suitable resistance management strategies to preserve the efficacy of existing tools. The characterisation of malaria vectors will maximize the impact of control intervention. The elucidation of resistance profiles and mechanisms underlying this resistance will improve resistance management for a better choice of control tools. The lack of such information has prevented evidence-based implementation of control intervention limiting the outcome of control.

Furthermore, the characterization of the impact of resistance on the effectiveness of control tools is also a key knowledge to guide control programs. Such an assessment of resistance impact needs approaches such as experimental hut trials. Use of experimental huts since it provides an opportunity to measure entomological outcomes, such as relative mortality and feeding success of resistant and susceptible vectors in controlled conditions is one of the best approaches to assess the impact of resistance on the efficacy of control intervention (Churcher et al., 2016b; N'Guessan et al., 2007b; Ngufor et al., 2017a).

This chapter presents a full characterization of the main malaria vectors in the area selected for experimental hut trial, including their species diversity, susceptibility profiles. It investigates the molecular basis of the resistance to insecticides and their *Plasmodium* infection rate. Characterization of the vectors present in the area will help to improve the design of vector control interventions, to better understand the resistance threats and then help assess the extent to which resistance even impacts the outcome of control intervention.

3.2. Methods

3.2.1 Study sites

Adult *Anopheles* mosquitoes were collected from Mibellon (6°46'N, 11°70'E), a village in Cameroon located in the Adamawa region, Mayo Banyo Division and Bankim Sub-division. The Adamawa region is in the mountainous area forming a transition between Cameroon's forested south and savanna north. Mibellon is located in close proximity to permanent water bodies, including a lake and swamps, which provide suitable breeding sites for mosquitoes. Human activities are mainly fishing, hunting, and subsistence farming, including maize, watermelon, and coffee plantations. A survey in the area revealed a high usage of insecticides in the coffee and watermelon farms. These insecticides are mainly pyrethroids, neonicotinoids, and carbamates (Menze et al., 2018b).

3.2.2 Mosquito collection and rearing

The blood-fed *Anopheles* mosquitoes were collected every morning between 06:00 and 11:00 in the houses. Mosquitoes were collected using this method since *An. funestus* which is the major vector in the area, has breeding sites difficult to access. Mosquitoes were collected and reared, as describes in Chapter 2.

3.2.3 Species identification

The field-caught females were morphologically identified according to morphological keys as previously described (Gillies and Coetzee, 1987). After genomic DNA extraction using the Livak method (Livak, 1984), PCR species identification was performed as described (Koekemoer et al., 2002) to identify *Anopheles funestus* group members as described in chapter 2, whereas the SINE PCR (Santolamazza et al., 2008) was used to differentiate members of the *An.gambiae* s.l complex as described by (Santolamazza et al., 2008).

3.2.4 *Plasmodium falciparum* infection rate

Sixty collected *An. funestus* females were dissected in two parts: the head and thorax together and the abdomen separately. Genomic DNA was extracted separately from the head/thorax and abdomen and the oocyst and the sporozoite rate evaluated using the TaqMan assay (Bass et al., 2008) in MX 3005 (Agilent, Santa Clara, CA, USA). For *An. gambiae*, due to the low number of mosquitoes, the whole mosquito was used, and only the overall *Plasmodium* infection rate estimated as described in method chapter 2.

3.2.5 Insecticide susceptibility assays

Following WHO protocol (WHO, 2016), F_1 *An. funestus sensu stricto* (*s.s.*) and *Anopheles gambiae s.s.* mosquitoes aged between 2 and 5 days were exposed to different classes of insecticides. These included the Type I (permethrin; 0.75%) and type II (deltamethrin; 0.05%) pyrethroids and the pseudo-pyrethroids, etofenprox (0.05%), the organochlorine DDT (4%), the organophosphate malathion (5%) and the carbamates: bendiocarb (0.1%) and propoxur (0.1%). For each test, mosquitoes exposed to untreated papers were used as control. The assay was carried out at temperatures of 25° C ± 2° C and 80% ± 10% relative humidity, as described in chapter 2.

3.2.5.1 PBO synergist assays

Piperonyl butoxide function as an inhibitor of P450s. To investigate the possible involvement of cytochrome P450s in the observed resistance, female *An. funestus s.s.* were pre-exposed to 4% PBO for 1 hr and immediately exposed to DDT (4%), permethrin (0.75%) and deltamethrin (0.05%) for 60 min. The mortality was assessed after 24 hrs and compared with mortality obtained for mosquitoes not pre-exposed to PBO.

3.2.5.2 Cone assays

To assess the efficacy of common insecticide-treated nets, cone assays were performed using five types of LLINs (Olyset Plus, Olyset Net, Yorkool, PermaNet 2.0, and PermaNet 3.0 (side).

These tests were carried out using pieces of LLINs (30 cm × 30 cm). These nets were selected based on the fact we wanted to test pyrethroid-only and also PBO-based nets. Before being involved in the study, the quality of bednets was assessed by exposing them to a susceptible lab strain. Five replicates of ten unfed mosquitoes (*An. funestus s.s.* and *An. gambiae s.s.)* females aged 2 to 5 days were introduced into each cone placed on the LLIN for 3 min (WHO, 2013a). After exposure, the mosquitoes were removed from the cones using a mouth aspirator and then transferred into paper cups and fed with 10% sugar solution. Numbers of mosquitoes knocked-down were recorded after 60 min. A negative control (untreated net) was included in each of the LLIN cone tests. Post-exposure mortality was recorded after 24 hr of observation. The assay was carried out at a temperature of 25^{o}C ± 2^{o}C and 80% ± 10% relative humidity.

3.2.6 Genotyping of L119F-*GSTe2*: metabolic resistance marker in *An. funestus* s.s.

The L119F-*GSTe2* mutation was genotyped to assess the role played by the glutathione S-transferase gene in the DDT and pyrethroid resistance observed in Mibellon as described previously (Riveron et al., 2014a). An allele-specific PCR (Tchouakui et al., 2019b) was used to detect the 3 genotypes of the L119F-*GSTe2* mutation. See details in chapter 2.

3.2.7 Genotyping of A296S-*RDL* in *An. funestus* s.s.

TaqMan genotyping assays were performed in 10 µl volume containing 1× Sensimix (Bioline), 80× primer/probe mix and 1 µl genomic DNA. The probes were labeled with two distinct fluorophores FAM and HEX, FAM to detect the resistant allele and HEX to detect the susceptible allele. The assay was performed on an Agilent MX3005 real-time PCR machine with cycling conditions of 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min as described previously (Djouaka et al., 2016a). See more details in chapter 2.

3.2.8 L1014F and L1014S *kdr* genotyping in *An. gambiae*

The L1014F-*kdr* mutation and the L1014S-*kdr* responsible for DDT and pyrethroid resistance in *An. gambiae s.l.* were genotyped in Mibellon mosquitoes. The reaction mixture of 10 µl final

volume containing 1× Sensimix (Bioline), 80× primer/probe mix and 1 µl template DNA was used for this assay. The probes were labeled with two distinct fluorophores: FAM to detect the resistant allele and HEX to detect the susceptible allele. The test was performed on an Agilent MX3005 real-time PCR machine with cycling conditions of 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min as previously described (Bass et al., 2007).

3.2.9 G119S *ace-1* genotyping in *An. gambiae*

The G119S *ace-1* is responsible for organophosphate and carbamate resistance in *An. gambiae* s.l. was genotyped in Mibellon mosquitoes. Ten µl volume containing 1× Sensimix (Bioline), 80× primer/probe mix, and one µl template DNA. Probes were labeled with two specific fluorophores FAM and HEX, FAM to detect the resistant allele, HEX to detect the susceptible allele. The assay was performed on an Agilent MX3005 real-time PCR machine with cycling conditions of 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min (Bass et al., 2010).

3.3 Results

3.3.1 Field collection

A total of 722 adult female *Anopheles* mosquitoes were collected indoor at Mibellon, out of which 584 (80.1%) were morphologically identified as *Anopheles funestus* group, and the remaining 138 (19.9%) identified to be *Anopheles gambiae* complex.

3.3.2 Species diversity among *An. funestus* group and *An. gambiae* complex

Out of the 120 *An. funestus* s.l. mosquitoes randomly selected and tested, *An. funestus* s.s. was found to be the only member of the group present at Mibellon. For *An.gambiae* s.l., out of 89 samples analyzed all of them were found to be *An. gambiae* s.s. (formerly S form).

3.3.3 Insecticide susceptibility bioassays with samples from Mibellon

Susceptibility profile of *An. funestus* s.s*.* A total of 2,700 F_1 mosquitoes were tested to assess the resistance profile to seven insecticides (Figure. 3.1). *An. funestus s.s.* (females and males) were resistant to type I and type II pyrethroids. For permethrin (Type I), mortality was 48.88 ± 5.76% for females and 90.72 ± 3.77% for males. For deltamethrin (Type II), mortality was 38.34 ± 5.79% for females and 53.96 ± 11.37% for males. However, mortality was higher for the pseudo-pyrethroid etofenprox, with a mortality rate of 82.9 ± 8.7% for females and 97.83 ± 2.17% for males (Figure 3.1A). For the organochlorine DDT, resistance was observed with a mortality rate of 55.28 ± 8.28% for females and 83.78 ± 3.13% for males (Figure 3.1A). For the carbamates, a possible resistance was observed against bendiocarb with 90.67 ± 4.3% mortality for females and 95.06 ± 1.97% for males (Figure 3.1A) whereas a near full susceptibility was observed for propoxur with 98.41 ± 1.59% mortality for females and 100% mortality for males (Figure 3.1A). Full susceptibility was observed for the organophosphate malathion with 100% mortality for both sexes (Figure 3.1A).

PBO synergist assays with *Anopheles funestus* s.s*.* Pre-exposure of *Anopheles funestus s.s.* to PBO led to the recovery of the susceptibility to both pyrethroids type I and II with increased mortality observed after PBO exposure from 48.88 ± 5.76% to 98.81 ± 3.77% for permethrin and from 38.34 ± 5.79% to 96.54 ± 1.16% mortality for deltamethrin (Figure 3.1B). Partial recovery was observed for DDT with increased susceptibility from 55.28 ± 8.28% to 84.16 ± 5.37% mortality after exposure to PBO (Figure 3.1B).

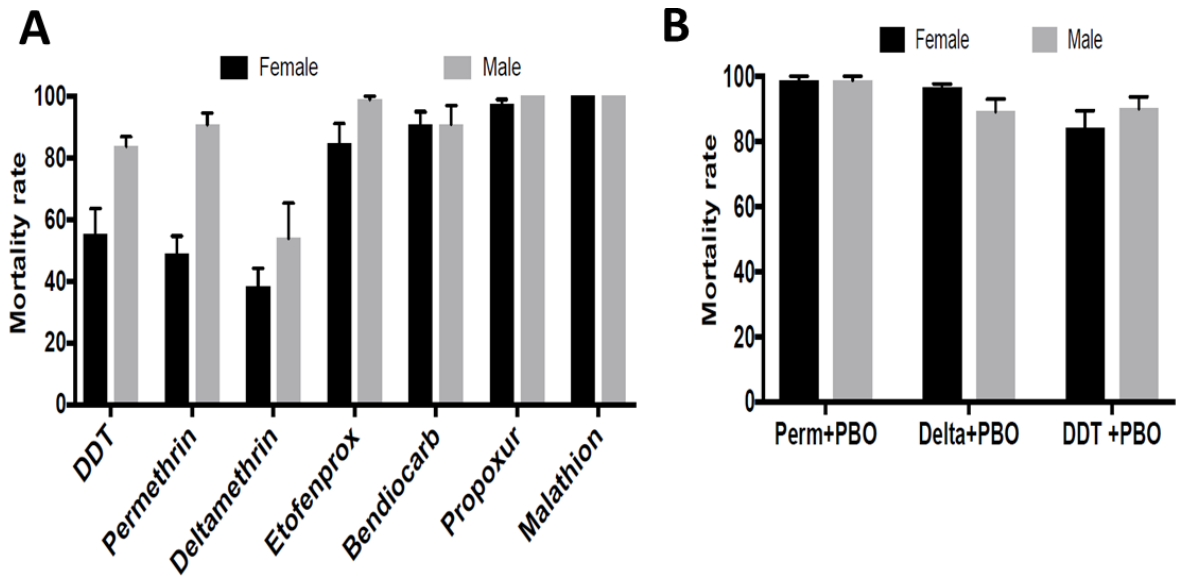

Figure 3.1: Susceptibility profile of *An. funestus* to insecticides

A) Recorded mortalities following 60-min exposure of *An. funestus* s.s. from Mibellon to different insecticides. Data are shown as mean ± SEM. **B)** Activities of PBO combined to permethrin, deltamethrin, and DDT on *An. funestus* s.s. from Mibellon. Data are shown as mean ± SEM.

Susceptibility profile of *An. gambiae* s.s. A total of 971 *An. gambiae* s.s. were tested to assess the resistance profile to seven insecticides. The *An. gambiae* s.s. population was highly resistant to type I and type II pyrethroids (Figure 4.2). For permethrin, mortality was 0 ± 0% for females and 4.44 ± 2.42% for males (Figure 3.2). A very high level of resistance was also observed with deltamethrin with no mortality for females and 1.52 ± 1.52% for males (Figure 3.2). Resistance was also observed for the pseudo-pyrethroid etofenprox with a mortality rate of 9.92 ± 7.66% for females and 13.46 ± 3.93% for males (Figure 3.2). A high level of resistance to the organochlorine DDT was observed with a mortality rate of 1.39 ± 1.39% for females and 1.85 ± 1.85% for males (Figure 3.2). For the carbamate bendiocarb, mortality rates of 66.23 ± 2.6% for females and 59.2 ± 3.73% for males were recorded. However, full susceptibility was observed for propoxur (Figure 3.2). For the organophosphate malathion, a near-full susceptibility was found with a mortality rate of 98.25 ± 1.75% for females and 98.48 ± 1.52% for males (Figure 3.2).

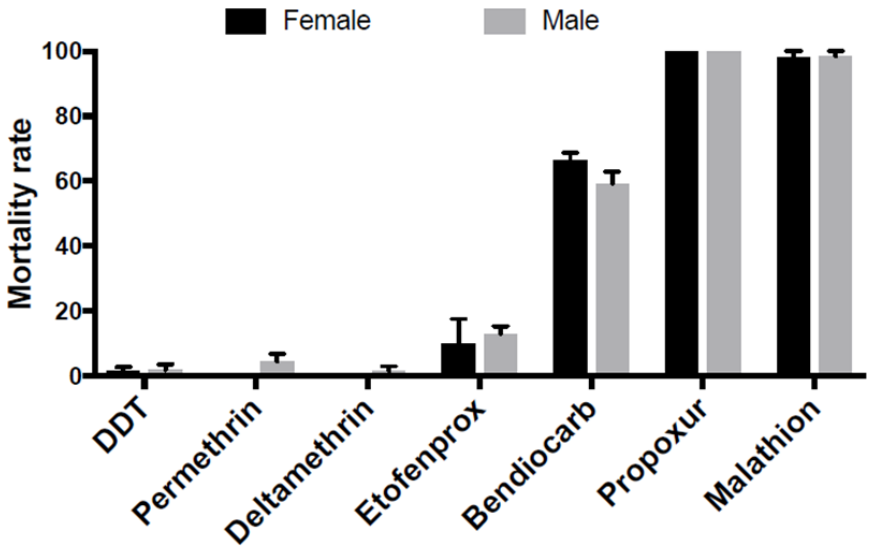

Figure 3.2: Susceptibility profile of *Anopheles gambiae* to insecticides. Recorded mortalities following 60-min exposure of *Anopheles gambiae* s.s. Mibellon to different families of insecticide. Data are shown as mean ± SEM.

3.3.4 Assessment of bed net efficacy with cone assays for *An. funestus*

Test of the efficacy of pyrethroid-only and PBO based-nets was performed with cone assays in *Anopheles funestus* s.s showing very low mortality rates recorded against most of the nets tested. The mortality rates were 2.78 ± 2.78%, and 2.5 ± 2.5%, for Olyset Net and Olyset Plus, respectively; 0 ± 0% and 48.33 ± 6.49% for PermaNet 2.0 and PermaNet 3.0 side, respectively; and, 0 ± 0% for Yorkoll (Figure 3.3A).

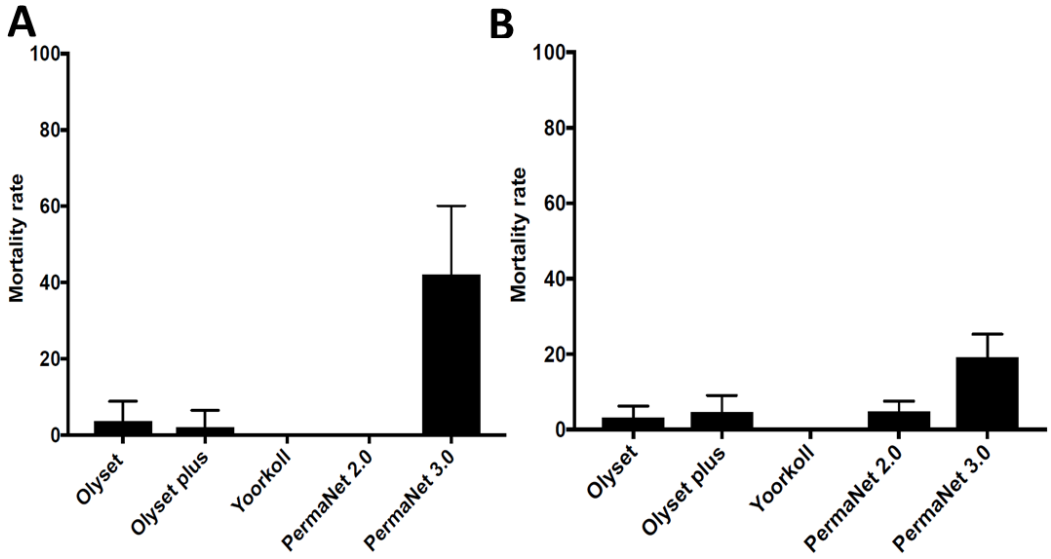

Figure 3.3: Cone assays with various nets for *An. funestus* and *An. gambiae*

(A) Recorded mortalities following 3-min exposure by cone assay of *An. funestus* s.s. (B) *An. gambiae* s.s. from Mibellon to Olyset, Olyset Plus, Yorkool, PermaNet 2.0, and PermaNet 3.0 (side). Data are shown as mean ± SEM.

3.3.5 Assessment of bed net efficacy with cone assays in *An. gambiae* s.s

Similarly, *Anopheles gambiae s.s* showed low mortality rates with the bed nets tested. The mortality rates were 3.13 ± 3.13% and 4.55 ± 4.55% for Olyset Net and Olyset Plus, respectively; 4.77 ± 2.76% and 19.09 ± 6.22% for PermaNet 2.0 and PermaNet 3.0 side, respectively; and, 0 ± 0% mortality for Yorkool (Figure 3.3B).

3.3.6 Molecular basis of the insecticide resistance in *An. funestus* s.s. population

L119F-*GSTe2* detection in *An. funestus* s.s. From 110 F_0 females collected from the field, 7 were homozygous resistant (RR) (6.3%), 48 were heterozygous (RS) (43.2%), and 55 were homozygous susceptible (SS) (49.5 %). Overall, the frequency of the 119F resistant allele was 28 and 72% for the L119 susceptible allele (Figure 3.4). The Hardy-Weinberg equilibrium has been checked for the GSTe2. It has been found that the population was not in Hardy-Weinberg equilibrium ($x^2 = 0.75$; ddl=2; α=0.1). This population is under a pressure that has disrupted the equilibrium of the population in favour of the homozygote resistant mosquitoes.

296S-*RDL* detection in *An. funestus* s.s*.*** Out of the 92 samples genotyped, 3 were RR (3.2%), 12 were RS (13.1%), and 75 were SS (83.7%) with the frequency of resistant allele of only 9.7% and susceptible allele of 90.3% (Figure 3.4). The Hardy-Weinberg equilibrium has been checked for the RDL. It has been found that the population is in Hardy-Weinberg equilibrium (x^2 = 7.28; ddl=2; α=0.025).

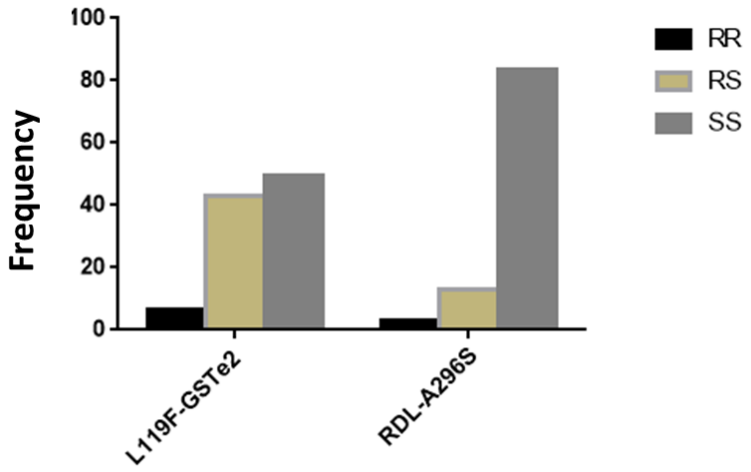

Figure 3.4: Genotypic frequencies of *RDL* and *GSTe2* mutation in *Anopheles funestus* population in Mibellon.

3.3.7 Molecular basis of insecticide resistance in *An gambiae* s.s.

L1014F/L1014S *kdr* detection in *Anopheles gambiae* s.s*.*** Out of 72 samples genotyped, 22 were RR (30.5%) 48 were RS (66.6%), and 2 were SS (2.7%) with a frequency of the resistant allele 1014F of 63.9% and the susceptible L1014 allele of 36.1%. Out of the 76 samples genotyped for the L1014S marker, 3 were RS (3.9%), and 73 were SS (96.1%). Thus a very low frequency of 1.97% was recorded for the resistance allele (Figure 3.5). For the L1014F, the Hardy-Weinberg equilibrium has been checked for the RDL. It has been found that the population is in Hardy-Weinberg equilibrium (x^2 = 7.28; ddl=2; α=0.005. For the L1014S, it was not not possible to calculate the Hardy-Weinberg equilibrium since the number RR individual in the population was equal to zero.

G119S *ace-1* detection in *Anopheles gambiae* s.s. Out of 50 samples genotyped, neither RR nor RS were detected as all the samples were found to be SS (Figure 3.5). For the G119S ace-1, it was not not possible to calculate the Hardy-Weinberg equilibrium since the number of RR and RS individual in the population was zero.

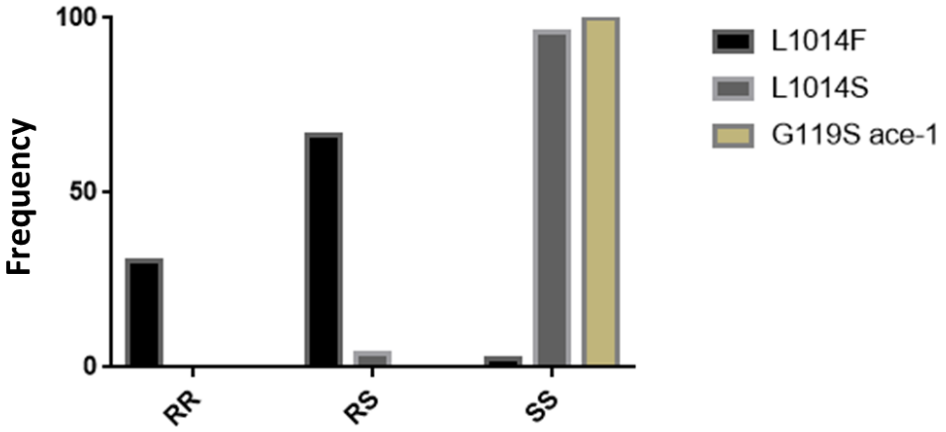

Figure 3.5: Genotypic frequencies of *Kdr*- L1014F, L1014S, and G119S *ace-1* mutation in *Anopheles gambiae* population in Mibellon.

3.3.8 *Plasmodium* infection rate in the *An. funestus* s.s. population in Mibellon

A total of 60 *An. funestus* from Mibellon were tested for *Plasmodium* infection using TaqMan from the head-thorax and the abdomen separately. The analysis of the head and thorax revealed 3 (5%) mosquitoes infected, which included 2 (3.3%) *Plasmodium falciparum* and 1 (1.7%) infection which is either *Plasmodium ovale*, *Plasmodium vivax* or *Plasmodium malariae.* However, 8 (15%) mosquitoes were detected infected when the abdomen was examined, including 5 (8.3 %) *Plasmodium falciparum*, 3 (5%) infections which are either *Plasmodium ovale*, *Plasmodium vivax* or *Plasmodium malariae* and 1 (1.7%) mixed infections (Figure. 3.6).

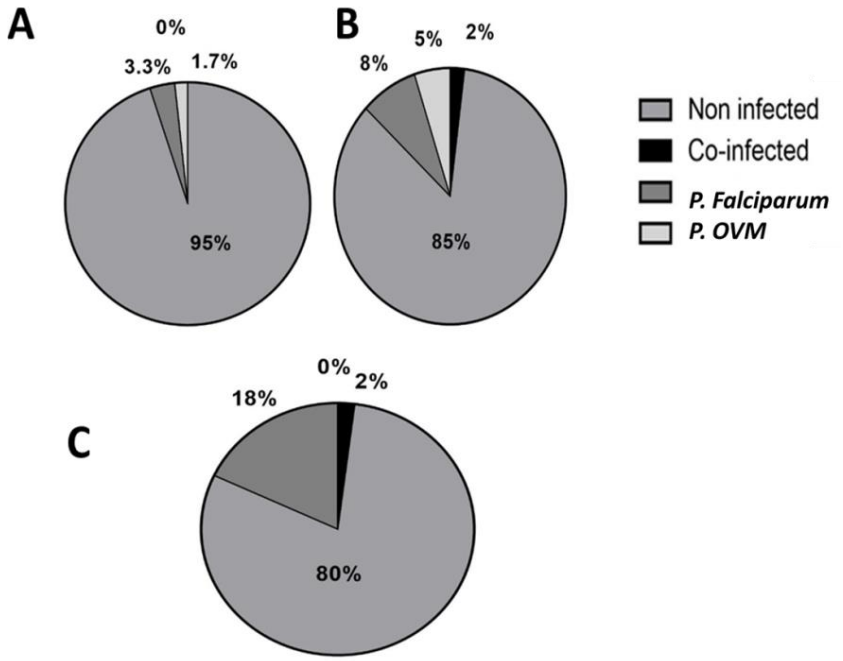

Figure 3.6: *Plasmodium* infection rate in malaria vectors from Mibellon in 2017 **(A)** *Plasmodium* infection rate in *An. funestus* s.s. with head and thorax. **(B)** *Plasmodium* infection rate in *An. funestus* s.s. with abdomen only. **(C)** *Plasmodium* infection rate in *An. gambiae* s.s. for whole mosquitoes.

3.3.9 *Plasmodium* infection rate in the *An.s gambiae* s.s. population in Mibellon

A total of 60 *An. gambiae* were tested for *Plasmodium* infection from the whole body. The analysis revealed 12 (20%) mosquitoes infected, which included 11 (18.3 %) *P. falciparum* and 1 (1.7%) mixed infection (Figure. 3.6C).

3.4 Discussion

Characterization of malaria vector populations is a prerequisite for the implementation of successful vector control interventions and for assessing the impact of insecticides resistance. This study comprehensively characterized the main malaria vectors in Mibellon, a location in Cameroon chosen for the implementation of experimental huts to assess the efficacy of insecticide-based interventions.

3.4.1 Species composition

Out of the nine species of *An. funestus* group described, only *Anopheles funestus* s.s. was detected in Mibellon. This result is similar to the one observed in the northern part of Cameroon, in Gounougou, where *Anopheles funestus s.s*. was 99.5% of the total collection and *Anopheles leesoni* was 0.5% (Menze et al., 2016). The predominance of *Anopheles funestus s.s*. in the *Anopheles funestus* group is also mentioned in West Africa, as observed at Kpome in Benin (Djouaka et al., 2016a). The focus on indoor blood-fed mosquito collection in this study may have prevented from collecting the outdoor resting members of the group. Nevertheless, this is in contrast with the distribution of members of this group observed in eastern and southern parts of Africa where several members are collected indoors. For instance, in Uganda *An. parensis* was found predominantly indoor (Mulamba et al., 2014a; Mulamba et al., 2014b). Similarly, many other species of the *An. funestus* group has been collected indoors in southern Africa, including *An. parensis, An. leesoni* and *An. rivulorum* (Hargreaves et al., 2000; Riveron et al., 2015; Vezenegho et al., 2013). For the *An. gambiae* complex*, An. gambiae s.s*. previously known as S form was the only species found. This result is similar to previous observations showing that *Anopheles gambiae s.s.* was the main species in localities south of the Adamawa mountains, characterized by humid savannah and forested areas (Simard et al., 2009; Wondji et al., 2005a).

3.4.2 The multiple insecticide resistance in both major vectors is a challenge for vector control

The multiple resistance pattern observed in this *An. funestus* population is similar to the pattern observed in the northern part of the country, with the exception of bendiocarb resistance, which was higher in the north (Menze et al., 2016). This result suggests that resistance in *Anopheles funestus* is widespread in Cameroon and could be a concern for effective insecticide-based control tools against this vector. *An. funestus* is highly resistant to both types I and II pyrethroids. The resistance observed in Mibellon seems higher than in Gounougou (2012). This difference could be due to the length of time between the two studies as resistance could have increased since 2012, when the study at Gounougou was carried out.

It could also be evidence that the pyrethroid resistance level in *An. funestus* in Cameroon is rising. This increase could be due to the massive distribution of LLINs implemented by the Cameroonian Government in the past five years. Mibellon is also located in an area where agriculture is the main activity, and agricultural use of pesticides could be another factor that is driving the increase in resistance level. A similar level of increase of pyrethroid resistance was observed in southern Malawi (Riveron et al., 2015) and also reported in Uganda for *Anopheles funestus* (Mulamba et al., 2014b). The high recovery of susceptibility observed after PBO exposure for deltamethrin, permethrin, and DDT suggests that cytochrome P450 genes are playing a significant role in these resistance patterns. Altogether, this resistance to pyrethroids should be of great concern for malaria control programs in Cameroon. If no strong action is taken to manage it, there is a risk that the massive distribution of pyrethroid-impregnated LLINs taking place in Cameroon could be jeopardized. The possible resistance observed to the carbamate bendiocarb in *Anopheles funestus* population shows that such insecticide should be ruled out as an alternative to pyrethroid for IRS. The full susceptibility to the organophosphate malathion, as also demonstrated across the continent so far, suggests that this insecticide class is the most suitable for IRS against this species. The President Malaria Initiative's plan to perform IRS in Cameroon should, therefore, consider using organophosphates such as pirimiphos-methyl (Actellic) to maximize the impact of such intervention.

Very high level of resistance to several classes of insecticides, including organochlorine, pyrethroid, and carbamate, was also observed in *Anopheles gambiae s.s.* population. This high resistance in *Anopheles gambiae* is in line with the increased resistance reported in this species in several sites across Cameroon (Antonio-Nkondjio et al., 2011; Antonio-Nkondjio et al., 2017; Etang et al., 2003; Fossog Tene et al., 2013; Nwane et al., 2013). The resistance in *Anopheles gambiae* was higher than in *An. funestus* for most insecticides (e.g., no mortality against permethrin in *An. gambiae* vs 40.9% in *An. funestus*) suggesting a greater selection operating in *An. gambiae*. This could be explained by a selection of resistance from breeding sites contaminated with insecticides used for the protection of the crops. As *An. gambiae* breeding sites are often located at the vicinity of crops, the selection could be greater in this species compared to *An. funestus* because of mosquito-breeding sites in Mibellon, where there is a large lake that insecticides from farms drain into.

3.4.3 Bio-efficacy of LLINs in cone assays

The low mortality rates observed with permethrin and deltamethrin for both species in Mibellon correlated with the reduced bio-efficacy of most LLINs observed with cone assays, including PBO-based nets such as Olyset Plus. This loss of efficacy may be due to selection pressure induced by the massive distribution of bed nets by the government (MICs, 2014; PNLP, 2016) in addition to the use of pesticide for farming in the area. For both species, the mortality for all the nets is very low, except for PermaNet 3.0 (side). For *An. funestus*, the mortality rates for all LLINs are lower than has been recently reported from other countries such, as DR Congo (Riveron et al., 2018a). However, because cone assays could underestimate the efficacy of LLINs, as they do not assess their additional excito-repellent effect (WHO, 2013c), studies with experimental huts are needed to establish the impact of resistance on LLINs in this region .

3.4.4 Predominance of metabolic resistance in *An. funestus* contrasts with a high frequency of knockdown resistance in *An. gambiae*

The near-full recovery observed for pyrethroids in *Anopheles funestus* after pre-exposure to PBO indicates that resistance is mainly conferred by metabolic resistance, particularly by cytochrome P450s (Wondji et al., 2009). This is in line with previous reports of the absence of *kdr* in this species in Cameroon (Menze et al., 2016) and across Africa (Irving and Wondji, 2017). The frequency of the 119F-*GSTe2*-resistant allele in Mibellon field population (28%) is lower than in the northern part of Cameroon in Gounougou (52%) (Menze et al., 2016) or in Ghana (44.2 %) (Riveron et al., 2014b) and Benin (56.25%) (Djouaka et al., 2016a). The frequencies in Mibellon are closer to that observed in the eastern part of Africa in Uganda (20.4%) (Morgan et al., 2010; Mulamba et al., 2014b). *GSTe2* has been shown to confer cross-resistance between DDT and pyrethroids (Riveron et al., 2014b). The partial recovery of DDT susceptibility after PBO assays suggests that *GSTe2* is probably playing a role in the resistance in this *An. funestus* s.s. population.

The frequency of the 296S-*RDL*-resistant allele is only 9.7%, which is lower than was observed in the northern region at Gounougou. This low frequency could be as a result of recovery to susceptibility for dieldrin after this insecticide was removed from public and agricultural sectors in Cameroon, as observed in Gounougou where the frequency of this mutation went down from 80% in 2006 to 40% in 2012 and 14.6% in 2015 (Menze et al., 2016; Wondji et al., 2011). Such a reversal of resistance is encouraging for the implementation of resistance management strategies.

The very high resistance levels to pyrethroids in *Anopheles gambiae* s.s. (no mortality to permethrin), correlates with the high frequency of the 1014F *kdr* allele (63.9%). This is in line with other reports from Africa where high pyrethroid resistance in *Anopheles gambiae s.l.* has been associated with nearly fixed *kdr* allele in the population, as observed recently in DR Congo (Riveron et al., 2018a), or previously in Côte d'Ivoire (Edi et al., 2012). However, this high level of *kdr* is in contrast to the very low frequency of the 1014S *kdr* allele in Mibellon is similar to previous reports across Cameroon showing that this marker, originally present in East Africa, has now migrated to Central and West Africa although still at very low frequencies (Nwane et al., 2011). Overall, the fact that 1014F *kdr* frequency is not fixed in Mibellon in the presence of such high pyrethroid and DDT resistance suggests that other mechanisms are playing an important role, probably metabolic resistance, as shown for other *Anopheles gambiae* s.l. populations in Cameroon (Antonio-Nkondjio et al., 2015; Etang et al., 2003; Fossog Tene et al., 2013). Further investigation of the resistance mechanisms will help elucidate the molecular basis driving resistance in this population. The total absence of the 119S *ace-1* mutation is in line with the susceptibility of this population to organophosphate and carbamate as this mutation is responsible for organophosphate and carbamate resistance (Edi et al., 2014; Weill et al., 2004). But as this allele has recently been detected in other regions of Cameroon (Elanga et al 2019), more attention should be paid to its occurrence in Mibellon in the future.

3.4.5 Roles of both *An funestus* and *An gambiae* in malaria transmission

This study further confirms the role of *An. funestus* in malaria transmission with a sporozoite infection rate of 5%. This is lower compared to that observed (Cohuet et al., 2004b) in Nkoteng

where *Plasmodium falciparum* infection rate was found to be 8.6%. In Benin (Kpome), *Anopheles funestus* population was found with high *Plasmodium* infection during the dry season (infection rate of 18.2 %), although from the whole mosquitoes (Djouaka et al., 2016a). The higher infection rate found in abdomen (15%) significantly differs (P=0.009) from the infection rate observed in the head and thorax (5%). This result further supports the significant barriers that the midgut plays in preventing oocyst migration to the salivary gland (Hillyer et al., 2007). The number of *Anopheles gambiae* s.s. infected with *Plasmodium* (20%) is higher compared to *Anopheles funestus* s.s. (15%). This rate is very high compared to previous results in Cameron (6.5-8.1%) (Cohuet et al., 2004a). This could be linked to the higher level of insecticide resistance in the *Anopheles gambiae* population, because they are resistant and live longer, which could increase their ability to be infected.

3.5 Conclusion

Multiple resistance observed in both *An. funestus* s.s. and *An. gambiae* s.s. at Mibellon in central Cameroon is a concern for ongoing insecticide-based interventions, although the full susceptibility to organophosphate offers an alternative to IRS. However, the impact of such multiple resistances on the effectiveness of insecticide-based control interventions needs to be evaluated, notably through experimental huts. The presence of resistance in both major vectors makes this area suitable for such studies.

Chapter 4: A comparative experimental hut evaluation of PBO-based and pyrethroid-only nets against the malaria vector *Anopheles funestus* reveals a loss of bed nets efficacy partially linked to *GSTe2* metabolic resistance

4.1. Context of the study

The scale-up of insecticide-based interventions, including Long Lasting Insecticidal Nets (LLINs) and Indoor Residual Spraying (IRS), has significantly contributed to the considerable reduction of malaria burden in the past decade(Bhatt et al., 2015). Unfortunately, growing insecticide resistance in malaria vectors is threatening these successes. However, the impact of resistance, notably metabolic resistance, on the effectiveness of vector control tools against pyrethroid-resistant mosquito populations, remains a topic of debate. Indeed, some studies have suggested that pyrethroid resistance does not yet impact the effectiveness of LLINs (Kleinschmidt et al., 2018b) whereas others have revealed a negative impact (Protopopoff et al., 2018b). Furthermore, to help manage resistance, novel LLINs with the piperonyl butoxide (PBO), an insecticide synergist, have been designed by various manufacturers (Corbel et al., 2010; N'Guessan et al., 2010a; Pennetier et al., 2013b). However, no assessment of how metabolic resistance impacts the efficacy of these nets has been performed. One of the key challenges of assessing the impact of metabolic resistance on the effectiveness of these insecticide-based interventions has been the lack of molecular markers for resistance, notably metabolic resistance, as the phenotype is not easily associated with the outcome of the interventions. Recent efforts have detected a key genetic marker in the glutathione S-transferase epsilon 2 gene (*GSTe2*) conferring a metabolic-mediated resistance to pyrethroids and Dichlorodiphenyltrichloroethane (DDT) in the major malaria vector *Anopheles funestus* in West and Central Africa (Riveron et al., 2014c). Besides cytochrome P450s and esterases, *GSTs* are, one of the main enzyme families conferring metabolic resistance to insecticides (Hemingway and Ranson, 2000) either through a direct metabolism or by catalyzing the secondary metabolism of substrates oxidized by cytochrome P450s (Hemingway and Ranson, 2000). Over-expression of GST epsilon 2 (*GSTe2*) has been associated with DDT and pyrethroids resistance in several mosquito species, including *An. gambiae* (Mitchell et al., 2014), *An. funestus* (Riveron et al., 2014c) and *Aedes aegypti* (Lumjuan et al., 2011). In *An. funestus*, genomic and structural analyses revealed that a leucine to phenylalanine amino acid change (L119F) in *GSTe2*, has enlarged the substrate-binding pocket of the enzyme, conferring DDT/pyrethroid resistance in West and Central African populations (Riveron et al., 2014b). This single amino acid change was used to design a simple field applicable DNA-based diagnostic tool. It was first a TaqMan assay (Riveron et al., 2014b), and later an allelic specific

PCR (Tchouakui et al., 2019c), providing the opportunity to address questions regarding the direct impact of GST-mediated metabolic resistance on insecticide-based interventions such as LLINs.

The pyrethroid resistance in *An. funestus* across Africa is driven by metabolic resistance (Riveron et al., 2013; Riveron et al., 2017b; Weedall et al., 2019; Weedall et al., In Press) as no knockdown resistance *(kdr)* has been reported so far for this species (Irving and Wondji, 2017). This predominance of the metabolic resistance mechanism in *An. funestus* through over-expression of detoxification genes such as *GSTe2* or *CYP6P9a/b* makes this vector suitable to assess the impact of metabolic resistance on control interventions. This has been done for the global resistance and kdr, and it is therefore important to evaluate the impact of metabolic resistance since these mechanisms are more likely to lead to control failure (Tchigossou et al., 2018; Weedall et al., 2019). The presence of the GST-mediated metabolic resistance in *An. funestus* populations such as in Cameroon (Menze et al., 2016; Menze et al., 2018a; Riveron et al., 2014b) also provides the opportunity to assess how the effectiveness of PBO-based nets is impacted when malaria vectors exhibit other type of metabolic resistance than cytochrome P450-based resistance which have been so far the only focus of synergists.

Two methods (epidemiological and entomological) are considered as useful methods to investigate the impact of insecticide resistance (WHO, 2012b). However, the epidemiological approach relaying on malaria cases investigation is not suitable since malaria transmission is affected by many confounding factors (Kleinschmidt et al., 2015; WHO, 2012b). The entomological approach since it provides an opportunity to measure entomological outcomes, such as relative mortality and feeding success of resistant and susceptible vectors in controlled conditions remains the method of choice.(N'Guessan et al., 2007b; WHO, 2012b). It also provides the relevant samples to assess how metabolic resistance impacts the effectiveness of LLINs as done previously to determine the impact of target site mutations such as *kdr* (Ngufor et al., 2014a).

To better inform countries of the usefulness of PBO nets to control pyrethroid-resistant mosquitoes in the face of the greater cost of PBO nets, here we compared the performance of conventional pyrethroid-only nets (Olyset, PermaNet 2.0 and Yorkool) versus PBO-based LLINs (Olyset Plus and PermaNet 3.0) against a pyrethroid-resistant *An. funestus* population from Cameroon using experimental huts. Furthermore, we took advantage of the L119F-

GSTe2 DNA-based diagnostic assay (Riveron et al., 2014b), to assess the impact of GST-mediated metabolic resistance on the performance of these five LLINs.

4.2 Material and Methods

4.2.1 Study area

The experimental hut station is located in Mibellon (6°4'60''N, 11°30'0''E), a village in Cameroon located in the Adamawa Region; Mayo Banyo Division and Bankim Sub-division as described in Chap 3. At the experimental station, 12 huts built following the World Health Organisation (WHO) standard, are available for a wide range of experimental hut trials. *An. funestus* s.s. is the main malaria vector in the area (Menze et al., 2018b). *Mansonia* sp*,* and *Culex sp* are also present in the area*.* Mosquitoes in the area are highly resistant to pyrethroid and DDT, as described in chapter 3. The trial was carried out for 12 weeks during the rainy season from 10th July to 16th October 2016.

4.2.2 Experimental hut construction

My experience as a PhD student started with the construction of the experimental station. The section 4.2.2.1 describes the positioning of the hut in the field. The section 4.2.2.2 describes the plan and the dimension of the hut and the The section 4.2.2.3 describes the different steps of the construction.

4.2.2.1 Hut position on the land

The plan, including several huts in many rows, was built in mosaic since we do not want the huts to face each other (Figure 4.1). The huts are built in the direction of the bresding sites. If the huts are facing each other, the front row may hide the entrance of the second row. This is reason why we suggested to put them in mosaic formation.

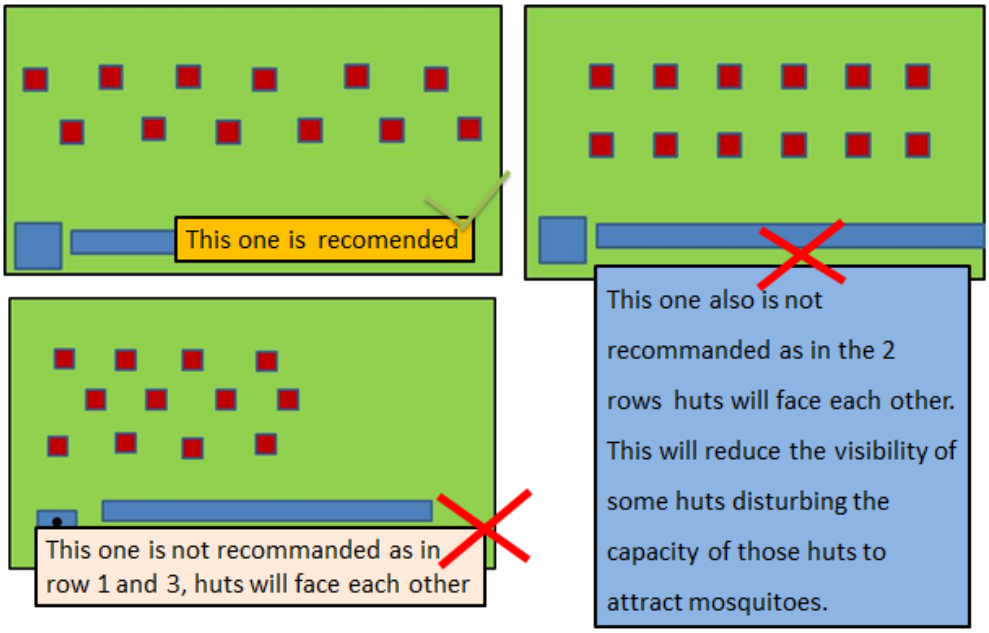

Figure 4.1: Disposition of huts on the land before the construction. Image explaining how huts should be positioned on the ground.

4.2.2.2 Plan and design of the hut

Construction plan and design of huts came from the prototype recommended by WHO for West African. The hut is constructed on a concrete base measuring 5.6 m x.5.6m surrounded by a drain channel (Figure 4.2).

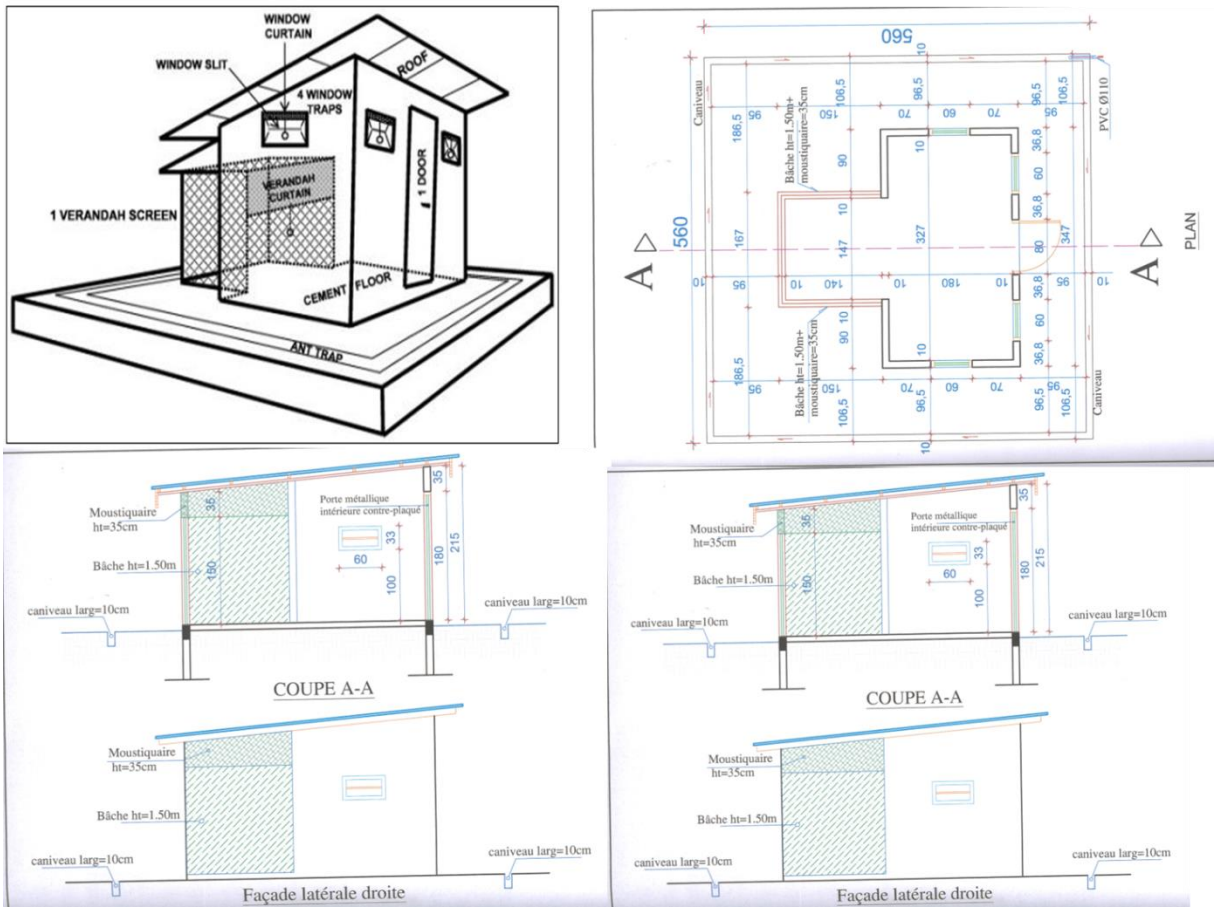

Figure 4.2: Dimensions considered during the construction of the experimental station in Mibellon Cameroon. The figure also describes each side of the hut.

4.2.2.3 Construction of the hut

The walls were made from concrete bricks and plastered inside and outside with a plaster made from a mixture of cement and sand. The roof is made from corrugated iron, and the ceiling is made from plywood (Figure 4.3).

Figure 4.3: Different steps of hut construction. **A)** Huts are built on a concrete base surrounded by a narrow moat to exclude scavenging ants. **B)** Construction following the plan and the dimensions. **C)** Plastering of walls made from a mixture of cement. **D)** The final version of the hut.

4.2.2.3 Window design and construction

The 4 windows located on three sides of the hut are designed to create an angle with a 2 cm gap, which will facilitate the entry of mosquitoes flying upward and prevent the mosquitoes from escaping once they have entered the hut (Figure 4.4).

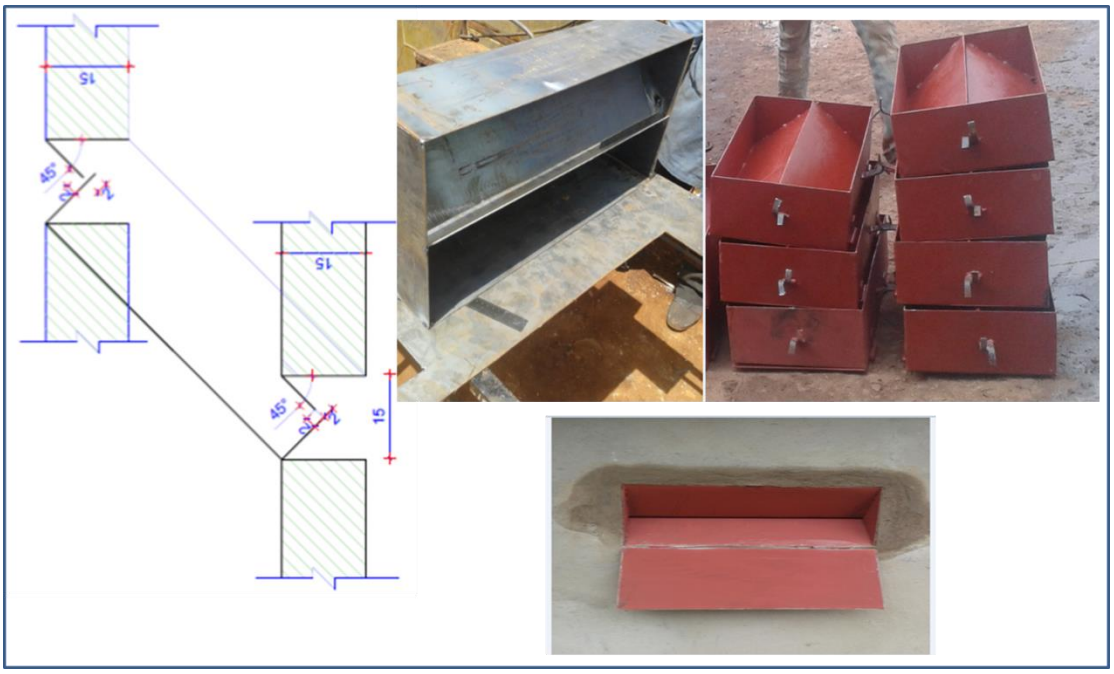

Figure 4.4: Design of windows for experimental following a specific design.

4.2.2.4 Selection of sleepers and functioning of hut.

A curtain is used as a separation between the veranda and the rest of the hut (Figure 4.5). Before bedtime, each sleeper is required to raise the curtain to allow mosquitoes to take refuge in the veranda. In the morning, it is recommended that the sleeper lowers the curtain before starting the collection to allow a separate collection of mosquitoes in the veranda and in the hut. Sleepers were selected from the inhabitant of Mibellon. The selection was made according to gender and age. It was not advised to work with people aged less than 17 years old. After the screening, volunteers were trained and evaluated before the trial. The sleepers signed an informed consent form. The volunteers were regularly followed in the health care center of the village and treat when malaria cases were found.

Figure 4.5: Curtain separating the veranda trap from the rest of the hut.

4.2.3 Net treatment / Arm comparison

During the experimental hut trial, five LLINs and one untreated net as negative control were compared (Table 4.1). These included three pyrethroid-only LLINs (PermaNet 2.0, Olyset, and Yorkool) and two PBO-based nets (PermaNet 3.0 and Olyset Plus) (Table1) (Figure 4.6). Manufacturers provided all nets except Yorkool, which was locally purchased. Each net was holed according to WHO protocol (WHO, 2013b), generating six holes (4 cm × 4 cm) per net, two on each of the long sides, and one on each of the short sides (Figure 4.7). The purpose of making holes in the nets is to facilitate the assessment of the bloodfeeding inhibition (BFI).

Figure 4.6: Photos of some nets packaging used during the study.

Figure 4.7: Net holed according WHO recommendations. Six holes (4 cm × 4 cm) per net, two on each of the long sides and one on each of the short sides.

Table 4. 1: Description of the long-lasting insecticidal nets used

Treatment arm	Description	Manufacturer
Untreated	100% polyester with no insecticide	Local market
Olyset	8.6×10^{-4} kg/m2 (2%) of permethrin incorporated into polyethylene	Sumitomo Chemical
Olyset Plus	8.6×10^{-4} kg/m2 (2%) of permethrin and 4.3×10^{-4} kg/m2 (1%) of PBO incorporated into polyethylene	Sumitomo Chemical
PermaNet 2.0	100% polyester coated with 1.8 g/kg of deltamethrin	Vestergaard Frandsen
PermaNet 3.0	Combination of 2.8 g/kg of deltamethrin coated on polyester with strengthened border (side panels) and deltamethrin (4.0 g/kg) and PBO (25 g/kg)	Vestergaard Frandsen
Yorkool	100% polyester coated with 1.8 g/kg of deltamethrin	Yorkool International Co

4.2.3 Hut effect

Before the study, the hut effect was assessed to evaluate any specific attractiveness of huts. It is essential to know if some huts are more attractive than others, even if this can be corrected by the Latin square design rotation or during the data analysis. Untreated nets were hung in the six huts used for the study, and for three weeks, between the 10th and 29th May 2016, volunteers sleeping underneath collected mosquitoes each morning at 6 am.

4.2.4 Bioassays

Cone bioassays were performed at the beginning of the study using the *An. coluzzii* susceptible Ngousso laboratory strain. This was done to confirm the quality of the 5 bed nets used in the study. Cone bioassays were also done in the insectary using F1 progeny from field-collected *An. funestus* from Mibellon (See details of rearing and cone test in Chapter 2 methods). For PermaNet 2.0, PermaNet 3.0 (side and top), Olyset and Olyset Plus beside an untreated control net, 5 batches of 10 unfed females, 2-5 days old, were exposed to each bed net using WHO cone assays for three minutes (WHO, 2013d). They were then transferred into a holding paper cup container, and the knockdown was checked after 60 min and the mortality after 24 hours post-exposure during which mosquitoes were provided a 10 % sugar solution. Bioassays were also performed to generate highly resistant (alive after 90 min exposure) and highly susceptible (dead after 30 min) mosquitoes against 0.75% permethrin, 0.05% deltamethrin, and 4% DDT WHO papers. The 30- and 90-minutes exposure tests were performed in separate tubes, then scored 24hr post-exposure, and the samples used to assess the association between L119F-*GSTe2* genotypes and resistance to these insecticides.

4.2.5 Experimental hut trial

The experimental hut trial was done following the protocol described in the guidelines for laboratory and field-testing of long-lasting insecticidal nets (WHO, 2013b) (See methods in chspter 2). To correct any specific attractiveness observed during the hut effect assessment, bed nets were rotated according to the Latin design square rotation (Figure 4.8) (WHO, 2013b) (Figure 4.8) so that at the end of the study, each net would have spent six days in each hut. Six volunteer males were selected to sleep in the room from 20:00 GMT to 5:00 GMT in the morning. The sleepers were also rotated so that at the end of the week, each sleeper would

have spent one night in each hut. The rotation of the sleepers was done to correct any bias due to any specific attractiveness from the sleepers (Ngufor et al., 2014b).

	Hut 1	Hut 2	Hut 3	Hut 4	Hut 5	Hut 6
Week 1	Control	Olyset	Olyset Plus	PermaNet 2.0	PermaNet 3.0	Yorkool
Week 2	Olyset	Olyset Plus	PermaNet 2.0	PermaNet 3.0	Yorkool	Control
Week 3	Olyset Plus	PermaNet 2.0	PermaNet 3.0	Yorkool	Control	Olyset
Week 4	PermaNet 2.0	PermaNet 3.0	Yorkool	Control	Olyset	Olyset Plus
Week 5	PermaNet 3.0	Yorkool	Control	Olyset	Olyset Plus	PermaNet 2.0
Week 6	Yorkool	Control	Olyset	Olyset Plus	PermaNet 2.0	PermaNet 3.0

Figure 4.8: Bed nets rotation according to the Latin design square rotation.

4.2.6 Mosquito collection

Mosquitoes were collected every morning, using hemolysis tubes from: (i) inside the nets, (ii) in the room: floor, walls and roof, and (iii) in the veranda exit trap. Mosquitoes collected from each compartment were kept separately in a bag to avoid any mixing between samples from different compartments. (Figure 2.2). Samples were transferred to the laboratory for identification. After identification, samples were then classified as dead, alive, blood-fed, or unfed (Appendix 1). The 'alive' samples were kept in the paper cup and provided with sugar solution for 24 hours and mortality was monitored. Dead mosquitoes were then kept adequately in labelled Eppendorf tubes with silica gel and live mosquitoes stored in RNA later (Figure 2.3).

4.2.7 Bed nets performance assessment

The performance of the bed nets was expressed relative to control (untreated nets) (Appendix 1) in term of:

i-Deterrence/entry rate: the reduction in hut entry relative to control. Deterrence (%) = 100 x (Du – Dt) / Du, where Du is the total number of mosquitoes found in the untreated hut (control), and Dt is the total number of mosquitoes in the treated hut.

ii-Entry rate (%) = 100 x (Ht/Hn) where Ht is the total number of mosquitoes found in the hut, and Hn is the total number of mosquitoes collected in all the six huts.

iii-Exophily: the proportion of mosquitoes found exited in the veranda trap.

Exophily (%) = 100 x (Ev/Et) where Ev is the total number of mosquitoes found in veranda, and Et is the total number of both inside the hut and veranda.

iv-Blood feeding rate (BFR). This rate was calculated as follows: Blood feeding rate = (N mosquitoes fed) x 100 / total N mosquitoes. Where "N mosquitoes fed" was the number of mosquitoes fed, and "total N mosquitoes" was the total number of mosquitoes collected.

v-Blood-feeding inhibition (BFI): the reduction in blood-feeding in comparison with the control hut. Blood feeding inhibition is an indicator of personal protection (PP). More precisely, the personal protection effect of each bed net is the reduction of blood-feeding percentage induced by the net when compared to control. The protective effect of each bed net can be calculated as follows: Personal protection (%) = 100 x (Bu – Bt) / Bu, where Bu is the total number of blood-fed mosquitoes in the huts with untreated nets and Bt is the total number of blood-fed mosquitoes in the huts with treated nets (WHO, 2013).

vi-Immediate and delayed mortality: the proportion of mosquitoes entering the hut that is found dead in the morning (immediate death) or after being caught alive and held for 24 h with access to sugar solution (delay mortality) (WHO, 2013). In this study, we focused on the overall mortality calculated as follows: Mortality (%) = 100 x (Mt/MT) where Mt is the total number of mosquitoes found dead in the hut and MT is the total number of mosquitoes collected in the hut.

4.2.8 Ethical clearance

The national ethics committee for health research of Cameroon approved the protocol of the study (ID:2016/03/725/CE/CNERSH/SP) (Appendix 6). Written, informed and signed consent was obtained from sleepers before starting the trials. The consent form provided all the information and the evaluation process about the study. Information was translated in local

language when needed. All the volunteers involved in the study were followed-up and treated when showing malaria symptoms.

4.2.9 Data analysis

Experimental hut trial: To calculate the proportion of each entomological outcomes and the level of significance between the treatments and between the control for each entomological outcomes, the XLSTAT software (Addinsoft, Berkeley, CA, USA) was used. To conteract the problem of multiple comparison, the bonferroni correction was apply using an alpha of 0.01 as the cut off for significance.

The effects of the treatments on each of the main proportional entomological outcomes (exophily, blood-feeding, and mortality) were assessed using binomial generalized linear mixed models (GLMMs) with a logit link function, fitted using the 'lme4' package for R 3.6 (Toe et al., 2018a; WHO, 2013a). A separate model was provided for each outcome and each mosquito species. In addition to the fixed effect of each treatment, each model included random effects to account for the following sources of variation: between the six huts used in the studies; between the six sleepers who slept in the huts; between the six weeks of the trial.

Test of association between L119F-*GSTe2*-mutation and the entomological outcomes: To investigate the association between the L119F-*GSTe2* mutation and the ability of the mosquitoes to survive, blood feed or escape, the online Vassar stats software were used to estimate the Odds ratio based on a fisher exact probability test with a 2x2 contingency table.

Hut effect analysis: The one-way analysis of variance (ANOVA) using Prism 7.0 was used to determine whether there were any statistically significant differences between the means of the mosquitoes collected from the 6 huts.

4.2.10 Impact of the L119F-*GSTe2* mutation on insecticide-treated nets

The samples collected during the investigation on the performance of nets were grouped in several categories: dead, alive, blood-fed, unfed, room and veranda, and inside nets. The L119F-*GSTe2* mutation was genotyped in each group using an Allele Specific-PCR. This allows

a direct measure of the relative survival and feeding success of resistant and susceptible insects in the presence of the different bed nets.

Genotyping

Samples classified as dead, alive, blood-fed, unfed, (Appendix 6) room, and veranda were used for DNA extraction using the Livak protocol (Livak, 1984). The L119F-*GSTe2* mutation was genotyped to assess how the glutathione S-transferase gene, *GSTe2*, impacts the performance of the bed nets. An allele-specific PCR (Tchouakui et al., 2019d; Tchouakui et al., 2018) was used to detect the three genotypes of the L119F-*GSTe2* mutation (RR, RS, and SS). (See chapter 2;section 2.4.3 for more details).

4.3 Results

4.3.1 Cone assays using the *An. coluzzii* susceptible lab strain Ngousso

At the beginning of the study, each bed net was exposed to the *An. coluzzii* susceptible lab strain Ngousso using WHO cone assays. Olyset Plus and PermaNet 3.0 showed a mortality of 100±00 %, PermaNet 2.0 showed 93.3±3.33% mortality and Olyset and Yorkool gave mortality rates of 96.97±3.33% and 96.3±3.7 % respectively. In contrast, no death was recorded for the untreated net (Figure. 4.9A).

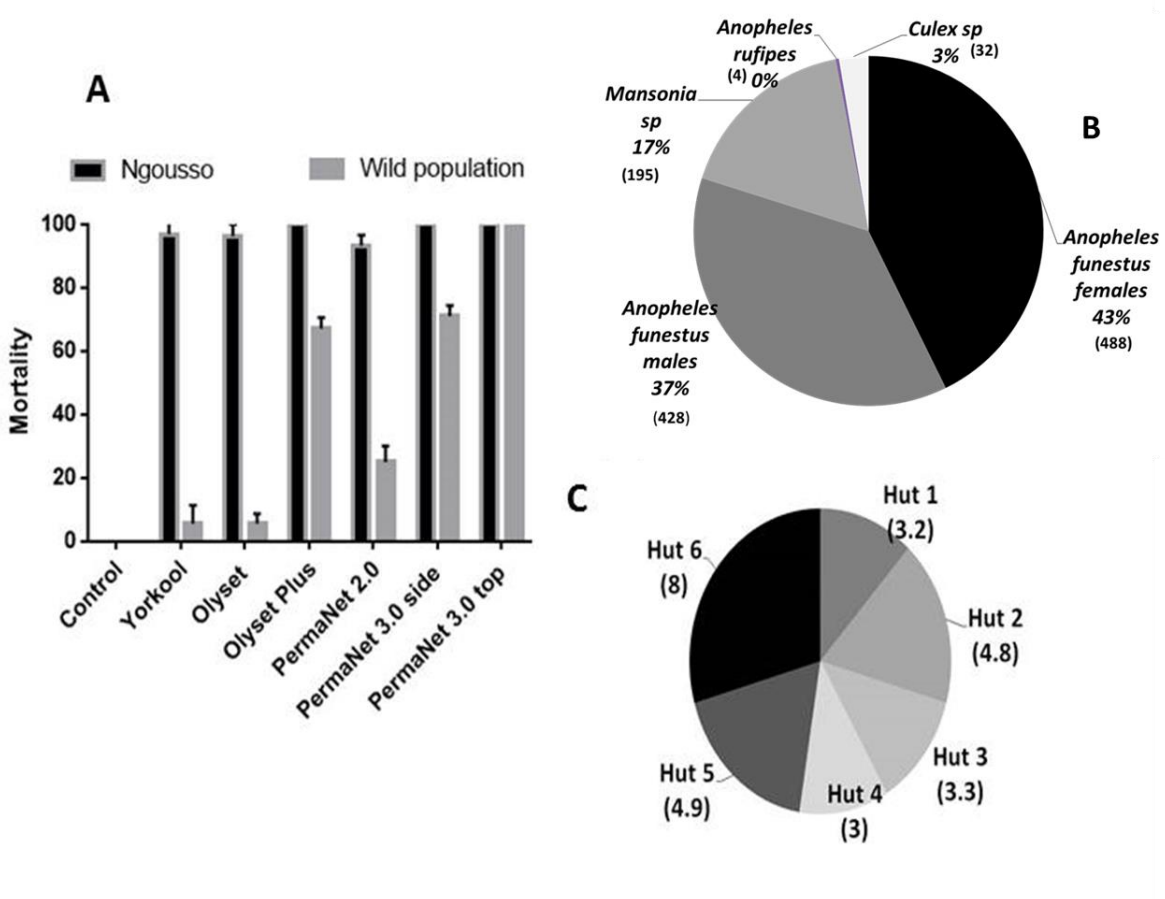

Figure 4.9: Quality control before the experimental hut trial in Mibellon**:** Species composition in Mibellon. **A)** Quality control of the efficacy of all the five nets checked against the susceptible laboratory strain of *An. coluzzii* Ngousso and LLINs efficacy testing using cone assays against the pyrethroid-resistant *An. funestus* population from Mibellon, Cameroon. **B)** The number of mosquitoes collected during the hut effect assessment; **C)** Average of *An. funestus.* ss collected by hut during the 18 days of the hut effect investigation.

4.3.2 Cone assays with *An. funestus* from the field (Mibellon)

Olyset and Olyset plus showed mortality of 5.63±3.2% and 67.23±3.4%, respectively, when exposed to the F_1 population of *An. funestus* from Mibellon using WHO cone assays. PermaNet 2.0 showed 25.06±5.06% mortality, and PermaNet 3.0 side and PermaNet 3.0 top gave 71.04±3.33 % and 100±0 % respectively whereas no mortality was recorded for the Yorkool and the untreated net (Figure 4.9A).

4.3.3 Hut effect

After 18 days of collections, a total of 1,147 mosquitoes were collected in the huts with the untreated net. Out of the 1,147, 488 were females *An. funestus*, 428 *An. funestus* males, 195 *Mansonia* spp, 32 *Culex* sp*,* and 4 *An. rufipes.* The average of *An. funestus* females collected per room after 18 days ranged from 3 to 8 per day (Figure. 4.9C). No significant difference (P=0.09; DF=5) between the numbers of mosquitoes collected in the different huts was observed (Figure. 4.9C).

4.3.4 Mosquito abundance

A total of 5,015 mosquitoes were collected in 6 huts by human volunteers sleeping in the huts for 12 weeks, corresponding to 432 man-nights. Out of the mosquitoes collected, 1,458 (29%) were *An. funestus.* s.s. females, 2,421 (48%) were *An. funestus* males, 1,063 (21%) were *Mansonia* spp, 03 (0.06%) were *An. gambiae* ss*.,* 69 (1.4%) were *Culex* sp and 04 (0.08%) were *Aedes* sp (Figure 4.10A). However, only female *An. funestus* and *Mansonia* sp, due to its significant nuisance in this area, were considered for analysis (Table 4.2 and 4.3).

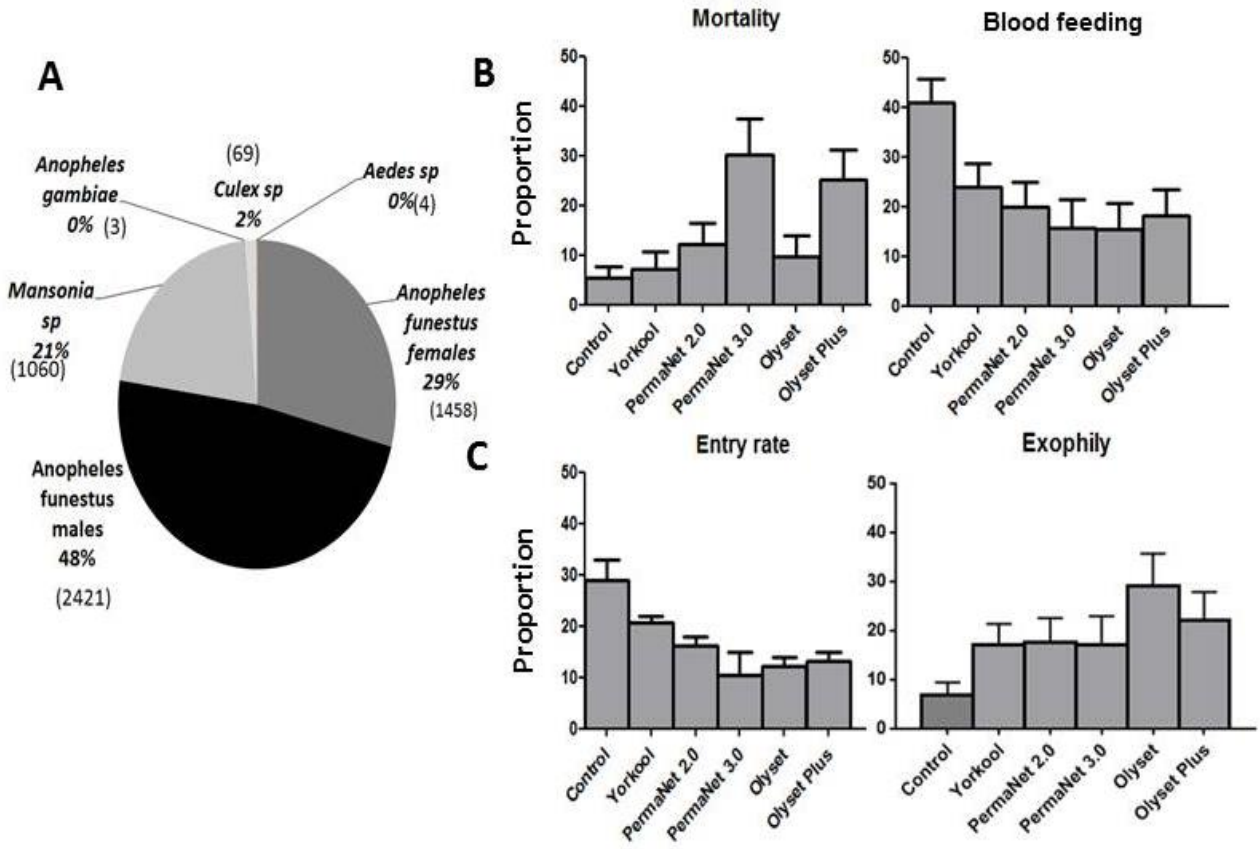

Figure 4.10: Performance of the five LLINs in experimental hut trials against pyrethroid-resistant *An. funestus* in Cameroon. **A)** Mosquito species composition during the experimental hut study. **B)** Assessment of deterrence (entry rate) and exophily of free-flying *An. funestus* in Mibellon for the five LLINs during the trial. **C)** The proportion of mortality and blood-feeding rate for the five LLINs against *An. funestus.*

Table 4.2: **Results of the performance of the five brands of LLINs against wild *An. funestus* females in experimental huts**

	Treatments					
	Control	**Yorkool**	**PermaNet 2.0**	**PermaNet 3.0**	**Olyset**	**Olyset Plus**
Females caught	390	303	237	153	176	199
Exophily%	6.9	17.2	17.7	17.0	29.0	22.1
95% Confidence limits	4.40-9.44)	(12.92-21.41)	(12.85-22.58)	(11.04-22.94)	(22.27-35.68)	(16.34-27.88)
P value		<0.001	<0.001	<0.001	<0.001	<0.001
Blood fed (%)	40.8	23.8	19.8	15.7	15.3	18.1
95% Confidence limits	(35.89-45.65)	(18.97-28.55)	(14.75-24.91)	9.92-21.45)	10.02-20.67)	(12.74-23.44)
P value		<0.001	<0.001	<0.001	<0.001	<0.001
Blood feed inh. (%)	0.0	41.71	51.36	61.52	62.37	55.63
Personal protection (%)	0.0	54.71	70.44	84.90	83.01	77.35
Overall mortality (%)	5.4	7.6	12.2	30.1	9.7	25.1
95% Confidence limits	(31.14-7.62)	(4.61-10.57)	(8.06-16.41)	(22.80-37.33)	(5.29-14.02)	(19.10-31.15)
P value		ns	<0.01	<0.001	ns	<0.001
Immediate mortality	2.6	4.6	5.4	27.6	6.1	18.3
P value	-	ns	ns	<0.001	<0.05	<0.001
Entry rate	29.3	20.78	16.25	10.4	12.07	13.6
P value		<0.001	<0.05	<0.001	<0.0001	<0.001
Deterrence (%)	0.0	22.3	39.2	60.8	54.9	49.0

PV, P-value; CI, Confidence interval; ns=Non Significant

Table 4.3: Results of the performance of the five brands of LLINs against wild *Mansonia* spp in experimental huts

	Control	**Yorkool**	**PermaNet 2.0**	**PermaNet 3.0**	**Olyset**	**Olyset Plus**
Total	342	159	142	216	141	163
Exophily%	42.4	26.4	37.3	31.9	28.4	31.3
95% Confidence limits	(37.16-47.64)	(19.56-33.27)	(29.37-45.28)	(25.73-38.16)	(20.93-35.81)	(24.17-38.41)
P-value		<0.001	ns	<0.01	<0.01	<0.01
Blood feeding (%)	30.1	22.0	16.2	9.3	15.6	20.2
95% Confidence limits	(25.25-34.98)	(15.57-28.45)	(10.14-22.26)	(5.39-13.12)	(9.61-21.59)	(14.08-26.41)
P-value		ns	<0.01	<0.001	<0.001	<0.01
Blood feeding inhibition. (%)	0.0	26.91	46.22	69.26	48.19	32.78
Personal protection (%)	0.0	66.01	77.66	80.53	78.64	67.96
Overall mortality (%)	37.4	49.1	51.4	65.5	40.4	68.1
P-value		<0.01	<0.01	<0.001	ns	<0.001
Immediate mortality	36,8	48,4	46,5	62	39	65
P-value		ns	ns	<0.001	<0.001	<0.001
Entry rate	29.3	12.1	14.1	12.2	18.56	13.6
P-value		<0.001	<0.001	<0.01	<0.001	<0.001
Deterrence (%)	0.0	53.5	58.5	36.8	58.8	52.3

PV, P-value; CI, Confidence interval;ns=Non Significant

4.3.5 Performance of the nets against *An. funestus* s.s. population

4.3.5.1 Deterrent effect/Entry rate

Overall, when compared to control, the three pyrethroid-only nets (PermaNet 2.0, Olyset, and Yorkool) showed different performance with PermaNet 2.0, presenting the highest mortality rate (54.9%) (Table 4.2). When comparing the two PBO nets (Olyset Plus and PermaNet 3.0), both demonstrated a high deterrent effect (49.0% and 60.8 %) (Table 4.2).
In comparison to control, the entry rate was significantly reduced in all the huts with the five LLINs with the deterrence rates ranging from 22.3% for Yorkool to 60.8% for PermaNet 3.0. PermaNet 3.0 had a significantly higher deterrence compared to PermaNet 2.0 (P=0.0001), but this was not the case for Olyset Plus (49%) over Olyset (54.9%) (P=0.09). (Table 4.2).

4.3.5.2 Induced exophily rate

Overall, when compared to control, the three pyrethroid-only nets (PermaNet 2.0, Olyset, and Yorkool) showed different performances with Olyset presenting the highest exophily rate (29.0%) compared to an average of 17% for PermaNet 2.0 and Yorkool (Table 4.2). When comparing the two PBO nets (Olyset Plus and PermaNet 3.0) in exophily, no significant difference was observed (P=0.1). A low exophily rate was recorded in the control hut for *An. funestus* s.s. (6.9%). However, the exophily rate was significantly higher in the hut with Olyset (15.3%; P=0.0001), Olyset Plus (18.1%; P=0.0001), PermaNet 2.0 (19.8%; P=0.0001), PermaNet 3.0 (15.7%; P=0.0004) and Yorkool (23.8%; P=0.0001) compared to the control hut (Figure. 5.2C and table 4.2). No significant difference is observed between the five tested LLINs. A significant high exophily activity was observed during week 2 and week 9 (P<0.05) (Appendix 7).

4.3.5.3 Mortality (overall mortality)

Overall, when compared to control, the three pyrethroid-only nets (PermaNet 2.0, Olyset, and Yorkool) showed different performance with PermaNet 2.0, presenting the highest mortality rate (12.2%) compared to 7.6% and 9.7% respectively for Yorkool and Olyset (Table 4.2). When comparing the two PBO nets (Olyset Plus and PermaNet 3.0) in mortality, no significant difference was observed (P=0.4).

Low mortality rates were recorded in the control hut for *An. funestus* s.s. (5.4%). However, the mortality rate was significantly higher in the hut with Olyset Plus (25.1%; P=0.001), PermaNet 2.0 (12.2%; p=0.002), and PermaNet 3.0 (30.1%; P=0.0001) compared to the control hut. The mortality rate was significantly higher with the two PBO-based nets than all the pyrethroid-only nets (P<0.001). However, no significant variation was observed between the mortality in the huts with Olyset (9.7 %; P=0.06) and Yorkool (7.6 %; P=0.2) compared to control (Fig. 4.10B; table 4.2).). It is also clear from our analysis that mortalities obtained were not influenced by the sleepers (P>0.05), by the huts (P>0.05) and by the weeks (P>0.05) (Appendix 8).

4.3.5.4 Blood feeding inhibition (BFI)

Overall, when compared to control, the three pyrethroid-only nets (PermaNet 2.0, Olyset and Yorkool) showed different performance with Olyset presenting the highest Blood Feeding Inhibition (BFI) rate (62.3.2%) compared to 41.71% and 51.36% respectively for Yorkool and PermaNet 2.0 (Table 4.2). When comparing the two PBO nets (Olyset Plus and PermaNet 3.0) in mortality, no significant difference was observed (P=0.5).

High blood feeding rates were recorded in the control hut (40.8%). However, the blood-feeding rate (BFR) was significantly lower in the hut with Olyset (BFR=15.3; BFI= 62.5%; P<0.0001), Olyset Plus (BFR=18.1%; BFI=55.6%; P=0.001), PermaNet 2.0 (BFR=19.8%; BFI=48.5%; P=0.0001), PermaNet 3.0 (BFR=15.7%; BFI=61.5%; P<0.0001) and Yorkool (BFR=23.8%; BFI=41.7%; P<0.0001) compared to the control hut (Figure. 4.10B; Table 4.2). PermaNet 3.0 had a higher BFI than PermaNet 2.0, but this was not the case for Olyset Plus compared to Olyset, although the differences were not significant (P=0.2). We noticed a significant reduction of the blood-feeding in hut 4 (P<0.05) and a significant increased during week 12 (P<0.05) (Appendix 9). These fluctuations were corrected by a rotation based on Latin square design (Table 4.2).

4.3.5.5 Personal protection (PP)

Overall, when compared to control, the three pyrethroid-only nets (PermaNet 2.0, Olyset and Yorkool) showed different performance with Olyset presenting the highest Blood Feeding Inhibition (BFI) rate (83.01%) compared to 54.71% and 70.44% respectively for Yorkool and

PermaNet 2.0 (Table 4.2). When comparing the two PBO nets (Olyset Plus and PermaNet 3.0) in mortality, no significant difference was observed (P=0.5).

The poorest performance in terms of PP was recorded with Yorkool (54.7%). PermaNet 3.0 provided a higher PP when compared to PermaNet 2.0 but not significant (84.9% vs 70.4%; P>0.05). No significant difference was observed when comparing the PP provided by Olyset and Olyset Plus (83 vs 77.3 %; P>0.05) (Table 4.2).

4.3.6 Performance of the nets against *Mansonia* spp. population

4.3.6.1 Deterrent effect

In comparison to control, the entry rate was significantly reduced for the five LLINs with deterrence rates ranging from 36.8% for PermaNet 3.0 to 58.8% for Olyset. Contrary to *An. funestus*, PermaNet 2.0 had a higher deterrence rate (58.5%) compared to PermaNet 3.0 (36.8%) (P=0.05) and similar for Olyset (58.8%) compared to Olyset Plus (52.3%) (Table 4.3).

4.3.6.2 Induced exophily rate

A higher exophily rate was recorded in the control hut for Mansonia spp (42.4%) than *An. funestus* (6.9%) (P<0.05). However, the exophily rate of *Mansonia* spp, was significantly lower in the hut with Olyset (28.4%; P<0.01), Olyset Plus (31.3%; P<0.01), PermaNet 3.0 (31.9%; P<0.01) and Yorkool (26.4%; P<0.001) compared to the control hut. For PermaNet 2.0 no significant difference was observed when compared to control (37.3%; P=0.3) (Table 4.3).

4.3.6.3 Mortality

The overall mortality rate recorded in the control hut for *Mansonia* spp was 37.4%. However, this rate was significantly higher in the hut with Olyset Plus (68.1%; P<0.001), PermaNet 2.0 (51.4%; P>0.05) and PermaNet 3.0 (65.3%; P<0.001) compared to the control. No significant variation was observed between the mortality in the huts with Olyset (40.4%; P>0.05) and Yorkool (49.1%; P>0.05) compared to control (Table 4.3). The two PBO nets PermaNet 3.0 and Olyset Plus had a significantly higher mortality rate than the pyrethroid-only nets PermaNet 2.0 and Olyset (Table 4.3).

4.3.6.4 Blood feeding inhibition (BFI)

Overall, when compared to control, the three pyrethroid-only nets (PermaNet 2.0, Olyset, and Yorkool) showed different performances with Yorkool presenting the highest Blood Feeding rate (22.0%) compared to 16.2% and 15.6% respectively for PermaNet 2.0 and Olyset (Table 4.3). When comparing the two PBO nets (Olyset Plus and PermaNet 3.0) in Blood feeding, a significant difference was observed (P=0.001). The blood-feeding rate recorded in the control hut for *Mansonia* spp was 30.1%. However, this rate was significantly lower in the hut with Olyset (15.6%; BFI=48.1%; P<0.01), Olyset Plus (20.2%; 32.7%; P=0.0009), PermaNet 2.0 (16.2%; BFI=42.6%; P<0.01) and PermaNet 3.0 (9.3%; BFI=69.26%; P<0.001) compared to the control hut. No significant variation was observed between the blood-feeding rate for Yorkool hut (22%; BFI=26.91%; P>0.05) compared to control (Table 4.3). PermaNet 3.0 had significantly higher BFI when compared to PermaNet 2.0 (P=0.02) and Olyset Plus (P=0.001).

4.3.6.5 Personal protection (PP)

The lowest PP was recorded with Yorkool (66.01%), whereas PermaNet 3.0 provided a higher PP but not significant when compared to PermaNet 2.0 (83.5% vs 77.6%; P>0.05) but higher than the Olyset nets (P=0.03). No significant difference was observed when comparing the PP provided by Olyset and Olyset Plus (48.1 % vs 32.7 %; P>0.05) (Table 4.3).

4.3.7 Validation of the association between *L119F-GSTe2* and resistance to pyrethroids and DDT in Mibellon

Before assessing the impact of *L119F-GSTe2* on LLINs, we first confirm its role in conferring pyrethroid/DDT resistance in this population by establishing the association between this marker and the ability to survive exposure to the pyrethroids used for Olyset (permethrin) and PermaNet (deltamethrin) nets besides DDT. Correlation analysis between L119F - *GSTe2* genotypes and deltamethrin revealed that homozygote resistant mosquitoes were significantly more likely to survive exposure to deltamethrin than both homozygotes susceptible SS (OR=4.6; P<0.001) and heterozygotes (OR=3.75; P<0.001). However, there was no significant difference between RS and SS (Figure 4.11D; Table 4.4). A similar pattern was observed for permethrin (Figure. 4.11E; Table 4.4). Analysis of the correlation with DDT

revealed a much stronger association between L119F and DDT resistance as RR exhibited a greater ability to survive exposure to DDT than SS (OR=66.7; P<0.001) and also more than RS (OR=4.8; P<0.001). Contrary to both pyrethroids heterozygote RS mosquitoes are also more able to survive exposure to DDT than homozygote susceptible (OR=14.0: P<0.001) (Figure 4.11F; Table 4.4).

Table 4.4. Impact of *GSTe2* on the ability of field population *An. funestus* to survive using samples from cone assays.

	Cone assays			
Olyset Plus 40 alive vs 40 dead		**OR**	**PV**	**CI**
	RR vs SS	1.1	>0.05	0.4-2.7
	RS vs SS	0.9	1.0	0.5-1.8
	RR vs RS	1.1	>0.05	0.4-2.7
	R v S	1.04	1.0	0.5-1.8
PermaNet 2.0 46 alive vs 34 dead	RR vs SS	2.09	<0.01	1.1-4.2
	RS vs SS	2.3	<0.001	1.2-4.4
	RR vs RS	0.8	>0.05	0.3-2.4
	R vs S	1.8	<0.01	0.9-3.5
PermaNet 3.0 39 alive vs 31 dead	RR vs SS	30.1	<0.001	0.8-5.3
	RS vs SS	2.4	<0.01	1.3-4.5
	RR vs RS	12.3	<0.001	1.5-98.6
	R vs S	3.8	<0.001	1.8-7.7
	WHO bioassays			
Deltamethrin 38 alive vs 38 dead		**OR**	**PV**	**CI**
	RR vs SS	4.6	<0.001	2.5-8.4
	RS vs SS	1.22	>0.05	0.69-2.15
	RR vs RS	3.75	<0.001	2.1-6.8
	R vs S	2.80	<0.01	1.54-5.1
Permethrin 28 alive vs 37 dead	RR vs SS	4.8	<0.001	2.66-8.8
	RS vs SS	1.6	>0.05	0.86-2.8
	RR vs RS	3.1	<0.001	1.7-5.5
	R vs S	2.99	<0.001	1.7-5.3
DDT 29 alive vs 30 dead	RR vs SS	66.7	<0.001	27.1-164
	RS vs SS	14.0	<0.001	6.3-31.2
	RR vs RS	4.8	<0.001	2.4-9.5
	R v S	15.3	<0.001	7.7-30.5

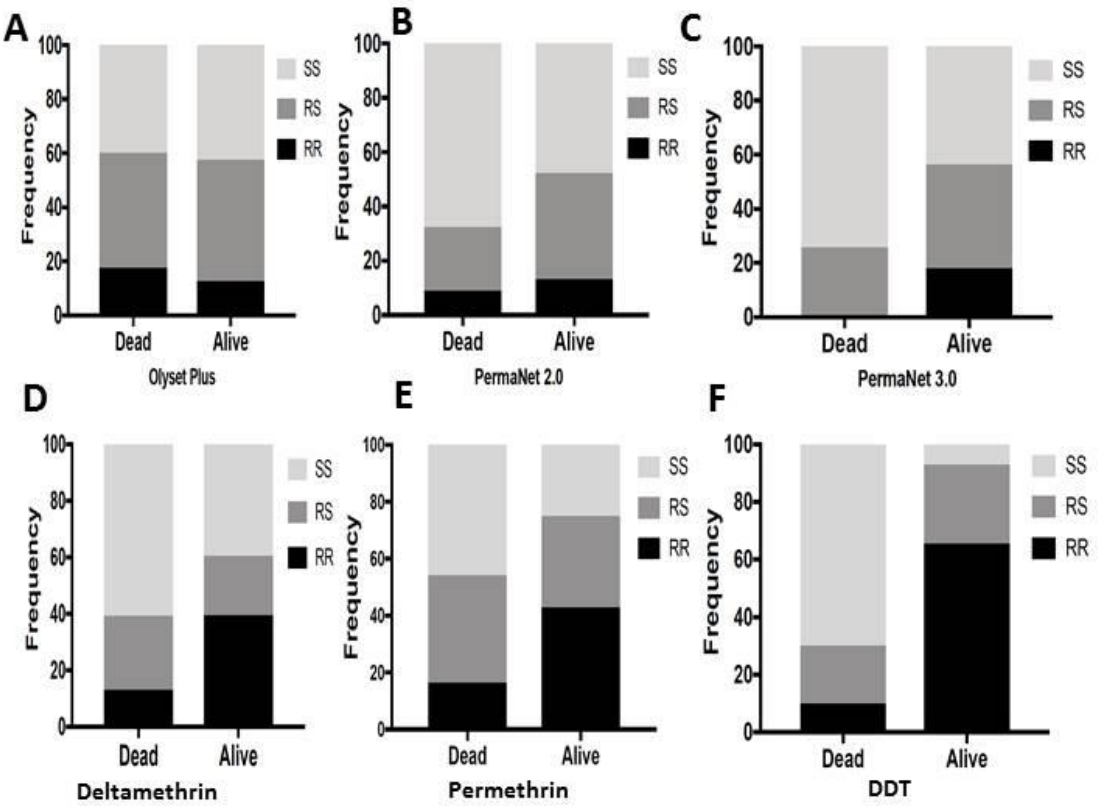

Figure 4. 11: Association between L119F-*GSTe2* mutation and ability to survive exposure to LLINs (cone assays) and WHO papers (bioassays): **A)** Genotype distribution of L119F-*GSTe2* between alive and dead mosquitoes after exposure to Olyset Plus showing no association; **B)** Genotype distribution of L119F-*GSTe2* between alive and dead mosquitoes after exposure to PermaNet 2.0 showing a significantly increased ability of resistant mosquitoes to survive (R vs S: P<0.008); **C)** Genotype distribution of L119F-*GSTe2* between alive and dead mosquitoes after exposure to PermaNet 3.0 showing a significantly greater ability of resistant mosquitoes to survive than that seen for PermaNet 2.0 (RR vs SS: OR= 30.1; P<0.0001). **D)** Genotype distribution of L119F-*GSTe2* between alive (90min) and dead (30min) mosquitoes after exposure to deltamethrin showing a significantly greater ability of homozygote resistant mosquitoes to survive (RR vs SS: OR=4.6; P<0.0001); **E)** Similarly for permethrin, homozygote resistant mosquitoes exhibit a significantly greater ability of to survive (RR vs SS: OR=4.8; P<0.0001); **F)** For DDT, a much greater ability of mosquitoes with the resistance allele to survive exposure to DDT papers (RR vs SS: OR=66.7; P<0.0001).

4.3.8 Comparative analysis of the impact of *L119F-GSTe2* mutation on the efficacy of pyrethroid-only and PBO-based nets

4.3.8.1 Impact on mortality

Due to the low number of dead mosquitoes (5), the impact of *GSTe2* resistance on mortality was not assessed for Olyset net. No correlation was observed between the presence of L119F-*GSTe2* mutation and the ability of mosquitoes to survive exposure to Olyset Plus (26 dead and 110 alive) when comparing the allelic frequency (OR=0.85; P=0.78; CI 0.45-1.59). A similar result was observed between RR vs SS (OR=0.61; P=0.39; CI 0.22-1.66), RS vs SS (OR=1.02; P=1; CI 0.56-1.8) and RR vs RS (OR=0.59; P=0.31; CI 0.21-1.68) (Figure. 4.12.A; Table 4.5).

Table 4.5: Impact of L119F-G*STe2* mutation on the ability of various insecticide-treated nets to kill mosquitoes

		Mortality		
	Genotype	OR	PV	CI
Olyset Plus	RR vs SS	0.61	>0.05	0.22-1.66
	RR vs RS	0.59	>0.05	0.21-1.68
	RS vs SS	1.02	1	0.56-1.8
	R vs S	0.85	>0.05	0.45-1.59
PermaNet 3.0	RR vs SS	3.47	>0.05	1-11.9
	RR vs RS	3.56	>0.05	1-12.7
	RS vs SS	0.97	1	0.53-1.76
	R vs S	1.42	>0.05	0.74-2.7
PermaNet 2.0	RR vs SS	/	/	/
	RR vs RS	/	/	/
	RS vs SS	1.1	1	0.6
	R vs S	1.18	>0.05	0.6-2.2

Similarly, no significant association was observed between the presence of the mutation and the ability to survive exposition to PermaNet 2.0 (24 dead and 89 alive mosquitoes) when using the allelic frequency (OR=1.18; P=0.51; CI 0.6-2.2) (Table 4.5) as well as the genotypic frequency of RS vs SS (OR=1.1; P=1; CI 0.6-6.6) (Figure. 4.12 B; Table 4.5).

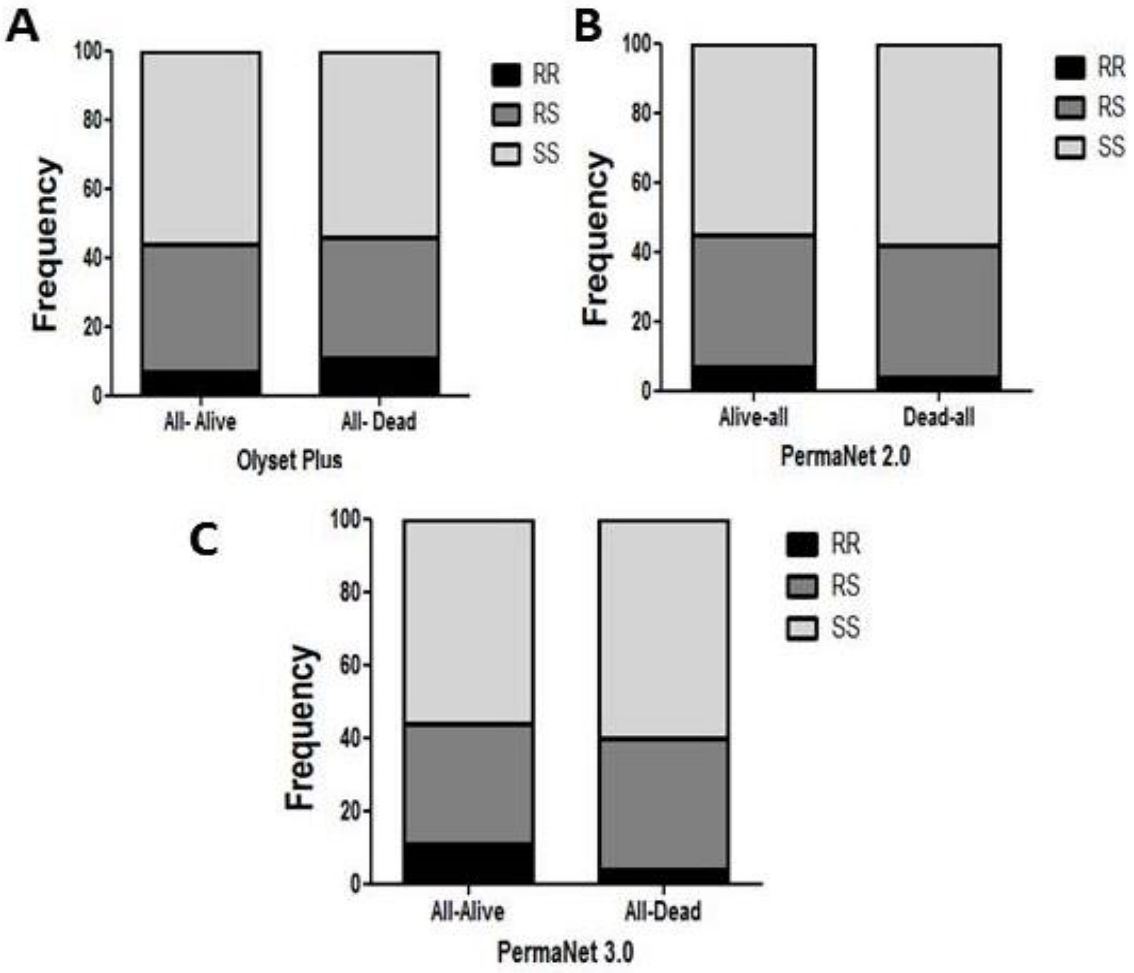

Figure 4.12: Impact of the *L119F-GSTe2* mediated metabolic resistance on bednet efficacy looking at the mortality rate after exposure to LLINs: **A)** Genotype distribution of L119F-GSTe2 between alive and dead mosquitoes after exposure to Olyset Plus showing no association; **B)** Genotype distribution of L119F-*GSTe2* between alive and dead mosquitoes after exposure to PermaNet 2.0 showing no association; **C)** Genotype distribution of L119F-GSTe2 between alive and dead mosquitoes after exposure to PermaNet 3.0 showing a trend of increased ability to survive in resistant mosquitoes but (P=0.08). Only 5 dead mosquitoes were recorded for Olyset, preventing assessment for this LLIN.

For PermaNet 3.0, no significant association was observed between the mutation and the ability to survive when using the allelic frequency (OR=1.42; P=0.65; CI 0.74-2.7) (Table 4.5) but comparing the genotypic frequency of RR vs SS provided a higher Odds ratio close to significance (OR=3.47; P=0.08; CI 1-11.9) (Figure. 4.12C).

4.3.8.2 Impact on blood feeding

For Olyset net, a significant association was observed between the 119F_*GSTe2* resistance allele and an increased ability to blood feed (21 blood-fed and 92 unfed) when comparing the allelic frequencies (OR=2; P=0.047; CI 1.06-3.7) and the genotypic frequency of RS v SS (OR=2.97; P<0.001; CI 1.6-5.3) (Figure. 4.13A; Table 4.6).

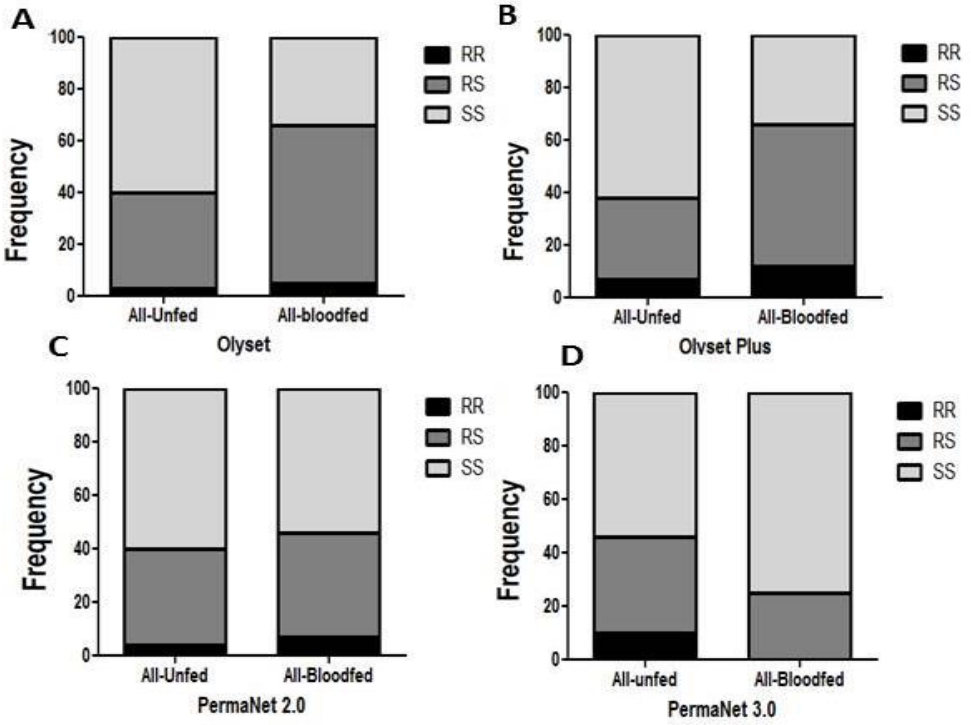

Figure 4.13: Impact of the L119F-*GSTe2* mediated metabolic resistance on bednet efficacy for blood-feeding ability: **A)** Genotype distribution of L119F-*GSTe2* between blood-fed and unfed mosquitoes after exposure to Olyset showing a significant increased ability to blood feed for resistant mosquitoes; **B)** Genotype distribution of L119F-*GSTe2* between blood-fed and unfed mosquitoes after exposure to Olyset Plus showing a significant increased ability to blood feed for resistant; **C)** Genotype distribution of L119F-*GSTe2* between blood-fed and unfed mosquitoes after exposure to PermaNet 2.0 showing an inverse marginal increased ability to

blood feed for homozygote susceptible SS compared to homozygote resistant RR (P<0.05) ; **D)** Genotype distribution of L119F-*GSTe2* between blood-fed and unfed mosquitoes after exposure to PermaNet 3.0 showing an increased ability to blood feed of susceptible mosquitoes compared to resistant mosquitoes (R vs S: OR=0.29 P=0.0014).

Table 4.6: Impact of *L119F-GSTe2* mutation on the efficacy of various bed nets to prevent blood feeding.

	Genotype	OR	PV	CI
Olyset Plus	RR vs SS	3.3	0.037	1.19-9.2
	RR vs RS	1.07	0.87	0.38-3.02
	RS vs SS	3.08	0.0016	1.67-5.66
	R vs S	2.23	0.018	1.2-4.1
	Mosquitoes in room			
	RR vs SS	12.3	0.0004	2.5-60.4
	RR vs RS	1.46	0.69	0.29-7.2
	RS vs SS	8.42	0.0001	4.37-16.2
	R vs S	4.56	0.0001	2.26-9.2
olyset	RR vs SS	2.6	0.39	0.6-11.7
	RR vs RS	0.89	0.64	0.2-3.9
	RS vs SS	2.97	0.0002	1.65-5.35
	R vs S	2	0.047	1.06-3.7
	Blood feeding in room			
	RS vs SS	3	0.0002	1.67-5.4
	R vs S	1.71	0.07	0.89-3.3
PermaNet 3.0	RR vs SS	inf		
	RR vs RS	inf		
	RS vs SS	0.5	0.04	0.26-0.92
	R vs S	0.35	0.011	0.17-0.73
	Blood feeding in room			
	RS vs SS	0.41	0.015	0.21-0.77
	R vs S	0.29	0.001	0.14-0.63
PermaNet 2.0	RR vs SS	0.4	0.04	0.1-1.56
	RR vs RS	0.48	0.33	0.12-1.9
	RS vs SS	0.8	0.65	0.47-1.5
	R vs S	1.31	0.2	0.6-2.5

For the Olyset Plus net, when considering the blood feed and unfed samples from the room only (23 blood-fed and 49 unfed), a significant association was found between L119F-*GSTe2*

and an increased ability to blood feed when comparing the allelic frequencies (OR=4.5; P=0.0001; CI 2.26-9.2). An even stronger association was observed when comparing the genotype frequencies between RR vs SS (OR=12.3; P=0.0004 CI 2.5-60.4) and RS vs SS (OR=8.42; P<0.001; CI 4.37-16.2). But no association was established when comparing RR v RS (OR=1.46; P=0.69; CI 0.29-7.2) (Figure. 4.13.B; Table 4.6). No association was also obtained when comparing RR v RS (OR=1.07; P>0.05; CI 0.38-3.02) when using the total number for blood-fed and unfed mosquitoes collected in all the compartments of the hut (33 blood-fed and 103 unfed).

For PermaNet 2.0, no association was observed when comparing the allelic frequency R vs S (OR=1.05; P=0.5; CI 0.55-2.01) (Figure. 4.13.C; Table 4.6). But an association was observed when comparing genotypic frequency RS vs SS (OR=1.4; P=0.0064; CI 0.77-2.53).

For PermaNet 3.0, a negative association was observed between the mutation and the ability to blood feed as resistant mosquitoes significantly blood-fed less than susceptible when comparing the allelic frequency (OR=0.35; P<0.05; CI 0.17-0.73) and the genotypic frequency of RS vs SS (OR=0.5; P=0.04; CI 0.26-0.92) (Figure 4.13D; Table 4.6).

4.3.8.3 Impact on exophily

For Olyset, no association was observed between the *L119F-GSTe2* mutation and exophily (67 in the room and 48 in veranda) when comparing the allelic frequency (OR=1.17; P=0.89; CI 0.6-2.2) (Table 4.6) and the genotypic frequency RS vs SS (OR=1.18; P=0.77; CI 0.6-2.1) (Figure. 4.14A; Table 4.7).

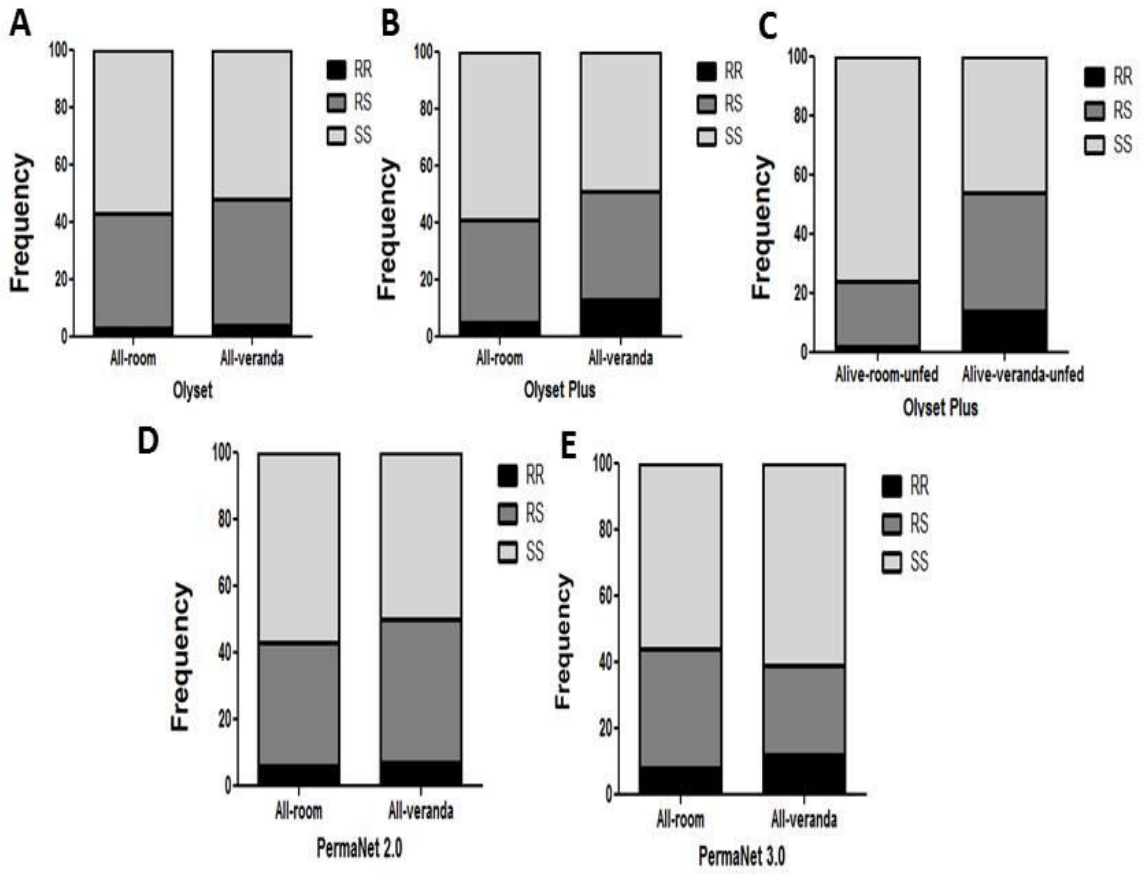

Figure 4.14: Impact of the L119F-*GSTe2* mediated metabolic resistance on bednet efficacy - exophily: **A)** Genotype distribution of L119F-*GSTe2* between indoor (Room) and outdoor (verandah) mosquitoes after exposure to Olyset showing no association; **B)** Genotype distribution of L119F-*GSTe2* between indoor (Room) and outdoor (verandah) mosquitoes after exposure to Olyset Plus showing a significant increased ability to exit the room when considering all mosquitoes; **C)** A greater association is observed with exophily with Olyset Plus when only analyzing unfed mosquitoes. **D)** Genotype distribution of L119F-*GSTe2* between indoor (Room) and outdoor (verandah) mosquitoes after exposure to PermaNet 2.0 showing a significant association (RS vs SS: OR=1.35; P<0.01) **E)** Genotype distribution of L119F-*GSTe2* between indoor (Room) and outdoor (verandah) mosquitoes after exposure to PermaNet 3.0 showing no association.

Table 4.7. Impact of L119F-*GSTe2* mutation on the efficacy of various bed nets in repellency

Exophily				
	Genotype	OR	PV	CI
Olyset Plus	RR vs SS	3.1	0.09	1.1-9
	RR vs RS	2.48	0.13	0.83-7.42
	RS vs SS	1.25	0.29	0.67-2.26
	R vs S	1.1	0.3	0.6-2.2
	unfed alive			
	RR vs SS	11.76	0.003	2.59-53.4
	RR vs RS	3.9	1	0.8-18.59
	RS vs SS	2.99	0.001	1.58-5.6
	R vs S	3.4	0.0008	1.67-6.9
Olyset				
	RS vs SS	1.18	0.77	0.6-2.1
	R vs S	1.17	0.89	0.62-2.2
PermaNet 3.0	RR vs SS	1.22	1	0.46-3.2
	RR vs RS	1.68	0.3	0.6-4.7
	RS vs SS	0.59	0.09	0.31-1.12
	R vs S	0.94	1	0.5-1.8
PermaNet 2.0	RS vs SS	1.35	0.009	0.75-2.43
	R vs S	1.22	0.77	0.65-2.3
	unfed alive			
	RS vs SS	3.37	0.0001	1.84-6.17
	R vs S	1.8	0.13	0.99-3.38

For Olyset Plus, an association was observed between the mutation and the ability to escape to the veranda when considering the allelic frequency (OR=3.4; P=0.0008; CI 1.67-6.9) (Table 4.6). An even stronger association was observed between the mutation and a preference for the veranda when comparing RR vs SS (OR=11.76; P=0.003; CI 2.59-53.4), RS vs SS (OR=2.99; P=0.0013; CI 1.58-5.65). But no significant association was observed between RR vs RS (OR=3.9; P=1; CI 0.8-18.59) (Figure. 414-C; Table 4.7).

For PermaNet 2.0, an association was observed between the L119F-*GSTe2* mutation and the ability to exit the room when comparing the genotypic frequency RS vs SS (OR=1.35; P=0.009; CI 0.75-2.3). A stronger association was observed when assessing the impact only among the unfed mosquitoes for RS vs SS (OR=3.37; P=0.0001; CI 01.84-6.17) but not at the allelic level (Fig 14-D; Table 4.7).

For PermaNet 3.0, no association was observed between the L119F-*GSTe2* mutation and a preference for the room or the veranda when comparing the allelic frequency (OR=0.94; P=1; CI 0.5-1.8) (Table 4.6). The same trend was observed when comparing the genotypic frequency RR vs SS (OR=1.22; P=1; CI 0.46-3.2), RS vs SS (OR=0.59; P=0.09; CI 0.31-1.12) RR vs RS (OR=1.68; P=0.3; CI 0.6-4.7) (Figure 4.14E; Table 4.7).

4.3.9 Correlation between L119F-*GSTe2* and mortality from cone assays

Due to the low number of dead mosquitoes obtained from the experimental huts, the impact of the L119F-*GSTe2* on the ability of mosquitoes to survive exposure to various nets was assessed using samples from cone assays. Only few dead were obtained for Yorkool and Olyset preventing the assessment of these nets.

Olyset Plus: No association was observed between the mutation and the mortality (40 dead vs 40 alive mosquitoes) at both allelic level (OR=1.04; P=1; CI 0.5-1.8) and genotypic for RR vs SS (OR=1.1; P>0.05; CI 0.4-2.7) (Figure4.11A; Table 4.4).

PermaNet 2.0: Resistant 119F-*GSTe2* mosquitoes exhibited a greater ability to survive than susceptible mosquitoes (34 dead and 46 alive) at both allelic level (OR=1.8; P<0.01; CI 0.9-3.5) and genotypic frequency RR v SS (OR=2.09; P<0.01; CI 1.1-4.2) (Figure 11B; Table 4.4).

PermaNet 3.0: Resistant 119F-*GSTe2* mosquitoes, when exposed to PermaNet 3.0, showed a greater ability to survive than susceptible mosquitoes even than with PermaNet 2.0 at allelic level (OR=3.8; P<0.001; CI 1.8-7.7). A higher correlation was further observed when comparing the genotypic frequencies for RR vs SS (OR=30.1; P<0.001; CI 3.8-234), RS vs SS (OR=2.4; P<0.01; CI 1.3-4.5) and RR vs RS (OR=12.3; P<0.001; CI 1.8-7.7) (Figure. 4.11C; Table 4.4). This result applies only to PermaNet 3.0 side net as no mosquito survived exposure to PermaNet top containing PBO.

4.4 Discussion

There is a growing concern that pyrethroid resistance will reduce the effectiveness of LLINs. However, the extent of such impact remains unknown, particularly in the context of metabolic resistance such as it is the case in the malaria vector *An. funestus*. This study has taken advantage of the dominating presence of metabolic resistance in *An. funestus* and the recent availability of a simple DNA-based diagnostic tool for GST-mediated resistance to pyrethroids, to establish the impact of metabolic resistance on the effectiveness of both pyrethroid-only and PBO-based LLINs against *An. funestus*.

4.4.1 PBO-based nets are more effective than pyrethroid-only nets against pyrethroid resistant *An. funestus* in experimental hut

4.4.1.1 Mortality

The mortality rates obtained in this study with experimental huts were generally low (7.3 to 30.1%). But such mortality levels are similar to those observed in other experimental hut studies, including in Benin (N'Guessan et al., 2010a) or recently in Burkina Faso, where the mortality rates ranged from 9.5% to 46.1% in two locations of high pyrethroid resistance in *An. gambiae* (Toe et al., 2018b). However, higher mortality has been observed for another highly resistant pyrethroid-resistant population of *An. gambiae* in Ivory Coast (Yaokoffikro) for PermaNet 3.0 (54%) and PermaNet 2.0 (34.7%) (Koudou et al., 2011) than seen here for the *An. funestus* population in Mibellon, which could potentially be associated with differences in resistance mechanisms. The low level of mortality observed is likely due to the level of pyrethroid resistance in *An. funestus* in Mibellon with permethrin mortality around 48.88 ± 5.76% and deltamethrin mortality around 38.34 ± 5.79% (Menze et al., 2018a). Nevertheless, despite the ongoing resistance, both PBO-based nets induced significantly higher mortality than the pyrethroid-only LLINs against both *An. funestus* and *Mansonia* spp. This higher mortality of PermaNet 3.0 and Olyset Plus with *An. funestus* is also in line with the cone assay results. However, PermaNet 3.0 (Top) presented higher mortality for both huts and cone assays than Olyset Plus due to higher concentration of PBO than in olyset as also seen for *An. gambiae* in Burkina Faso (Toe et al., 2018b). Overall, the higher mortality with both PBO-nets

suggests that, in areas of pyrethroid resistance, these new generation LLINs (PermaNet 3.0 and Olyset Plus) provide better protection against both malaria vectors and nuisance mosquitoes such as *Mansonia* spp..

4.4.1.2 Blood feeding

The blood-feeding rate of all the five nets, an indicator of the personal protection provided by the net, was significantly lower (≤23.8%; P<0.001) compared with the untreated control (40.8%). However, the blood-feeding inhibition rate was higher for PermaNet 3.0 (61.5%) than PermaNet 2.0 (51.4%) this was not the case between Olyset Plus (55.6%) and Olyset (62.4%). Similar results have been obtained for *An. gambiae* in Benin (Pennetier et al., 2013b) where comparable blood feeding inhibition rates were observed for Olyset Plus (85%) and Olyset (82%) despite the greater mortality rate with Olyset Plus (81%) vs 42% for Olyset. The same pattern of blood feeding inhibition was observed here for *An. funestus* and *Mansonia spp*. with PermaNet 3.0 providing the highest inhibition level in *Mansonia* spp. whereas Olyset Plus was even lower than Olyset.

Over all, synergist nets with PBO (PermaNet 3.0) tend to provide better personal protection in terms of BFI when compared to pyrethroid only nets. This may be explained by the fact mosquitoes are killed by PBO nets before blood feeding. As recommendation to the Cameroon government which relies mainly on LLINs to control malaria, the message is that even if PBO nets are performing better that pyrethroid only nets, the personal protection provided is still very low (around 30%). We recommend next generation nets like Royal guard which prevent the mosquitoes from egg laying or IRS using organophosphate.

4.4.1.3 Exophily

The excito-repellency effect of all the nets was at least twice higher compared to control, showing that *An. funestus* mosquitoes were affected by the repellent effect of pyrethroids. These results are in line with previous findings demonstrating the repellent effect of pyrethroid nets against *Anopheles* mosquitoes (Koudou et al., 2011; N'Guessan et al., 2007b). No significant difference was observed in the level of exophily between the conventional nets compared to the synergist suggesting that PBO did not impact the repellent effect of

pyrethroids. Comparing *An. funestus* to *Mansonia* spp., revealed a significant difference in terms of exophily with only 6.9% of *An. funestus* exiting the hut with untreated net whereas 42.4% *Mansonia* spp. exited the room with a control net. This is different from the previous observation in Ivory Coast, where a similar exophily (~35%) was observed for both *An. gambiae* and *Mansonia* spp. in experimental huts (Koudou et al., 2011). Furthermore, while all the 5 LLINs significantly increased the exophily rate for *An. funestus*, this was not the case in *Mansonia* spp with no significant change observed probably as *Mansonia* spp are more exophilic (Kumar et al., 1992).

4.4.2 *GSTe2* mediated metabolic resistance is reducing the efficacy of LLINs: Case from tube test

With the design of the L119F-*GSTe2* diagnostic tool (Riveron et al., 2014b), this study has investigated for the first time, the impact of GST-mediated metabolic resistance on the efficacy of LLINs. The significant correlation observed in this population between genotypes of this marker and resistance to both pyrethroids and also DDT further confirms the major role played by *GSTe2* in the resistance against these insecticides. This highlights the fact that metabolic resistance should not be tackled only by focusing on cytochrome P450s.

4.4.3 *GSTe2* mediated metabolic resistance is reducing the efficacy of LLINs: analysis done with samples from experimental hut trial

A significant association was observed between the blood-feeding ability of *An. funestus* and the *L119F-GSTe2* mutation as mosquitoes with the 119F resistance allele have significantly higher blood-feeding rates compared to those with L119 susceptible allele against Olyset Net ($P<0.001$) and Olyset plus ($P<0.001$). This suggests that L119F-*GSTe2* mutation likely contributes to an increased malaria transmission as every additional bite increases the chance of sporozoite to be passed to the populations. This is particularly a concern as 119F-RR resistant mosquitoes have also been shown to live longer (Tchouakui et al., 2019d; Tchouakui et al., 2018) and to have a greater vectorial capacity to transmit *Plasmodium* (Tchouakui et al., 2019a) . However, it is noticeable that the impact of L119F-*GSTe2* on blood-feeding ability is mainly seen for Olyset Plus and Olyset LLINs impregnated with permethrin but presented an

opposite effect for PermaNet 3.0 and 2.0 both impregnated with deltamethrin. The cause of such difference between permethrin- and deltamethrin-based nets is unclear at this point since the greater blood-feeding in the Olyset net is not associated with greater survivorship. This is particularly true as a similar significant association was observed between L119F genotypes and resistance to both insecticides in this *An. funestus* population. Further work will be needed to establish the cause of this difference between permethrin and deltamethrin nets. Similar genotype/phenotype studies performed to assess the impact of the knockdown target-site resistance (*kdr*) on resistance did not reveal such a significant association with blood-feeding potentially since *kdr* frequency was already very high in tested *An. gambiae* population (Ngufor et al., 2014a). However, the indirect comparison of *kdr* effect in two separate populations of *An. gambiae* in Benin; one with high *kdr* frequency (Ladji) and another with low frequency (Malanville) did suggest that *kdr* could also impact the efficacy of LLINs (N'Guessan et al., 2007a).

Interestingly, the L119F-*GSTe2* mutation was also associated with an increased exophily for Olyset Plus and PermaNet 2.0, suggesting that the increased expression of *GSTe2* could impact mosquito's behavior, helping them avoid exposure to insecticides. Similarly, over-expression of carboxylesterase in *Culex pipiens* (Berticat et al., 2004) has been previously associated with a behavioral change as resistant mosquitoes were found to have reduced mobility (Berticat et al., 2004) supporting that metabolic resistance could impact mosquitoes' behavior in the presence of insecticide-based interventions.

No significant association was observed between L119F-*GSTe2* and mortality in the experimental huts samples for all LLINs potentially due to the low number of dead mosquitoes, which did not provide statistical power coupled with a lower frequency of RR in this location. However, a trend was observed for PermaNet 3.0 with an increased proportion of 119F homozygote resistant mosquitoes able to survive exposure to this LLIN than both homozygote susceptible L119-SS (OR= 3.47; P=0.08) and heterozygotes (OR=3.46; P=0.1).

4.4.4 Impact of the *GSTe2* mediated metabolic resistance on the efficacy of LLIN using cone bioassays

This trend for a correlation was supported by results with samples from cone assays where a robust association was observed between L119F-*GSTe2* and the ability to survive exposure to

PermaNet 3.0 (side) (OR=30.1) and PermaNet 2.0 although at lower extent (OR=2.09). The lack of association observed with Olyset Plus could be explained by the effect of PBO, which for this net is incorporated on the entire net, whereas for PermaNet 3.0, PBO is only on the top. The PermaNet 3.0 used here is only the side without PBO. Despite that, it is surprising that a greater impact was seen on PermaNet nets as contrary results were observed for blood-feeding where *GSTe2* impacted more the Olyset nets but not the PermaNet. One reason why a greater association is found with PermaNet 3.0 (side) could be the fact that with higher deltamethrin concentration than PermaNet 2.0, mosquitoes surviving exposure to PermaNet 3.0 are those likely to be much more resistant because of a greater expression of *GSTe2* and other cytochrome P450s. So, the higher dose of deltamethrin in PermaNet 3.0 has further selected mosquitoes with 119F-*GSTe2* resistant alleles than with the lower dose in PermaNet 2.0. This is also likely the reason why a trend of a greater ability to survive was seen in the experimental hut for PermaNet 3.0 (Tableau 4.5).

It is also a reminder that mosquitoes displaying several resistance mechanisms than just P450-based resistance could avoid even PBO-based nets as when cytochrome P450 enzymes are inhibited by PBO, mosquitoes bearing other mechanisms, as in this case the 119F resistance allele, are able to better survive exposure to PBO-based nets. However, this cannot explain why such results were not observed with Olyset Plus, the other PBO-net. Further work is needed to elucidate the full interaction of *GSTe2* and LLINs. However, the spread of a *GSTe2*-mediated metabolic resistance-conferring the ability of mosquitoes to survive exposure to synergist LLINs will constitute a concern at a time when such nets are gradually being introduced to fight pyrethroid-resistant mosquitoes. GST resistance will also need to be taken into consideration than just cytochrome P450 resistance.

4.5 Conclusion

This study has revealed a loss of efficacy of LLINs against pyrethroid-resistant populations of *An. funestus* in Cameroon using experimental huts. However, PBO based nets (PermaNet 3.0 and Olyset Plus) are more efficient than conventional pyrethroid-only nets. Noticeably, the GST-mediated metabolic resistance not impacted by PBO is a severe threat to the continued effectiveness of the PBO-based LLINs, and the impact of this mechanism also needs to be taken into account to maximize the efficiency of LLINs. One option could be to generate new

mosaic LLINs, also incorporating the Diethyl Maleate (DEM) beside PBO to inhibit GST metabolic activity and further increase the efficacy of these nets.

4.6 Ethics approval and consent to participate

The national ethics committee for health research of Cameroon validates the protocol of the study N° 2016/03/725/CE/CNERSH (Appendix 6).

Chapter 5: Duplicated *CYP6P9a* and *CYP6P9b* cytochrome p450 genes significantly reduced the efficacy of bed nets against pyrethroid-resistant *Anopheles funestus* in an experimental hut trial.

5.1 Context of the study

Despite the recent reduction in Africa's malaria burden (Dhiman, 2019), insecticide resistance poses a serious threat to the continued effectiveness of the major control tools such as insecticide-treated nets (Churcher et al., 2016b). Metabolic resistance has been shown to provide a higher risk of control failure (Hemingway et al., 2016; Riveron et al., 2019), notably the one driven by cytochrome P450s. In the previous chapter, we showed that GST-mediated metabolic resistance could impact of the efficacy of bed nets, but nothing is known for P450-based resistance before this study because of the lack of suitable DNA-based markers for this resistance. Thanks to the recent design of molecular markers for the duplicated *CYP6P9a/b*, we are in a great position for the first time to assess how P450-based pyrethroid resistance is impacting the efficacy of nets, including pyrethroid-only and PBO nets.

P450s are playing a role of high importance in insecticide resistance, notably pyrethroid resistance in malaria vectors. P450s are consistently found over-expressed in relation to resistance (Amenya et al., 2008; Djouaka et al., 2008; Riveron et al., 2014a; Riveron et al., 2013; Wondji et al., 2009). Several P450s such as *CYP6P9a/b, CYP6M7, CYP6AA1, CYP6P4a/b, CYP6P2,* and *CYP6P5* have been found overexpressed in multiple resistant populations of *An. funestus* across Africa, althought most of them are still waiting to be validated (Ibrahim et al., 2016; Mulamba et al., 2014a; Riveron et al., 2014a). In *An. gambiae* from West Africa, P450s such as *CYP6P3* and *CYP6M2* were found overexpressed (Djouaka et al., 2008). *CYP6P9a/b* genes were found to be the major ones involved in pyrethroid resistance in the southern part of Africa. *CYP6P9a* is more expressed in Malawi in both Resistant (R)-Susceptibe (S) (Fold Change (FC) 62.7) and Control (C)-S (FC69.7) than in Mozambique (FC 38.5 and FC 37.9 for R-S and C-S, respectively). The *CYP6P9b* pattern was observed in Mozambique in both R-S (FC50.2) and C-S (FC64.9) (Riveron et al., 2013). Comparative functional analysis of *CYP6P9a* and *CYP6P9b* confirmed that both genes can confer resistance independently to both types I (permethrin) and type II (deltamethrin) pyrethroids (Figure 5.1) (Riveron et al., 2013).

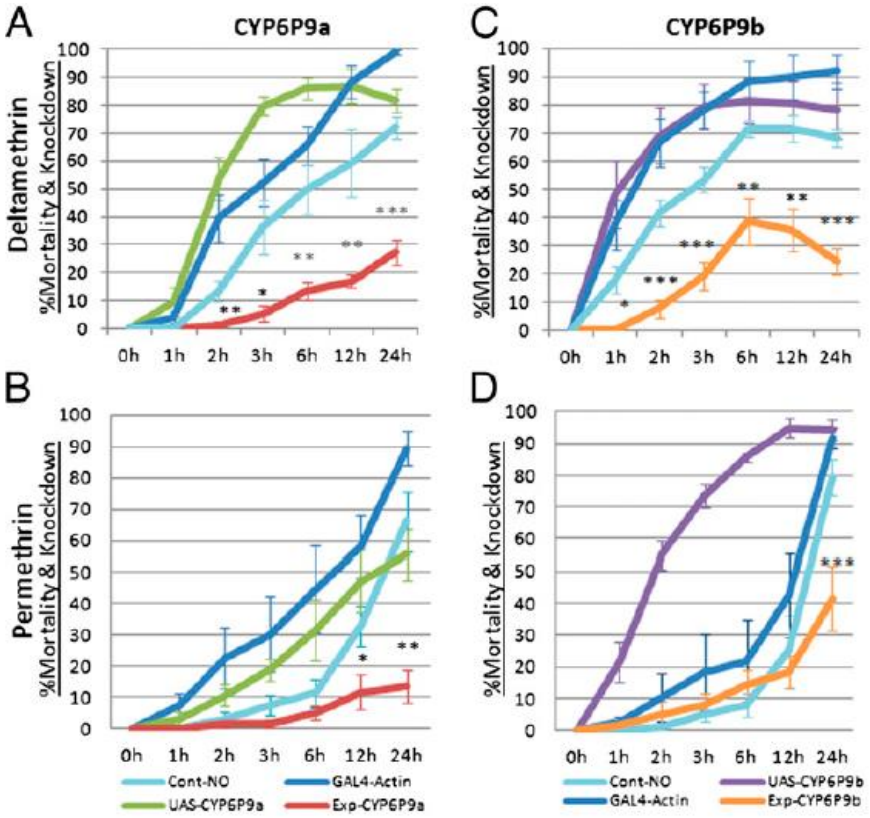

Figure 5.1: Bioassays results with transgenic strains for *CYP6P9a* and *CYP6P9b*. **A)** Test with deltamethrin on transgenic line **B)** Test with permethrin on transgenic Act5C-CYP6P9a. **C)** Test with deltamethrin on transgenic Act5C-*CYP6P9b*. **D)** Test with permethrin on transgenic Act5C-*CYP6P9b*. It is adapted from (Riveron et al., 2013).

Work started early by Amenya et al 2008 and, more importantly, Wondji et al 2009) detected the duplicated *CYP6P9a* and *b* using quantitative trait locus (QTL) mapping sequencing of Bacterial artificial chromosomes (BAC) clones and gene expression. The genomic organization of these genes and the evidence of duplication have been demonstrated. However, their expression is not always the same, so they do not always act as a single unit. This is highlighting the need of assessing the effect of these duplicated genes separately (Amenya et al., 2008; Riveron et al., 2013; Wondji et al., 2009).

Based on all these observations on *CYP6P9a/b*, further investigations indicated that mosquitoes from southern Africa consistently showed different polymorphism when compared to other Africa regions. This polymorphism included an AA insertion 8 bp upstream of a putative CCAAT box through an A/C substitution. This was used to generate the *CYP6P9a* marker. Concerning the *CYP6P9b*, an indel of 3 bp (AAC) was consistently observed with a

deletion in the resistant FUMOZ and presence in susceptible FANG. Based on this, a DNA-based diagnostic assay to detect the *CYP6P9b*-mediated resistance was designed (Mugenzi et al., 2019; Weedall et al., 2019).

Elucidating how insecticide resistance is changing the effectiveness of LLINs using these markers is key to established appropriate insecticide resistance management strategies to prevent the resurgence of malaria (Hemingway, 2017). The probability that increasing insecticide resistance will affect the efficacy of these interventions cannot be ignored (Churcher et al., 2016b; Strode et al., 2014; WHO, 2018). However, assessing the impact of metabolic resistance is critical since it can lead to control failure. Some malaria vectors such as *An. funestus* where pyrethroid resistance is mainly metabolic (Irving and Wondji, 2017) provide a suitable opportunity to assess this. The impossibility to evaluate the impact of insecticide resistance on the effectiveness of control tools such as LLINs due to the lack of molecular marker for metabolic resistance in all the major malaria vectors in Africa, including *An. gambiae* and *An. funestus* is a major obstacle to develop evidence-based resistance management strategies.

Using theses markers, a significant association was observed between permethrin resistance and the *CYP6P9a/b* genotype. For *CYP6P9b* as example, a significant high correlation was observed when comparing the surviving ability RR vs. SS (OR = infinity; P = 1.1 × 10−50) and RS vs. SS (OR = 715; P = 2.4 × 10−45). These data validating of the role of *CYP6P9a/b* in the resistance is the reason why we have relied on such test for our study investigating the impact of insecticide resistance.

This study aims to assess the impact of P450- resistance on the performance of pyrethroid-only and PBO-based nets against the pyrethroid-resistant *An. funestus* mosquitoes using a hybrid laboratory strain. This study revealed that P450-based metabolic resistance significantly reduces the effectiveness of both LLIN types but more for pyrethroid-only LLINs.

5.2 Methods

5.2.1 Location of the experimental station used for the releasing

The experimental station is located at Mibellon (6°4'60''N, 11°30'0''E), a village in Cameroon located in the Adamawa Region; Mayo Banyo division and Bankim Sub-division. (See method Chapter 2, Section 2.1.1).

5.2.2 Laboratory strain: Fumoz/Fang crossing

The crossing was done using two laboratory strain. *An. funestus* from Angola (FANG) and *An. funestus* from Mozambique (FUMOZ). During rearing, the pupae of each strain were kept individually in Falcon tubes (15ml) locked with a piece of cotton. After the emergence of pupae in the Falcon tube, the males and the females were separated. 50 males and 50 females that emerged separately were then released in a cage for the crossing. More details about the strains are available in the paragraph 5.2.2.1 and 5.2.2.2

5.2.2.1 FUMOZ

FUMOZ is a resistant laboratory strain selected from *An. funestus* s.s. Resistance in that strain has been selected over generations after exposure to 0.1% lambda-cyhalothrin. WHO tube assays in successive generations F2 to F4 showed increased resistance with each successive generation (Figure 5.2) (Hunt et al., 2005).

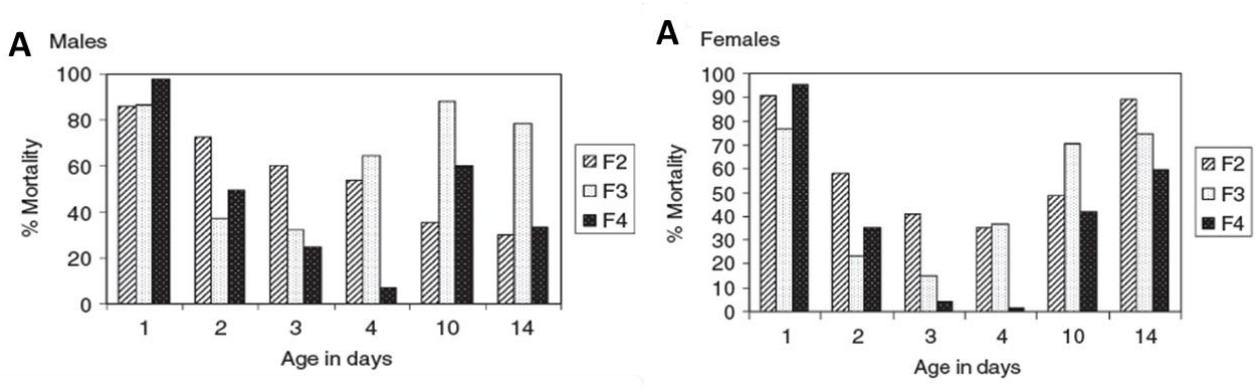

Figure 5.2: Recorded mortalities following 60-min exposure and 24-h post-exposure observation for three generations of *An. funestus* selected after exposure to 0.1% lambda-cyhalothrin. **A)** Males: F2 (n = 1592), F3 (n = 1023) and F4 (n = 910). **B)** Females: F2 (n = 1566), F3 (n = 1020) and F4 (n = 1037). Adapted from (Hunt et al., 2005).

5.2.2.2 FANG

FANG is a susceptible laboratory strain selected from *An. funestus* s.s and colonized from Calueque in southern Angola since 2002 as mentioned by (Wondji et al., 2005b).

5.2.2.3 Crossing

The hybrid strain was obtained after a crossing of FUMOZ (resistant lab strain with resistance mediated by *CYP6P9a* and *b* at the fixed level) and Fang (susceptible lab strain). After the initial F1 generation obtained from the reciprocal crosses of 50 males and 50 females of both strains, the hybrid strain was reared to F_5 and F_6 generation, which were used for the releasing in the huts (Figure 5.3). The Crossing was done in both direction, female FUMOZ x male FANG (Figure 5.3.A) and female FANG x male FUMOZ (Figure 5.3.B).

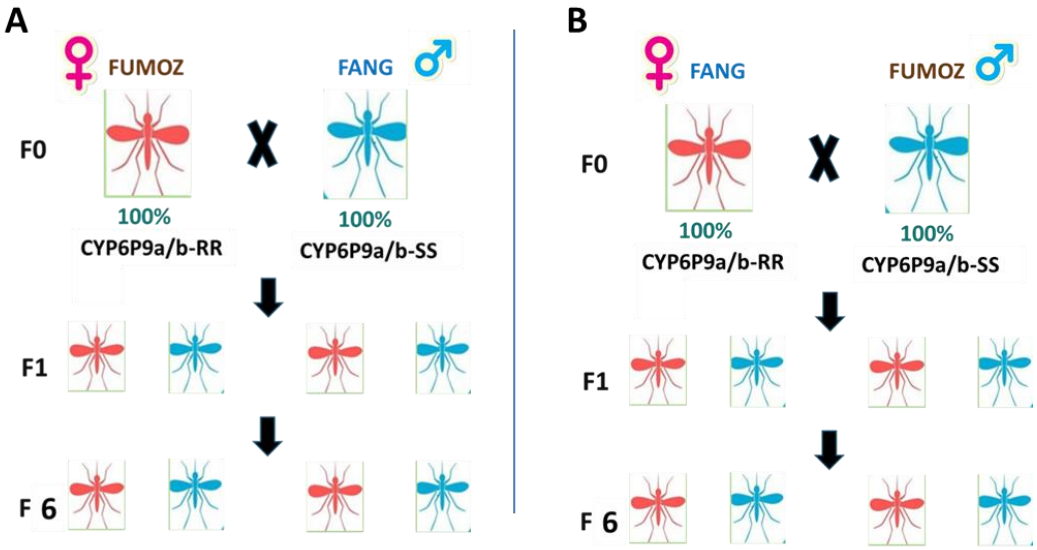

Figure 5.3: reciprocal crossing between the highly pyrethroid-resistant strain FUMOZ-R (originally from Mozambique) and the fully susceptible FANG strain (originated from Angola). **A)** Female FUMOZ crosses with male FANG. **B)** Male FUMOZ crosses with female FANG.

5.2.3 Experimental hut design

The huts were built following the prototype recommended by WHO for the West African region (See Chapter 2, Section 2.1.1).

5.2.4 Hut treatment / Arm comparison

During the experimental hut trial, four insecticide treated nets were used: Olyset, Olyset Plus, PermaNet 2.0 and PermaNet 3.0. An untreated nets were used in the control hut.

5.2.5 Bioassays

Cone bioassays were performed at the beginning of the study using susceptible laboratory strain *Anopheles coluzzii* from Ngousso. This was done to confirm the quality of the five bed nets involved in the study. Cone bioassays were also done in the insectary using the reciprocal hybrid strain to confirm the role of *CYP6P9a/b* in resistance. A fragment of PermaNet 2.0, PermaNet 3.0, Olyset, and Olyset Plus was used for the cone test. See details of the protocol in chapter 2. Five batches of 10 unfed females, aged 2-5 days old, were exposed to each bed net using WHO cone assays for three minutes. They were then transferred into the holding paper cup containers. The knockdown was checked after 60 min and the mortality after 24 hours. WHO tube assays were also done, dead/ alive mosquitoes were obtained and genotyped to further to confirm the role of *CYP6P9a/b* in resistance.

5.2.6 Experimental hut trial

The experimental hut trial was done in this case not using the wild population. The hybrid lab strain Fumoz-Fang was reared in the insectary and released in the treated hut every evening. A number varying from 50 to 100 were released every night and collected in the morning.

5.2.6 1 Latin square design rotation and volunteer sleepers

Usually, the Latin square design rotation was applied to correct the specific attractiveness that some huts may have due to their position (WHO, 2013b). Because a fixed number of mosquitoes were released every night in the huts selected for the study, the rotation was not necessary in this case. Six volunteering men were selected to sleep in the room from 20:00 GMT to 5:00 GMT in the morning. The sleepers were rotated so that each sleeper would have spent one night in each hut at the end of the week. The sleeper's rotation was done to correct any bias due to any specific attractiveness from the sleepers.

5.2.6 2 Releasing and recapture

Every test night, each volunteer was installed in the different hut selected for the study. 50 to 100 mosquitoes from the lab strain Fumoz/Fang were equally released in the huts. Mosquitoes were collected every morning, using hemolysis tubes from: (i) inside the nets, (ii) in the room: floor, walls and roof, and (iii) in the veranda exit trap. Mosquitoes collected from each compartment were kept separately in a bag to avoid any mixing between samples from different compartments. (Figure 2.2). Samples were transferred to the laboratory for identification. After identification, samples were then classified as dead, alive, blood-fed, or unfed (Appendix 1). The ‘alive’ samples were kept in the paper cup and provided with sugar solution for 24 hours and mortalities monitored. Dead mosquitoes were then kept adequately in labelled Eppendorf tubes with Silicagel for dead and live mosquitoes stored in RNA later (Figure 2.3).

5.2.7 Bed nets performance assessment

The performance of the bed nets was expressed relative to control (untreated nets) (Appendix 1) in term of:

i-Deterrence/entry rate: the reduction in hut entry relative to control. Deterrence (%) = 100 x (Du – Dt) / Du, where Du is the total number of mosquitoes found in the untreated hut (control), and Dt is the total number of mosquitoes in the treated hut.

ii-Entry rate (%) = 100 x (Ht/Hn) where Ht is the total number of mosquitoes found in the hut, and Hn is the total number of mosquitoes collected in all the six huts.

iii-Exophily: the proportion of mosquitoes found exited in the veranda trap. Exophily (%) = 100 x (Ev/Et) where Ev is the total number of mosquitoes found in veranda, and Et is the total number of both inside the hut and veranda.

iv-Blood feeding rate (BFR). This rate was calculated as follows: Blood feeding rate = (N mosquitoes fed) x 100 / total N mosquitoes. Where "N mosquitoes fed" was the number of mosquitoes fed, and "total N mosquitoes" was the total number of mosquitoes collected.

v-Blood-feeding inhibition (BFI): the reduction in blood-feeding in comparison with the control hut. Blood feeding inhibition is an indicator of personal protection (PP). More precisely, the personal protection effect of each bed net is the reduction of blood-feeding percentage induced by the net when compared to control. The protective effect of each bed net can be calculated as follows: Personal protection (%) = 100 x (Bu – Bt) / Bu, where Bu is the total number of blood-fed mosquitoes in the huts with untreated nets and Bt is the total number of blood-fed mosquitoes in the huts with treated nets (WHO, 2013).

vi-Immediate and delayed mortality: the proportion of mosquitoes entering the hut that is found dead in the morning (immediate death) or after being caught alive and held for 24 h with access to sugar solution (delay mortality) (WHO, 2013). In this study, we focused on the overall mortality calculated as follows: Mortality (%) = 100 x (Mt/MT) where Mt is the total number of mosquitoes found dead in the hut and MT is the total number of mosquitoes collected in the hut.

5.2.8 Confirming role of *CYP6P9a/b* in conferring resistance in hybrid strains

Before assessing the impact of *CYP6P9a/b* on the effectiveness of LLINs using the experimental huts, the role of *CYP6P9a/b* in conferring resistance was evaluated in laboratory conditions via WHO tube tests and cone tests.

5.2.8.1 Bioassays

WHO bioassays were conducted with 0.75% permethrin and 0.05% deltamethrin for 30 min and 90 min. For each insecticide, four replicate of 25 mosquitoes from the hybrid strains were used (see more details regarding the bioassays in Chapter 2). Alive mosquitoes after 90 min of exposure and those dead after 30 min of exposition were then genotyped to establish the association between the *CYP6P9a/b*-R resistant allele and the ability of mosquitoes to survive to these insecticides.

5.2.8.2 Cone assays

Cone test bioassays were conducted with a fragment of Olyset, Olyset Plus, PermaNet 2.0 and PermaNet 3.0 (sides and top). The resistant hybrid strains FUMOZ-FANG were used (see more details regarding cone test protocol in Chapter 2). Alive and dead mosquitoes obtained after exposure were then genotyped. The association between the *CYP6P9a/b* resistant allele and the ability of mosquitoes to survive to these insecticides was established.

5.2.9 Impact of the duplicated P450 gene, *CYP6P9a* and *CYP6P9b* on the performance of bed nets

The samples collected during the investigation on the performance of nets were grouped in several categories: dead, alive, blood fed, unfed; room and veranda. The *CYP6P9a* and *CYP6P9b* were genotyped in each group using the RFLP-PCR. This allowed a direct measure of the relative survival and feeding success of resistant and susceptible insects in the presence of different bed nets.

5.2.9 1 Genotyping of *CYP6P9a/b* using sample from the laboratory

The *CYP6P9a* and *CYP6P9b* were genotyped using dead and alive mosquitoes obtained after cone test and WHO tube tests with the hybrid strain to confirm the role of *CYP6P9a/b* in the resistance observed. A PCR–RFLP (Mugenzi et al., 2019; Weedall et al., 2019) was used to detect the three genotypes for each gene as detailed in chapter 2 methodology.

5.2.9 2 Genotyping of *CYP6P9a/b* using sample from experimental hut

The *CYP6P9a* and *CYP6P9b* were also genotyped to assess how these P450 genes are impacting the performance of bed nets. A PCR–RFLP (Mugenzi et al., 2019; Weedall et al., 2019) was used to detect the three genotypes for each gene, as detailed in Chapter 2 methodology.

5.2.10 Statistical analysis

Experimental hut trial: To calculate the proportion of each entomological outcome and the level of significance between the treatments and the control, the XLSTAT software (Addinsoft, Berkeley, CA, USA) was used, as done previously (Badolo et al., 2014; Chouaibou et al., 2006). The numbers of mosquitoes collected in the huts with different treatments were analyzed by negative binomial regression. The effects of the treatments on each of the main proportional entomological outcomes (exophily, blood-feeding, and mortality) were assessed using binomial generalized linear mixed models (GLMMs) with a logit link function, fitted using the 'lme4' package for R 3.6. (R Development Core Team, 2019) (Ngufor et al., 2016a; Toe et al., 2018). A separate model was fitted for each outcome and each mosquito species. In addition to the fixed effect of each treatment, each model included random effects to account for the following sources of variation: between the huts used in the studies; between the sleepers who slept in the huts; between the ten weeks of the trial (Appendix 4).

Test of association between the genes (*CYP6P9a/b***) and the entomologicaloutcomes:** To investigate the association between the genes and the ability of the mosquitoesto survive, blood feed or escape, Vassar stats were used to estimate the odds ratio based ona fisher exact probability test with a 2x2 contingency table.

Hut effect analysis: The one-way analysis of variance (ANOVA) using Prism 7.0 (GraphPad, San Diego, CA, USA) was used to determine whether there were any statistically significant differences between the means of the mosquitoes collected from the six huts (Appendix 5). For all the analyses in this study, an alpha of 0.05 was used as the cut off for significance.

Construction of graph: Most of the graphs in this document were constructed using the software Prism 7.0 (GraphPad, San Diego, CA, USA).

5.2.11 Ethical clearance

The national ethics committee for health research of Cameroon approved the protocol of the study (ID:2016/03/725/CE/CNERSH/SP) (Appendix 6). Written, informed, and signed consent was obtained from sleepers before starting the trials. The consent form provided all the information and the evaluation process about the study. Information was translated into the local language when needed. All the volunteers involved in the study were followed-up and treated when showing malaria symptoms.

5.3 Results

5.3.1 Susceptibility test

Susceptibility profiles of the FUMOZ-FANG and FANG-FUMOZ strains: The WHO tube test performed with the reciprocal FANG-FUMOZ strains revealed that both hybrid strains were resistant to pyrethroids and carbamates and moderately resistant to DDT (93% mortality) (Figure 5.4). However, a significant difference was observed for deltamethrin with a higher mortality rate recorded for the strain generated from crossing females FUMOZ_R to males FANG (48.5%) than in the strain from females FANG and males FUMOZ_R (77.3%). In addition it was also noticed that FUMOZ (females) x FANG (males) were more resistant to deltamethrin than permentrin (48.5% vs 80.7%) (Figure 5.4). Based on the fact that both reciprocal strains are resistant to pyrethroid, only FUMOZ-FANG crossing will be used in this study.

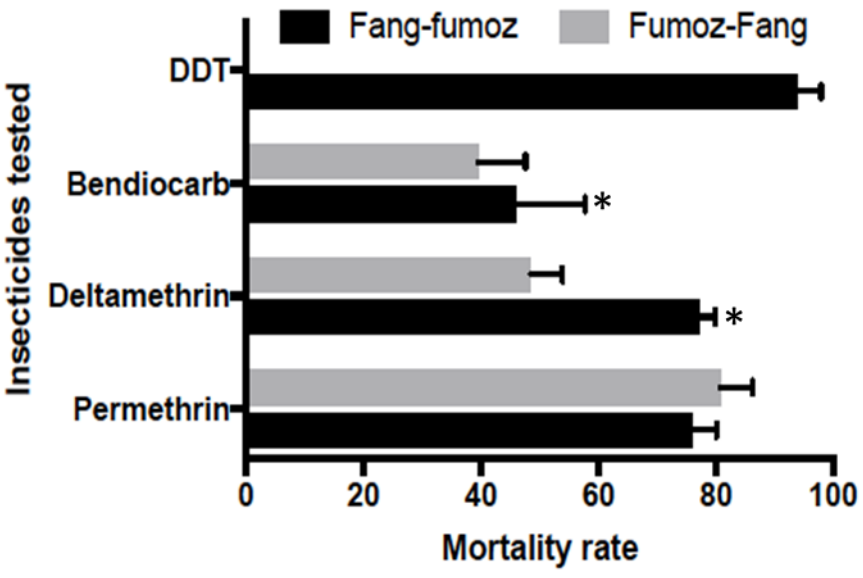

Figure 5.4: Susceptibility profile of both reciprocal the strain FUMOZ-FANG and Fang-Fumoz to insecticides. Recorded mortalities following 60-min exposure of both reciprocal strains to Permethrin, Deltamethrin, Bendiocard, and DDT.

Susceptibility profiles of the FUMOZ-FANG strains obtained after 30 and 90 min of exposition: The bioassays performed with the reciprocal FANG-FUMOZ strains revealed that the hybrid strains were resistant to permethrin with mortality of 81.30±6.59 % and 39.28±1.59% respectively for 90 min and 30 min. Resistance was also observed with deltamethrin with a mortality of 86.36±3.11 % and 42.28±2.48 %, respectively, for 90 min and 30 min. (Figure 5.5).

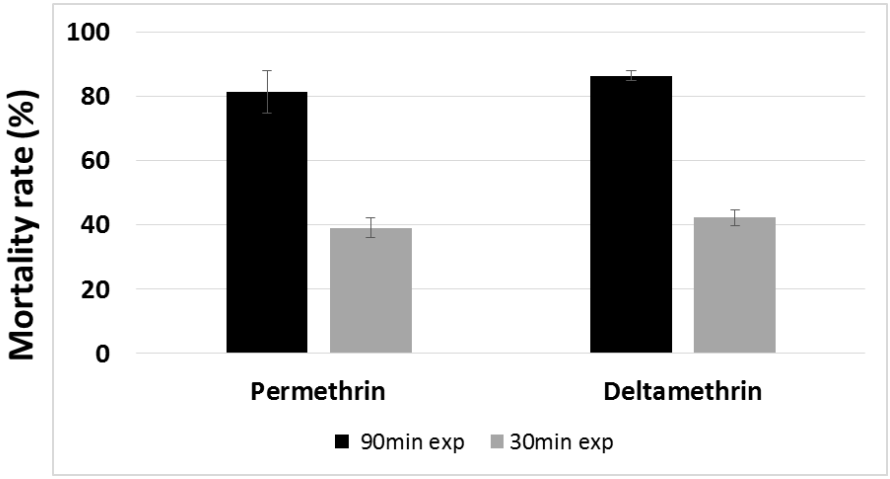

Figure 5.5: Susceptibility profile of the hybrid strain Fumoz-Fang to insecticides. Recorded mortalities following 30-min and 90-min exposure of the hybrid strain Fumoz-Fang to pyrethroid type I (permethrin) and to type II (deltamethrin). Data are shown as mean ± SEM.

5.3.2 Quality control and performance of the nets in the laboratory against the hybrid strain

All the four nets used in the study were first exposed to the susceptible lab strain Kisumu for quality control. The mortality for Olyset, Olyset Plus, Permanet2.0 and PermaNet 3.0 side and top was 100% (Figure 5.6). In the laboratory, *Anopheles funestus* from the hybrid strain was exposed to bed nets using WHO cone assay. Olyset and Olyset plus showed mortality of 30.09±11.52 %, and 48.91±14.78 % respectively. PermaNet 2.0 showed 31.07±3.75% mortality and PermaNet 3.0 side and PermaNet 3.0 top gave 40.66 ±6.69 % and 100±0 % mortalities respectively (Figure 5.6). Overall, PBO-synergist nets were more effective than pyrethroid-only nets. (Figure 5.6).

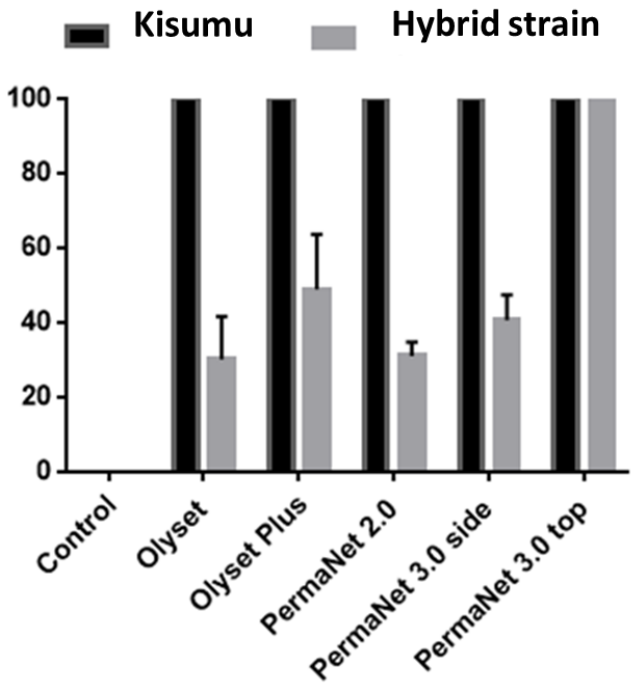

Figure 5.6: Assessment of net quality: Recorded mortalities following 3 min exposure to various nets with cone assays using Kisumu, *An. gambiae* susceptible lab strain and recorded mortalities following 3 min exposure by cone using *An. funestus* (crossing Fumoz-Fang-F5).

5.3.3 Mosquitoes released during the study.

A total number of 1385 mosquitoes from the hybrid strain FUMOZ-FANG, generation F5-F6 were released during the study. 356 samples were released in the control hut, 270 in the hut

treated with PermaNet 2.0, 322 in the hut treated with PermaNet 3.0, 224 for Olyset and 213 for Olyset Plus.

5.3.4 Validating the role of *CYP6P9a/b* in pyrethroid resistance in the hybrid FUMOZ-FANG strains before any field studies

5.3.4.1 Validating the role of *CYP6P9a*_R in pyrethroid resistance in the hybrid FUMOZ-FANG before the experimental hut

Using the hybrid FUMOZ/FANG strains, the role of the *CYP6P9a*_R allele in the observed pyrethroid resistance was confirmed. WHO bioassays showed a mortality of 39.0% and 42.3% after 30 minutes' exposure and mortality rates of 81.3% and 86.3% after 90 minutes' exposure, respectively to permethrin and deltamethrin (Figure 5.7). The odds ratio of surviving exposure to permethrin when homozygous for the resistant *CYP6P9a*_R allele (RR) was high at 693 (CI 88-5421; P<0.0001) compared to the homozygous susceptible (SS) (Figure 5.7). The OR was 131 (CI 27-978; P<0.0001) when comparing RR to RS, indicating that the resistance conferred by CYP6P9a is additive.

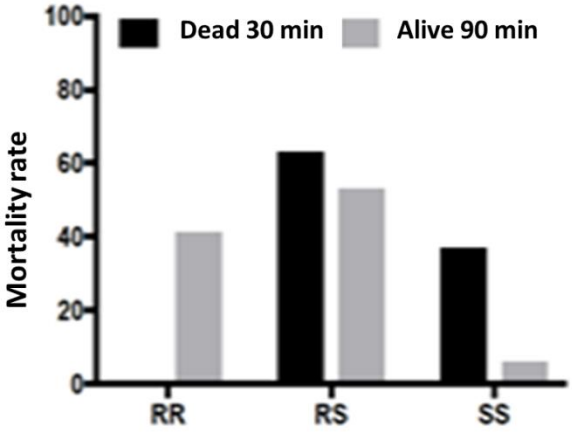

Figure 5.7: Role of *CYP6P9a* in pyrethroid resistance using Fumoz-Fang, the hybrid lab strain. Distribution of the *CYP6P9a* genotypes according to resistance phenotypes.

5.3.4.2 Validating the role of *CYP6P9b*_R in pyrethroid resistance in the hybrid FUMOZ-FANG strains before the experimental hut

To confirm the ability of *CYP6P9b*_R marker to foresee pyrethroid resistance phenotype in *An. funestus*, The hybrid strain FUMOZ-FANG at the F6 generation. The genotyping of the same strain that was used previously to validate the *CYP6P9a* (Weedall et al., 2019) shown that those remaining alive after exposure to permethrin for 180 minutes are mainly homozygote resistant (20.83%) and heterozygotes (75%) with only two being homozygote susceptible. Among the 47 mosquitoes found dead after 30 min exposure to permethrin (highly susceptible), 97.87% were found homozygote susceptible, and one remaining was a heterozygote (Figure 5.8). A strong association was perceived between permethrin resistance and the *CYP6P9b* genotype when comparing RR vs SS (OR= infinity; P=1.1x10^{-50}) and RS vs SS (OR=715; P=2.4x10^{-45}) (Figure. 5.8).

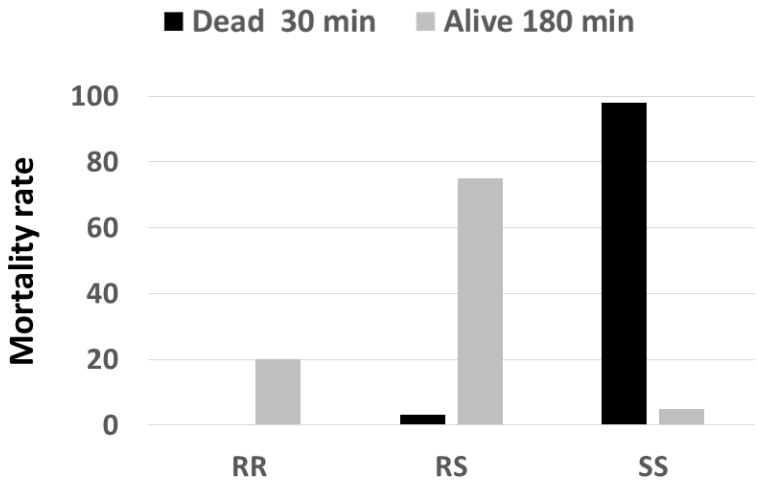

Figure 5.8: Role of CYP6P9b in pyrethroid resistance. Distribution of the *CYP6P9b* genotypes according to resistance phenotypes.

5.3.4.3 Validation of the role of *CYP6P9a* and *CYP6P9b* in conferring resistance using samples from cone assays

Using dead and alive obtained after cone test in the laboratory condition, CYP6P9a homozygous resistant mosquitoes (RR) and heterozygous (RS) were significantly more able to survive exposure to PermaNet 2.0 PermaNet 3.0 side; Olyset and Olyset Plus than homozygote susceptible (Table 5.1; figure 5.9) For example Olyset show a strong association between the gene and the ability to survive when comparing RR v SS (OR=35.1; P<0.00001) and RS v SS (OR=34.6; P<0.00001) for the *CYP6P9a*. The same trend when looking at the allelic frequency R v S (OR=7.7; p<0.05) (Table 5.1; figure 5.9). Concerning the *CYP6P9b* homozygous resistant

mosquitoes (RR) and heterozygous (RS) were significantly more able to survive exposure. A strong association between the gene and the ability to survive when comparing RR v SS (OR=32.4; P<0.00001) and RS v SS (OR=97.3; P<0.00001) (Table 5.1; figure 5.9).

Table 5.1: *CYP6P9a* and *CYP6P9b* genes reducing the efficacy of Olyset Net against pyrethroid resistant *An. funestus* after cone assay in the laboratory.

		OR	P value	CI	OR	P value	CI
Mortality							
Olyset			*CYP6P9a*			*CYP6P9b*	
	RR vs SS	35.1	<0.00001	11.0-111.2	32.4	<0.00001	10.8-97.3
	RS vs SS	34.6	<0.00001	14.4-83.52	97.3	<0.00001	34.2-277.1
	RR vs RS	0.9	1.0	0.3-2.8	3.0	>0.05	0.9-9.4
	R vs S	7.7	<0.001	3.9-15.2	11.1	<0.001	5.2-23.4
Olyset Plus	RR vs SS	6.4	<0.001	2.3-17.4	9.7	<0.001	3.2-29.1
	RS vs SS	7.2	<0.001	2.6-20.14	17.9	<0.001	5.7-55.8
	RR vs RS	1.1	>0.05	0.6-2.0	1.8	>0.05	0.9-3.5
	R vs S	2.0	0.02	1.1-3.7	2.2	<0.01	1.2-4.1
			CYP6P9a			*CYP6P9b*	
PermaNet 2.0	RR vs SS	239.0	<0.001	48.3-1182.9	NA	NA	NA
	RS vs SS	63.0	<0.001	15.6-254.1	NA	NA	NA
	RR vs RS	3.7	<0.01	1.3-10.3	7.5	<0.001	2.5-21.7
	R vs S	10.2	<0.0001	5.2-19.8	26.9	<0.05	12.6-57.1
PermaNet 3.0	RR vs SS	81.0	<0.001	20.5-319.7	NA	NA	NA
	RS vs SS	6.5	<0.01	1.7-25.4	NA	NA	NA
	RR vs RS	12.0	<0.001	5.5-25.9	15	<0.05	6.9-32.7
	R vs S	9.5	<0.001	5.2-19.8	11.9	<0.05	5.3-22.9

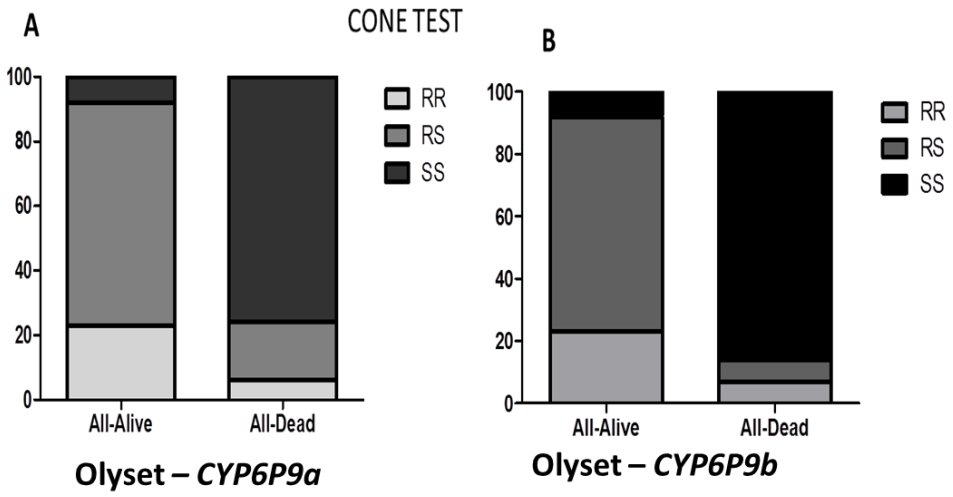

Figure 5.9: Association between the duplicated *CYP6P9a/b* genes and the ability to survive exposure to Olyset Plus net after cone assays: **A)** Genotype distribution of *CYP6P9a* between alive and dead mosquitoes after exposure to Olyset showing strong association (RR vs SS: P<0.001); **B)** Genotype distribution of *CYP6P9b* between alive and dead mosquitoes after exposure to Olyset showing strong association (RR vs SS: P<0.001).

5.3.4.4 Validating the role of *CYP6P9a* combined to *CYP6P9b* in pyrethroid resistance in the hybrid FUMOZ-FANG strains before the experimental hut

The role of *CYP6P9b* and *CYP6P9a* in pyrethroid resistance assessed revealed that both genes are combined additively to increased the level of resistance. All mosquitoes carrying the double homozygote resistant genotypes (RR/RR) were all alive after 180 minutes exposure. However, only half of the homozygote resistant for *CYP6P9b* were also homozygote resistant for *CYP6P9a* with the other half only heterozygotes for *CYP6P9a* (Figure 5.10.A). Double heterozygote mosquitoes (RS/RS) were mostly alive (66.7%) with 10.4% RR/RR and 16.7% RR/RS (Figure 5.10.B) suggesting possible fitness cost related with having double resistance alleles for both genes. All the dead mosquitoes were found to be RR/RR, RR/RS or RS/RS; this indicates a solid association with permethrin resistance with high OR (infinity; P<0.0001) (Figure 5.10.B).

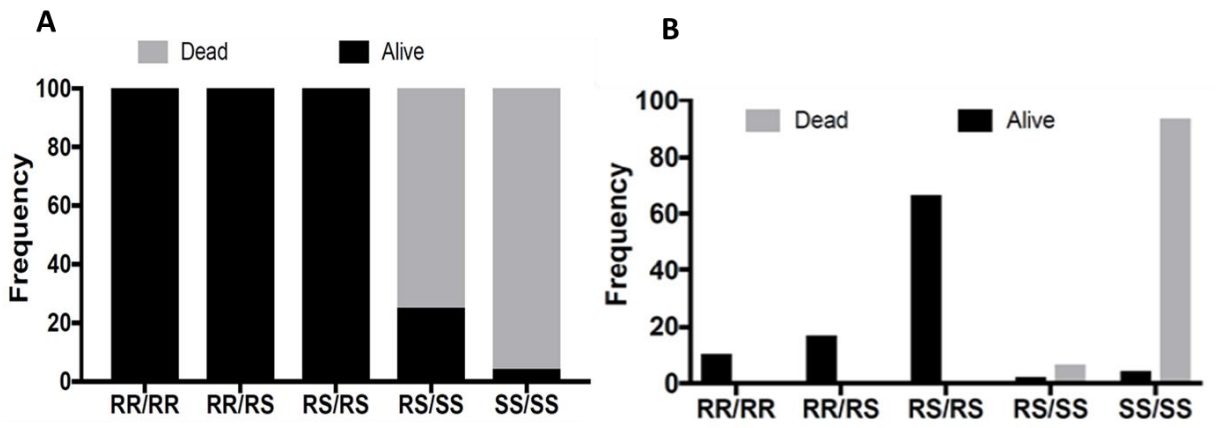

Figure 5.10: *CYP6P9a* and *CYP6P9b* combined additively to confer a higher level of resistance**.** XX/YY is the combination of both genes : XX represent the *CYP6P9a* and YY the *CYP6P9b* **A)** Distribution of the combined genotypes of both *CYP6P9a* and *CYP6P9b,* showing that both genotypes combined to increase the pyrethroid resistance. **B)** Frequency of various combinations of genotypes between *CYP6P9a* and *CYP6P9b* in F8 hybrid strain (FUMOZ_X_Fang) suggesting independent segregation of genotypes of both genes in this hybrid strain.

5.3.5 Bed nets performance with experimental hut

5.3.5.1 Mortality

Analysis of mortality rates revealed very high mortality of the hybrid FUMOZ-FANG strain against PBO-based nets with mortality of 99.1% for Olyset Plus and 98.8% for PermaNet 3.0. In contrast, lower mortality was observed for the pyrethroid-only nets with mortality rates of only 56.7% for Olyset and 31.4% for PermaNet 2.0. Very low mortality was observed in the untreated control net (9.9%) (Figure 5.11; Table 5.2). For the entire study, the PBO – based nets were found to be more effective with mortality closed to 100 % when compared to conventional nets. This is certainly due to the fact that in the hybrid strain the resistance is driven by the P450.

5.3.5.2 Blood feeding

The blood-feeding rate did not significantly differ when comparing control (7.8%) to Olyset (11.6% P>0.05) and Olyset Plus (5.6%; P>0.05), but the blood-feeding was higher in Olyset compared to Olyset Plus (11.6% v 5.6%; P=0.013) (Figure 5.11 and Table 5.2).

The blood-feeding was high in PermaNet 2.0 compared to PermaNet 3.0 (10.2% v 6.8%) but not in a significant way (P=0.1). PermaNet 3.0 provided a higher personal protection compared to PermaNet 2.0 but not significantly (79.04 % vs 73.34%; P= 0.09)(Figure 5.11). The same trend was already observed with Olyset and Olyset Plus. This is because the synergist net was faster in killing the mosquitoes before they blood fed.

For PermaNet Nets, blood feding was significant reduced in PermaNet 2.0 (10.2%; P<0.0001) and PermaNet 3.0 (6.8%; P<0.0001) when compared to control (27.8.8%) (Figure 5.11 and Table 5.2).

Table 5.2: Performance of Olyset, Olyset Plus, PermaNet 2.0 and PermaNet 3.0 Plus against *An. funestus* females (crossing Fumoz-Fang-F5) in experimental hut trial

Treatments						
	Control	**PermaNet 2.0**	**PermaNet 3.0**	**Control**	**Olyset**	**Olyset Plus**
Total	366	274	322	141	224	213
Exophily%	11.5	15.0	15.8	13.5	23.7*	7.5*
blood fed (%)	28.7	10.2*	6.8*	8.5	11.6^{NS}	5.6^{NS}
blood feed inhibition. (%)	0.0	64.38	76.18	0.0	NS	NS
Personal protection (%)	0.0	73.34	79.04	0.0	-53 ^{NA}	00 ^{NA}
Mortality (%)	9.9	31.4*	98.8*	9.9	56.7*	99.1*

NA: Sample size too low in the control to make the calculation. NS: Non Significant

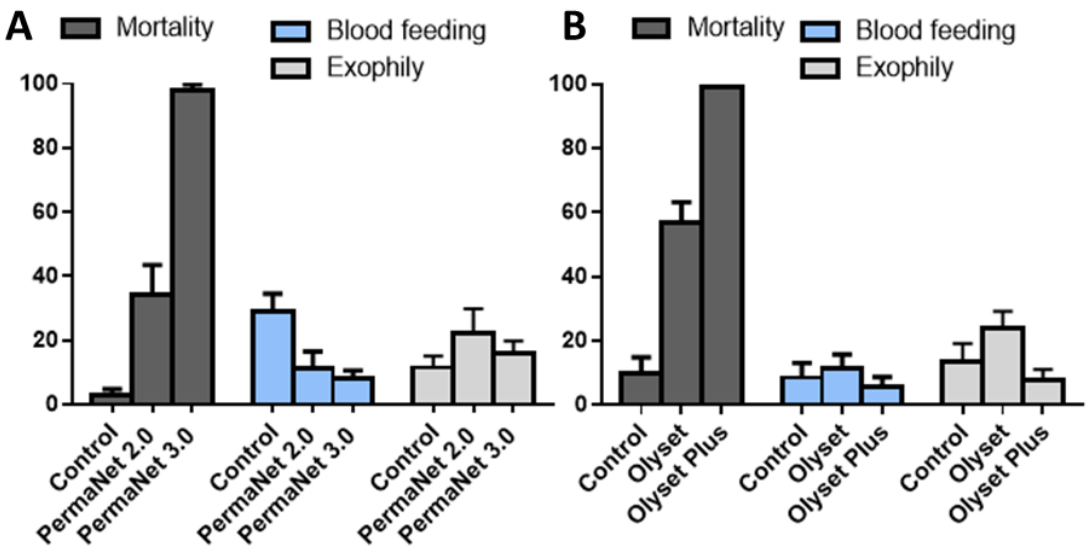

Figure 5.11: Performance of LLINs against the hybrid strain in Experimental hut. **A)** The proportion of mortality, blood-feeding and exophily rate for PermaNet 2.0 and PermaNet 3.0**. B)** The percentage of mortality, blood-feeding and exophily rate for Olyset, Olyset Plus.

5.3.5 *CYP6P9a* is reducing the efficacy of bed nets in Experimental hut trial.

5.3.5.1 *CYP6P9a* impacting Mortality

Genotyping of the *CYP6P9a* maker allowed us to assess the impact of P450-based metabolic resistance on the loss of efficacy of PermaNet 2.0. Because most of the mosquitoes released in the PermaNent 3.0 huts died, the impact of the CYP6P9a on the ability to survive exposure to LLIN was assessed only for PermaNet 2.0. To avoid confounding effects from blood-feeding, or net entry or exophily status, the distribution of the CYP6P9a genotypes was assessed firstly only among unfed mosquitoes collected in the room. This revealed a highly significant difference in the frequency of the three genotypes between the dead and alive mosquitoes ($P<0.0001$) (Figure 5.12). Analysis of the correlation between each genotype and mortality revealed that *CYP6P9a* homozygous resistant mosquitoes (RR) show an increased ability of the mosquitoes to survive exposure to the PermaNet 2.0 when compared to homozygote susceptible mosquitoes (SS) with (odds ratio=34.9; CI=15.8-77.1; $P<0.0001$) (Table 5.3).

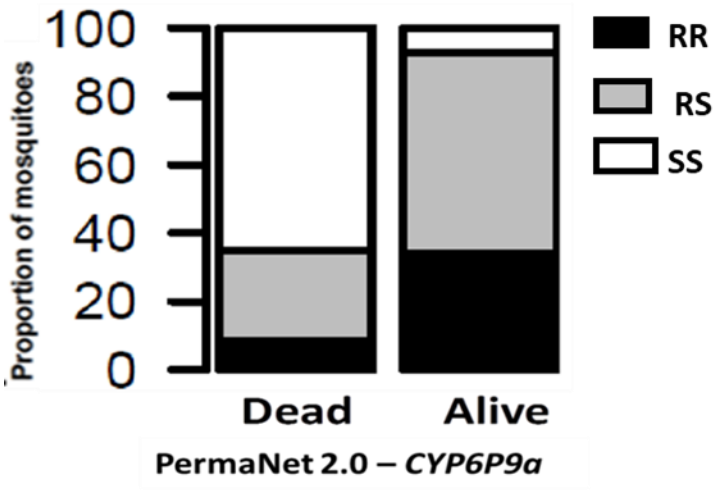

Figure 5.12: Association between duplicated *CYP6P9a* gene and ability to survive exposure to PermaNet 2.0 in experimental hut trial: Genotype distribution of *CYP6P9a* between alive and dead mosquitoes after exposure to PermaNet 2.0 showing strong association (RR vs SS: P<0.001).

Genotyping Olyset-exposed mosquitoes revealed that *CYP6P9a* homozygous resistant mosquitoes (RR) were also significantly more able to survive exposure to Olyset than homozygote susceptible (SS) (OR=5.0; P<0.001 for *CYP6P9a*) (Table 5.4; Figure 5.13)

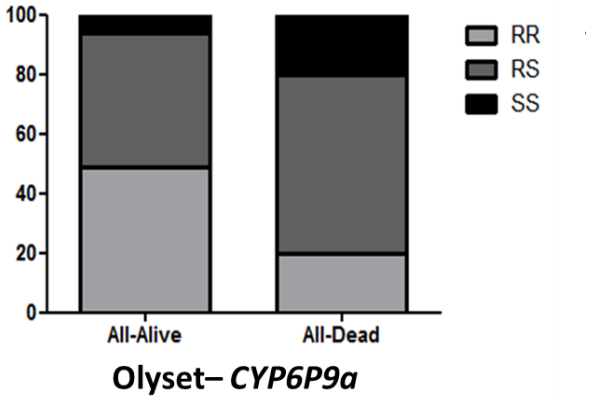

Figure 5.13: Association between duplicated *CYP6P9a* gene and ability to survive exposure to Olyset in experimental hut trial: Genotype distribution of *CYP6P9a* between alive and dead mosquitoes after exposure to Olyset showing strong association (RR vs SS: P<0.001);

5.3.5.2 *CYP6P9a* impacting the Blood feeding

Analysis of the impact of the *CYP6P9a* resistance on blood-feeding ability revealed that homozygote resistant mosquitoes (RR) were more likely to blood feed than homozygote susceptible (SS) but not significantly (OR= 1.75;P>0.05) when exposed to PermaNet 2.0 in experimental hut (Figure 5.14; Table 5.3).

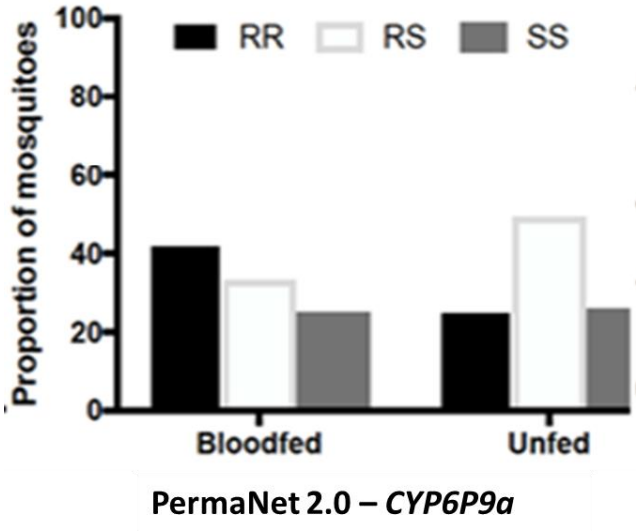

Figure 5.14: Association between duplicated *CYP6P9a* gene and ability to blood feed when exposed to PermaNet 2.0 in experimental hut trial. *CYP6P9a*-R allele increases the ability of resistant mosquitoes of taking a blood meal in contrast to susceptible ones when exposed to PermaNet 2.0.

The impact of the *CYP6P9a* gene on the blood-feeding ability of the mosquitoes exposed to Olyset was assessed. This revealed that homozygote resistant mosquitoes (RR) were significantly more likely to blood feed than homozygote susceptible (SS) (OR= 5;P=0.001) when exposed to Olyset in the experimental hut (Figure 5.15; Table 5.4).

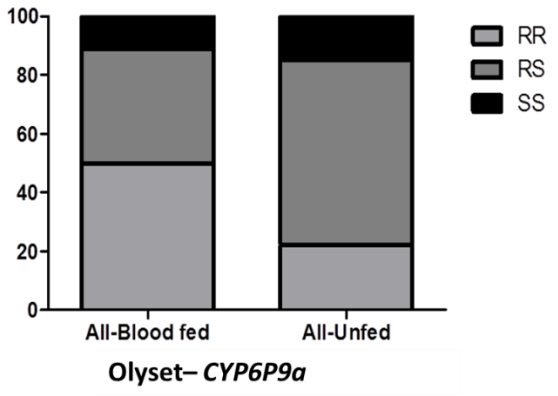

Figure 5.15: Association between duplicated *CYP6P9a* gene and ability to blood feed when exposed to Olyset in experimental hut trial. *CYP6P9a*-R allele increases the strength of resistant mosquitoes of taking a blood meal in contrast to susceptible ones when exposed to Olyset.

For PermaNet 3.0, the same observation was done when comparing RR v RS individuals (OR= 2.6; P<0.01) for PermaNet 3.0 (Table 5.3). For Olyset Plus Net, the impact of the *CYP6P9a* resistance on blood feeding ability was also assessed, revealing that homozygote RR mosquitoes are significantly more likely to blood feed than heterozygote RS (OR= 6.3; P<0.001). (Table 5.4).

Samples collected in the control hut were also genotyped. Genotypes distribution in control showed no difference between unfed and fed for genotypes proving that difference observed in other huts are not random (Figure 5.16).

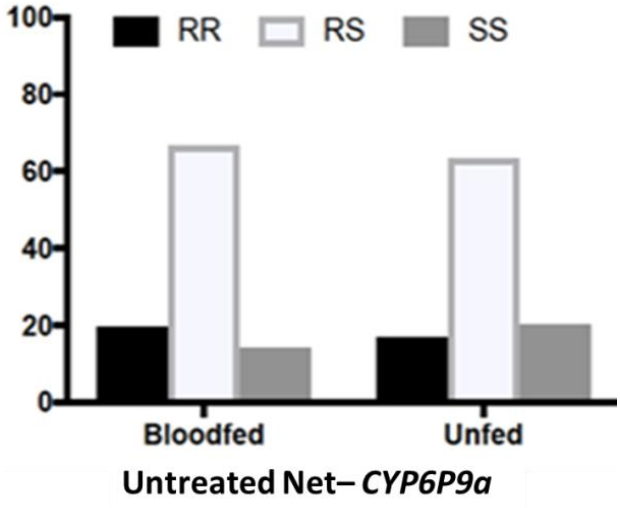

Figure 5.16: Mosquitoes exposed to untreated nets show no difference between unfed and fed for *CYP6P9a* genotypes.

Table 5.3: Correlation between genotypes of *CYP6P9a, CYP6P9b,* mortality and blood-feeding after exposure to PermaNet 2.0 and PermaNet 3.0 in experimental huts.

		OR	P value	CI	OR	P value	CI
Mortality							
PermaNet 2.0			*CYP6P9a*			*CYP6P9b*	
Unfed	RR vs SS	34.9	<0.001	15.8-77.1	109.3	<0.001	40.7-293.2
	RS vs SS	19.9	<0.001	9.7-40.9	34.7	<0.001	14.6-82.5
	RR vs RS	1.75	>0.05	0.81-3.8	3.14	<0.01	1.4-6.9
	R vs S	6.25	<0.001	3.3-11.7	7.2	<0.001	3.8-13.4
All samples	RR vs SS	10.8	<0.001	5.6-20.8	62.1	<0.001	23.6-163.6
	RS vs SS	5.3	<0.001	2.8-9.8	21.9	<0.001	8.7-55.1
	RR vs RS	2.04	<0.001	1.1-3.7	2.8	<0.01	1.48-5.2
	R vs S	3.17	<0.05	1.78-5.65	4.7	<0.001	2.6-8.7
Blood feeding							
PermaNet 2.0	RR vs SS	1.75	>0.05	0.82-3.7	1.29	>0.05	0.58-2.9
	RR vs RS	2.5	>0.05	1.09-5.75	0.92	>0.05	0.49-1.7
	RS vs SS	0.7	>0.05	0.28-1.7	1.68	1	0.79-3.5
	R vs S	1.43	>0.05	0.82-2.5	0.94	1	0.5-1.6
PermaNet 3.0	RR vs SS	4.54	<0.0001	2.3-8.7	5.04	<0.001	1.7-14.6
	RR vs RS	2.6	<0.01	1.43-4.7	2.4	<0.01	1.31-4.39
	RS vs SS	1.74	>0.05	0.87-3.47	2.1	>0.05	0.73-6.03
	R vs S	2.14	>0.05	1.17-3.19	4.1	<0.0001	2.2-7.5

Table 5.4: Correlation between genotypes of *CYP6P9a*, *CYP6P9b*, mortality and blood feeding after exposure to Olyset and Olyset Plus in experimental huts.

		OR	P value	CI	OR	P value	CI
Mortality							
Olyset			*CYP6P9a*			*CYP6P9b*	
Unfed room	RR vs SS	5.0	=0.001	2.01-12.4	15.0	<0.001	4.48-50.30
	RS vs SS	1.8	>0.05	0.8-3.9	6.0	<0.001	1.9-18.75
	RR vs RS	2.7	>0.05	1.6-6.08	2.4	1.0	1.2-4.8
	R vs S	2.04	<0.05	1.1-3.6	2.5	<0.01	1.4-4.5
All samples	RR vs SS	7.03	<0.01	2.5-19.4	5.1	<0.01	1.8-14.6
	RS vs SS	2.1	>0.05	0.8-5.7	1.9	1.0	0.7-5.3
	RR vs RS	3.1	≤0.05	1.6-6.1	2.5	<0.01	1.3-4.8
	R vs S	2.4	<0.01	1.3-4.3	2.4	<0.01	1.3-4.3
Blood feeding							
Olyset			*CYP6P9a*			*CYP6P9b*	
Room	RR vs SS	4.5	<0.01	1.9-10.3	10.01	<0.001	4.1-24.9
	RS vs SS	0.8	1.0	0.3-1.7	0.8	1.0	0.3-2.2
	RR vs RS	5.4	<0.001	2.6-10.88	11.1	<0.001	5.3-23.03
	R vs S	2.3	<0.01	1.3-4.1	4.7	<0.001	2.5-8.1
All samples	RR vs SS	3.1	<0.001	1.2-7.8	8.01	<0.001	2.4-25.9
	RS vs SS	1.2	<0.01	0.5-2.8	1.8	1.0	0.5-5.8
	RR vs RS	3.7	<0.001	1.9-7.1	4.3	<0.001	2.3-8.1
	R vs S	1.99	<0.05	1.1-3.5	2.9	<0.001	1.5-5.4
Olyset			*CYP6P9a*			*CYP6P9b*	
All samples	RR vs SS	2.05	>0.05	0.9-4.4			
	RS vs SS	0.3	<0.05	0.1-0.7			
	RR vs RS	6.3	<0.001	3.1-12.5			
	R vs S	2.0	<0.05	1.1-3.5			

5.3.6 *CYP6P9b* is reducing the efficacy of bed nets.

5.3.6.1 *CYP6P9b* impacting the Mortality

To measure the impact of *CYP6P9b* on the efficiency of LLINs, the effect was assessed on mosquitoes exposed to PermaNet 2.0 in experimental hut since the number of live mosquitoes collected in hut treated with PermaNet 3.0 was very low. The impact of *CYP6P9b* genotypes on the ability of mosquitoes to survive exposure to PermaNet 2.0 was firstly evaluated on the unfed samples collected only in the room. This was to exclude bias caused by feeding or exophiliy. This investigation showed that the three genotypes were detected and were significantly different in their distributions (P<0.0001) (Figure 5.17). The analysis also revealed that *CYP6P9b* homozygote resistant (RR) were significantly more able to survive exposure to PermaNet 2.0 than homozygotes-susceptible mosquitoes (SS) (OR=109.3; CI=40.7-293.2; P<0.0001) (Table 5.3). The association between the *CYP6P9b* and the survival ability was found to be more robust compared to the one observed for *CYP6P9a* using the same mosquito samples (OR=34.9; CI=15.8-77.1; P<0.0001) (Table 5.3).

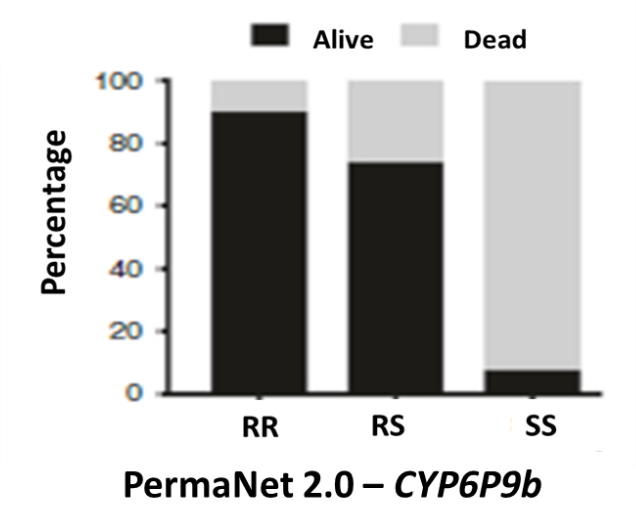

Figure 5.17: Distribution of *CYP6P9b* genotypes between dead and alive mosquitoes after exposure to PermaNet 2.0 net in experimental huts showing that *CYP6P9b*_R significantly allows mosquitoes to survive exposure to this insecticide-treated net.

Similarly, the heterozygote mosquitoes (RS) demonstrated a greater ability to survive compared to homozygote susceptible (SS) when exposed to PermaNet 2.0 (OR=34.7; CI=14.6-82.5; P<0.0001) (Table 5.3). The fact that the homozygote resistant mosquitoes (RR) present a greater ability to survive compared to heterozygotes (RS) (OR=3.14; CI=1.4-6.9: P=0.0058)

demonstrate the additive role of the resistant allele at the *CYP6P9b* locus in conferring resistance. In addition, a strong association was also observed between the gene and the ability to survive when comparing the allelic frequency (R vs S; OR=7.2; CI=3.813.4; P<0.0001) (Table 5.3). The same trend showing a strong association between the *CYP6P9b* genotypes and the mortality was also observed when analyzing all the samples dead and alive including the blood-fed and the one collected in the veranda (Table 5.3).

Genotyping of Olyset-exposed mosquitoes revealed that *CYP6P9b* homozygous resistant mosquitoes (RR) were also significantly more able to survive exposure to Olyset than homozygote susceptible (SS) (OR=15.6; P<0.001) (Table 5.4; Figure 5.18).

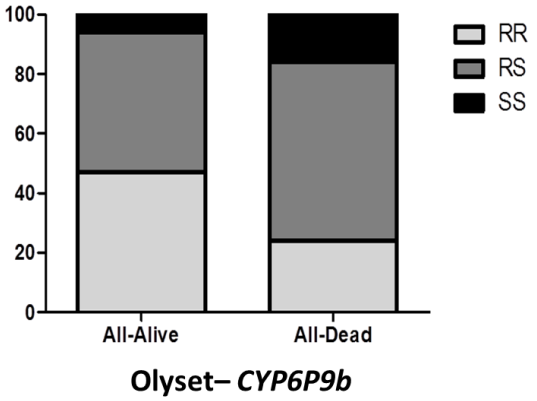

Figure 5.18: Association between *CYP6P9b* gene and the ability to survive when exposed to Olyset in experimental hut trial. *CYP6P9b*-R allele increases the ability of resistant mosquitoes to survive in contrast to susceptible ones when exposed to Olyset.

5.3.6.2 *CYP6P9b* impacting the Blood feeding

The effect of the *CYP6P9b* gene was measured on the aptitude to take the blood meal when exposed to PermaNet 3.0. A strong association was observed between *CYP6P9b* genotypes and the ability to blood-feed. Homozygote resistant mosquitoes (RR) were more able to bloodfeed when compared to homozygote susceptible mosquitoes (SS) (OR=5; CI=1.7-14.6; P=0.0001) (Table 5.3; Figure 5.19). Similar strong association was also observed when comparing homozygote resistant (RR) to heterozygotes (RS) (OR=2.4: CI=1.3-4.3; P=0.0085). When comparing RS to SS, no significant association was observed between the *CYP6P9b*

genotypes and an increased ability to bloodfed (OR=2.1; CI=0.73-4.39; P >0.05) (Table 5.3; Figure 5.19).

Nevertheless, for PermaNet 2.0 no association was observed between the *CYP6P9b* genotypes and the blood feeding (P >0.05) (Table 5.3; Figure 5.20.A) Samples from control hut were also genotyped and no difference between unfed and fed for genotypes were noticed proving that difference observed in treated huts are not random (Figure 5.20.B).

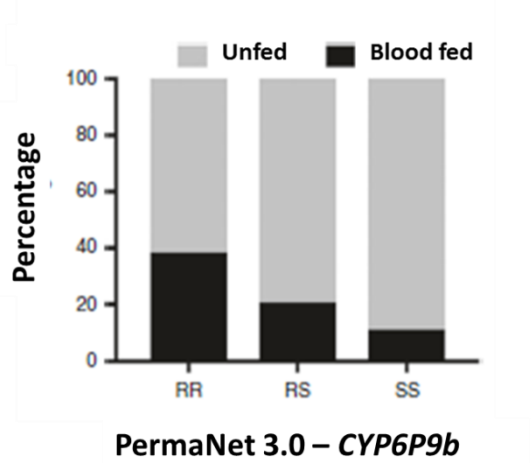

Figure 5.19: Distribution of *CYP6P9b* genotypes between blood-fed and unfed mosquitoes after exposure to the PBO-based net PermaNet 3.0 showing that *CYP6P9b*_R allele increases the ability to take a blood meal.

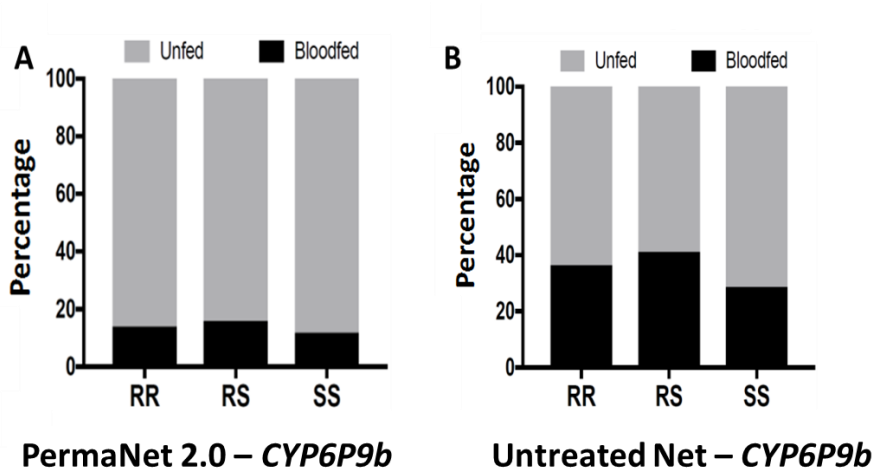

Figure 5.20: impact of the *CYP6P9b*-mediated pyrethroid resistance on blood feeding after exposure to insecticide-treated nets: **A)** Distribution of *CYP6P9b* genotypes between blood fed and unfed mosquitoes after exposure to the pyrethroid-only net PermaNet 2.0 showing no effect of *CYP6P9b* for this net. **B)** is for the untreated nets showing no correlation between *CYP6P9b* genotypes and blood feeding success.

Genotyping Olyset-exposed mosquitoes revealed that *CYP6P9b* homozygous resistant mosquitoes (RR) were also significantly more able to blood feed when exposed to Olyset than homozygote susceptible (OR=10.01; P<0.001) (Table 5.4; Figure 5.18).

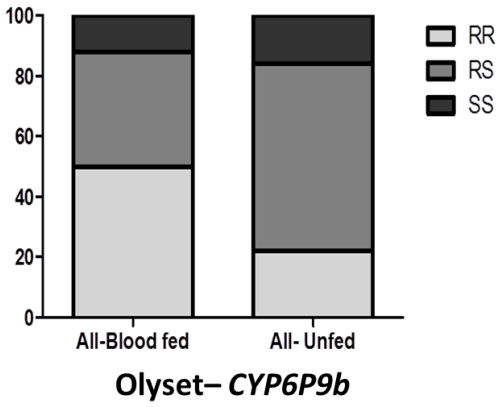

Figure 5.21: Association between the duplicated *CYP6P9b* gene and the ability to blood-feed when expose to Olyset in experimental hut trial. *CYP6P9b*-R allele increases the ability of resistant mosquitoes of taking a blood meal in contrast to susceptible ones when exposed to Olyset

5.3.7 *CYP6P9a* combines with *CYP6Pb* to further reduce the efficacy of bed nets

Since, it has been observed that *CYP6P9a* and *CYP6P9b* was not necessarily linked, the combined effect at both genes on mortality and blood feeding was assessed.

5.3.7.1 Impact of *CYP6P9a* combines with CYP6Pb on mortality

PermaNet 2.0 : Examination of the effect of combined genotypes on the mortality using samples exposed to PermaNet 2.0 has furthermore established the independent segregation of genotypes at both genes. Double homozygote resistant mosquitoes (RR/RR) at both genes demonstrate by far a greater capacity to survive when exposed to PermaNet 2.0 compared to all the other combinations (Figure 5.21. A). This result is indicating that mosquitoes double homozygote resistant at *CYP6P9a* locus combines with *CYP6Pb* act additively to provide a more resistance to pyrethroids causing at a high level the reduction of the performance of LLINs. In addition,the additive effect of both genes acting together was reinforced by the fact that mosquito double homozygote resistant (RR/RR) at both genes were more able to survive when compared to double susceptible (SS/SS) (OR=76.5 ; CI=15-387).

Table 5.5: Correlation between genotypes of *CYP6P9a*, *CYP6P9b*, mortality and blood feeding after exposure to PermaNet 2.0 and PermaNet 3.0 in experimental huts.

		Mortality		
		OR	PV	CI
PermaNet 2.0	RR/RR vs SS/SS	76.5	<0.00001	15.1-387.7
	RR/RR vs RS/SS	47.7	<0.00001	12.6-181.01
	RR/RR vs RR/SS	6.1	0.01	1.2-29.2
	RR/RR vs RR/RS	1.6	0.82	0.5-5.07
	RR/RR vs RS/RS	3.2	0.042	1.1-9.4
	RR/RS vs SS/SS	29.6	<0.00001	6.3-139.4
	RR/RS vs RS/SS	48.1	<0.00001	10.5-221.5
	RR/RS vs RR/SS	3.8	0.011	0.9-16.02
	RR/RS vs RS/RS	2	0.15	0.8-4.8
		Blood feeding		
		OR	PV	CI
PermaNet 3.0	RR/RR vs SS/SS	6.5	<0.0001	2.2-19.1
	RR/RR vs RS/SS	2.86	<0.00001	1.5-5.4
	RS/RS vs SS/SS	2.26	0.028	0.78-6.5

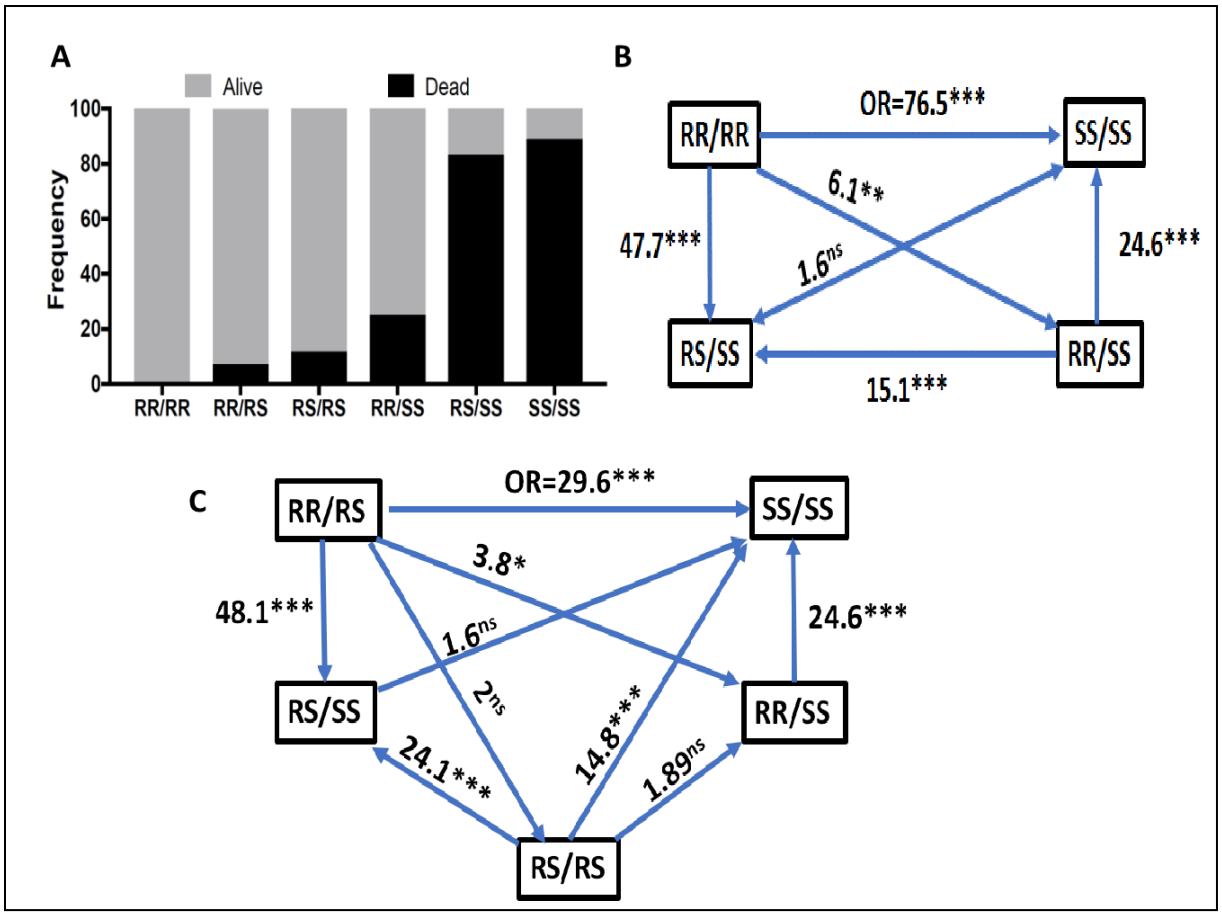

Figure 5.22: *CYP6P9a* combines with *CYP6P9b* to further reduce mortality when mosquitoes are exposed to insecticide-treated nets **A)** Combined genotype distribution in mosquitoes, demonstrating that double homozygote at both genes presents an increased survival rate when exposed to PermaNet 2.0. **B)** increased capacity to survive exposure to PermaNet 2.0 of the double homozygote resistant (RR/RR) at both genes compared to other genotypes supporting the additive resistance effect of both genes. **C)** increasing survival rate when exposed to PermaNet 2.0 when comparing RR/RS and RS/RS.

For Olyset: A comparison of the distribution of both sets of genotypes revealed that mosquitoes double homozygotes resistant (RR/RR) at both genes also had by far a significant ability to survive Olyset exposure to Olyset than all other combinations showing that both genes act additively to confer a greater resistance to pyrethroids with a higher reduction of Olyset efficacy (Table 5.6). The additive effect was further confirmed as RR/RR mosquitoes had the greatest ability to survive Olyseexposure when compared to double susceptible (SS/SS) (OR= 8.61; P<0.01) (Table 5.6; Figure 5.23). A significantly high ability to survive is also observed in RR/RR when compared to other combinations such as against RR/RS (OR= 12.2;

CI=3.0-49.9 P<0.0002) and RR/SS (OR= 2.0; CI=0.3-13.3 P<0.01) No significant difference was observed between RR/RR and RR/SS (OR= 1.8; CI=0.3-11.2 P>0.05) (Table 5.5).

Table 5.6: *CYP6P9a* and *CYP6P9b* acting together further increase the ability of *An. funestus* (crossing Fumoz-Fang-F5) to survive and blood feed against Olyset after experimental hut trial.

		CYP6P9a/b combined		
		Mortality all		
		OR	PV	CI
Olyset	RR/RR vs SS/SS	8.61	<0.01	1.8-39.1
	RR/RR vs RS/SS	1.8	>0.05	0.3-11.2
	RR/RR vs RR/SS	2.0	<0.01	0.3-13.3
	RR/RR vs RR/RS	12.2	<0.001	3.0-49.9
	RR/RR vs RS/RS	2.6	1.0	1.2-5.5
Olyset		Bloodfeeding all		
		OR	PV	CI
	RR/RR vs SS/SS	8.1	≤0.05	2.4-27.58
	RR/RR vs RS/SS	2.4	<0.05	0.5-11.1
	RR/RR vs RR/SS	1.4	1.0	0.2-8.2
	RR/RR vs RR/RS	1.8	>0.05	0.7-4.7
	RR/RR vs RS/RS	5.8	<0.001	2.8-12.05
		Bloodfeeding Room only		
	RR/RR vs SS/SS	9.3	<0.001	3.5-24.8
	RR/RR vs RS/SS	1.5	>0.05	0.2-8.9
	RR/RR vs RR/SS	1.5	>0.05	0.2-8.9
	RR/RR vs RR/RS	0.5	1.0	0.1-1.7
	RR/RR vs RS/RS	11.0	<0.001	4.9-24.47

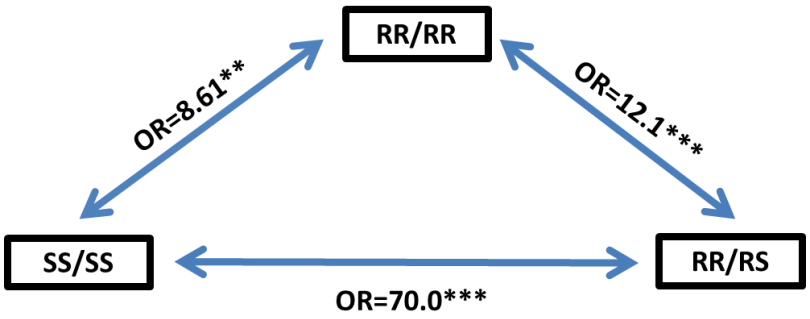

Figure 5.23: Increased capacity to survive exposure to Olyset of the double homozygote resistant (RR/RR) at both genes compared to other genotypes supporting the additive resistance effect of both genes.

5.3.7.2 Impact of *CYP6P9a* combined with *CYP6P9b* on Bloodfeeding

After the examination of the combined genotype distribution for blood feeding, a continuing reduction of the effect is perceived for genotypes RR/RR>RS/RS>SS/SS. This was using mosquitoes collected in room treated with PermaNet 2.0 (Figure 5.24.A). The analysis also has shown that double homozygote-resistant mosquitoes (RR) were significantly more able to blood feed when compared to double homozygote susceptible (SS) after exposition to PermaNet 3.0 in experimental huts (Table 5.5; Figure 5.24.B).

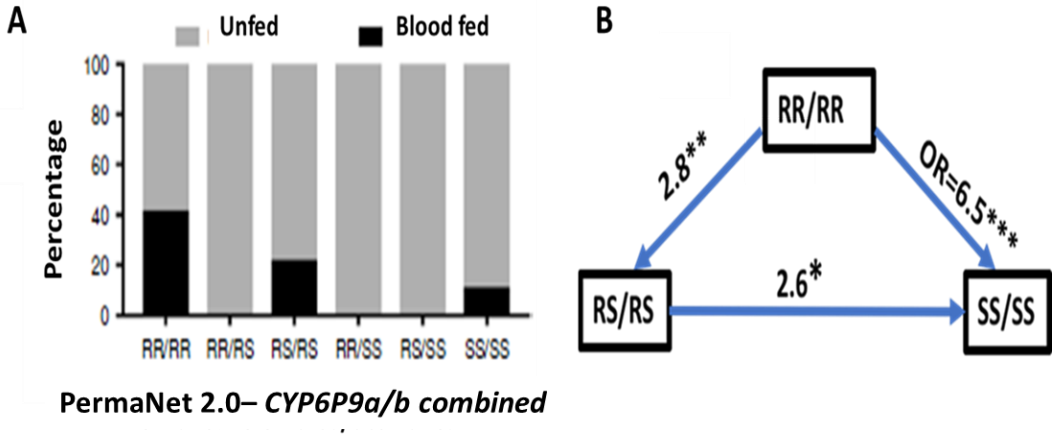

Figure 5.24: Distribution of the combined genotypes of both *CYP6P9a* and *CYP6P9b* after exposure to PermaNet Nets revealing an additive effect of both genes in increasing the ability to blood feed. **A)** For PermaNet 2.0 **B)** For PermaNet 3.0.

A comparison of the distribution of both sets of genotypes in mosquitoes from the hybrid strain exposed to Olyset revealed a significant increase ability to blood feed for double homozygote resistant mosquitoes with highest significance against double homozygote susceptible (OR= 9.3; CI=3.5-24.8 P<0.0001) (Table 5.6).

5.4 Discussion

The discovery of the resistance markers linked with P450-mediated resistance currently offers an exceptional tool to screen pyrethroid resistance in field populations of the major malaria vectors such as *An. funestus*.

Assessing the impact of insecticide resistance on the effectiveness of insecticide-treated control tools is crucial in order to implement suitable insecticide resistance management (IRM) strategies as recommended by the global plan for resistance management strategies(WHO, 2012a). The aim of this is to be able to fill the information gap required to plan appropriate IRM in order to preserve the efficacy of current and future insecticides. However, the extent of the impact of this resistance, notably, metabolic resistance on the effectiveness of bed nets is not well assessed due to the lack of markers to track and quantify the resistance. In this study, we use markers recently made available (Mugenzi et al., 2019; Weedall et al., 2019) to see how insecticide resistance is changing the efficacy of insecticide-based interventions such as LLINs. But more importantly, it also uniquely allows one to assess the interplay between P450 genes in the overall genetic variance to resistance as seen here between *CYP6P9a* and *CYP6P9b*.

5.4.1 Comparative assessement of efficacy of Pyrethroid-only vs PBO nets in the context of metabolic resistance

The performance of pyrethroid only nets was not similar. Olyset presented a higher mortality compared to PermeNet 2.0 (56.7% vs 31.4%; P<0.0001). The performance of the PBO nets Olyset Plus and PermanNet 3.0 was very similar (99.1%vs 98.8%).

When comparing the performance of conventional nets vs synergist ones, we observed a The better performance of PBO nets as observed in other studies (Pennetier et al., 2013a; Toe et

al., 2018a). The high mortality observed with PBO nets against the hybrid strain Fumoz/Fang is due to the fact the mechanisms underlying the resistance in this population are mainly *CYP6P9a* and *CYP6P9b* which are inhibited by PBO (Mugenzi et al., 2019; Riveron et al., 2013; Weedall et al., 2019). These results demonstrate that PBO-net are a solution for P450-resistance.

The efficacy of nets observed in this study confirm the loss in efficacy of the pyrethroid only nets against *An. funestus* s.s. as observed all over the continent. This loss of efficacy was observed in Mozambique (Glunt et al., 2015; Riveron et al., 2019) in Malawi (Riveron et al., 2015), in Congo (Riveron et al., 2018b) and Cameroon (Menze et al., 2020; Menze et al., 2018b). Overall, similarly to this study, it has been noticed that PBO-nets demonstrate a better performance compared to pyrethroid-only(Pennetier et al., 2013a; Toe et al., 2018a). The very high mortality observed in this study with Olyset Plus and PermaNet 3.0 against P450 resistant mosquitoes suggests that PBO nets can be the solution in an area where resistance is mediated by P450. In the absence of Kdr in *An. funestus* (Irving and Wondji, 2017), the loss of efficacy of PBO nets against P 450 resistant mosquitoes (Riveron et al., 2019) suggest that we should pay attention to other resistance mechanisms like cuticular resistance that may be taking place.

5.4.2 Impact of the *CYP6P9a/b* gene on the performance of Bed nets

Since the number of live mosquitoes collected in hut treated with PermaNet 3.0 and Olyset Plus was very low, it was not possible using samples from experimental hut to assess the association between the presence of the resistant alleles and the ability to survive when exposed to PBO nets. However, after cone test where the exposition time is just 3 minutes compared to overnight with experimental huts, we were able to obtained enough dead and alive after exposition to PBO nets. When comparing the impact of *CYP6P9a/b* on Pyrethroid only nets and PBO nets using samples from cone assays we noticed that the impact of *CYP6P9a* on Pyrethroid only nets was higher when compared to PBO nets. (Olyset, OR= 35.1; P<0.00001 vs Olyset Plus, OR= 6.4; P<0.001 and PermaNet 2.0, OR=239.0; P<0.001 vs PermaNet 3.0, OR=81.0; P<0.001). The same trend was observed with the *CYP6P9b* genes (Table 5.1). This can be explained by the fact that the addition of the PBO in the synergist net inhibits the cytochrome P450 enzymes (Amenya et al., 2008).

PermaNet nets from Vestergaard and Olyset from Sumitomo nets have shown reduced performance against *CYP6P9a* and *CYP6P9b* resistant mosquitoes. For both nets, strong association was observed between the resistant alleles and the increased ability of the mosquitoes to survive after exposure to these nets. Nevertheless, the impact of *CYP6P9a* and *CYP6P9b* seems to be higher on PermaNet nets which are impregnated with deltamethrin compared to Olyset nets impregnated with Permethrin (Olyset, OR= 35.1; P<0.00001 vs PermaNet 2.0, OR=239.0; P<0.001 and Olyset Plus, OR= 6.4; P<0.001 vs PermaNet 3.0, OR=81.0; P<0.001) (table 5.1). It seems that the hybrid strain is more resistant to type II insecticide. This is in correlation with what has been observed during the WHO bioassays (Figure 5.1) where the mosquitoes were more resistant to deltamethrin than permentrin (48.5% vs 80.7%).

It will be useful to assess the impact of those genes on the performance of nets impregnated with Alpha-cypermethrin like Duranet to check how *CYP6P9a* and *CYP6P9b* impact different pyrethroid type.

Looking at the odd ratio obtained, a stronger association was observed between *CYP6P9a*/*CYP6P9b* and the loss of efficacy of nets (result chapter 5) compared to *GSTe2* (Chapter 4). The impact was higher with P450 genes certainly because *CYP6P9a* and *CYP6P9b* are the primary resistance mechanism in the population used (Fumoz-Fang). On the other hand, in the *An. funestus* population from Mibellon, the *GSTe2* acts beside other mechanisms, since the *GSTe2* genotypic frequency for RR in Mibellon is around 20% (Chapter 3).

However, one of the key message from this study is that a greater reduction of bed net efficacy is observed when both *CYP6P9b* combines with *CYP6P9a* as double homozygote resistant mosquitoes by far were able to survive exposure to pyrethroid-only nets than all genotypes revealing the greater risk that Super resistance may caused to insecticide-based interventions and supporting the concern highlighted in the WHO global plan for insecticide resistance management that if nothing is done pyrethroid resistance could lead to increase the burden of malaria in Africa.

The impact of *CYP6P9a* and *CYP6P9b* is major concern for vector control program. It is calls for novel nets not relying on pyrethroids in the future. However, the impact of these resistance alleles on the efficacy of LLINs in natural populations remains to be established as we only

performed a test with hybrid strain from two laboratory strains. Such work must be done urgently particularly as the molecular tools are now available.

5.5 Conclusion

This study, through the experimental hut, reveals that insecticide resistance driven by *CYP6P9a* and *b* is reducing the efficiency of bed nets, mainly standard nets. The wide spread of Insecticide resistance and the way it changes the efficacy of nets is a threat for vector control and an urgent call to put out a new product for vector control. Despite the high mortality observed with PermaNet 3.0, the *CYP6P9a/b* resistant mosquitoes were able to blood feed before dying. This increased ability of resistant mosquitoes to blood-fed is critical because blood feeding is a key factor for malaria transmission. The greater reduction of efficacy of insecticide-treated nets observed in double resistant mosquitoes is a major concern for the sustainability of insecticide-based interventions relying on pyrethroid notably LLINs and where such resistance is present. Since the mechanisms underlying the resistance in the lab strain Fumoz-Fang are P450, PBO-nets should be preferably deployed for greater impact although ultimately novel insecticide-treated nets like Interceptor G2) (aphalcypermethrin and chlofenapyr and Royal Guard (alphacypermethrin and Pyriproxyfen) not relying on pyrethroids only should be envisaged.

Chapter 6: General conclusion and Perspectives

The WHO through GPIRM is emphasizing the necessity to investigate the impact of insecticide resistance to implement suitable insecticide resistance management strategies (IRM) to help preserve the efficacy of current and future insecticides. However, the extent of the impact of insecticide resistance, notably, metabolic resistance on the efficiency of vector control such as Long-lasting Insecticidal Nets (LLINs) remains unclear. Taking advantages of the markers recently made available, this study has investigated the impact of insecticide resistance on the effectiveness of vector control tools against *Anopheles funestus,* a major African malaria vector. During the project, the following questions were adressed: i) Is metabolic resistance to insecticides in *An. funestus* reducing the effectiveness of vector control tools such as LLINs and IRS? ii) Can metabolic resistance to pyrethroids alter the vectorial capacity of *An. funestus* by changing key factors such as mortality rate and the ability of the mosquito to take its blood meal in the presence of the insecticide, jeopardizing vector control programs? To answer these questions, we went through three mains objectives: 1) Determine the bionomics and insecticides resistance profiling of malaria vectors at a selected site for experimental hut trials in central Cameroon. 2) Evaluate using experimental hut, the performance of PBO-based and pyrethroid-only nets against the malaria vector *An. funestus* and to investigate how *GSTe2* metabolic mediated resistance is impacting that performance and 3) Evaluate the performance of PBO-based and pyrethroid-only nets against the hybrid lab strain Fumoz-Fang and to examine how duplicated CYP6P9a and *CYP6P9b* cytochrome P450 genes are impacting that performance (Chapter 5).

6.1 Characterization of malaria vectors to maximize the impact of control intervention

The lack of information such as resistance profiles and mechanisms involved has prevented evidence-based implementation of control intervention limiting the outcome of control. It was imperative to achieve the characterization of malaria vectors to maximize the impact of control intervention and to implement suitable resistance management strategies to preserve the efficacy of existing tools. Regarding the level of resistance in malaria vectors against bed nets in the area, we strongly suggest to the government for the future to consider IRS with organophosphate like Actellic. Furthermore, the characterization of the effects of resistance on the effectiveness of control tools is also a crucial knowledge to guide control programs.

This is the reason why we focused on the characterization of this area selected for the experimental hut trial.

Looking at the species diversity, *An. funestus* s.s. (80%) is the primary malaria vector in Mibellon, followed by *An. gambiae* s.s. (20%) previously known as S form. This study also confirms the role of *An. funestus* in malaria transmission with a sporozoite infection rate of 5%. Multiple resistance was observed in both *An. funestus* s.s. and *An. gambiae* s.s. at Mibellon. This is a concern for ongoing insecticide-based interventions although the full susceptibility to organophosphate offers an alternative to IRS. The insecticide resistance observed reemphasise the need to assess the impact of such multiple resistances on the effectiveness of insecticide-based control interventions to improve vector control strategies. The presence of resistance in both major vectors makes this area suitable for such studies.
We went further to investigate the mechanisms underlying the insecticide resistance observed. The recovery observed for pyrethroids in *An. funestus* after pre-exposure to PBO indicates that metabolic resistance, particularly cytochrome P450s, play a role in that resistance. The frequency of the 119F-*GSTe2*-resistant allele in Mibellon field population (28%) is lower than in the northern part of Cameroon in Gounougou (52%) and closer to that observed in the eastern part of Africa in Uganda (20.4%). However, *GSTe2* seems to play a role in the resistance found in Mibellon. The frequency of the 296S-RDL-resistant allele is only 9.7%. This low frequency could be as a result of recovery to susceptibility for dieldrin after this insecticide was removed from public and agricultural sectors in Cameroon. The very high resistance levels to pyrethroids in *Anopheles gambiae s.s*. (no mortality to permethrin), correlates with the high frequency of the 1014F *kdr* allele (63.9%). The total absence of the 119S *ace-1* mutation is in line with the susceptibility of this population to organophosphate and carbamate as this mutation is responsible for organophosphate and carbamate. Having all these pieces of information will undoubtedly guide the National Malaria control program to improve their strategies.

6.2 Performance of PBO nets vs pyrethroid only nets against *An.funestus* from Mibellon

To better inform countries of the usefulness of PBO nets to control pyrethroid-resistant mosquitoes in the face of the greater cost of PBO nets, here we compared the performance of conventional pyrethroid-only nets (Olyset, PermaNet 2.0 and Yorkool) versus PBO-based LLINs (Olyset Plus and PermaNet 3.0) against a pyrethroid-resistant *An. funestus* population from Cameroon using experimental huts. The mortality rate was significantly higher with the two PBO-based nets than all the pyrethroid-only This study has revealed a loss of efficacy of LLINs against pyrethroid-resistant populations of *An. funestus* in Cameroon using experimental huts. However, PBO-based nets (PermaNet 3.0 and Olyset Plus) are more efficient than conventional pyrethroid-only nets (P<0.001). These results demonstrate that the mass killing provided by PBO nets is higher compared to Pyrethroid only nets. In addition, the personal protection offered by PBO nets was also found to be higher. Noticeably, the GST-mediated metabolic resistance not impacted by PBO is a serious threat to the continued effectiveness of the PBO-based LLINs and the impact of this mechanism also needs to be taken into account to maximize the efficiency of LLINs. One option could be to generate new mosaic LLINs also incorporating the Diethyl Maleate (DEM) beside PBO to inhibit GST metabolic activity and further increase the efficacy of these nets.

6.3 *GSTe2* linked to the increased ability of *An. funestus* from Mibellon to Blood feed in the presence of bednets.

For Olyset net, a significant association was observed between the 119F_*GSTe2* resistance allele and an increased ability to blood feed (21 blood-fed and 92 unfed) when comparing the allelic frequencies (OR=2; P=0.047; CI 1.06-3.7) and the genotypic frequency of RS v SS (OR=2.97; P<0.001; CI 1.6-5.3) (Figure. 4.4A; Table 4.5). For the Olyset Plus net, when considering the blood feed and unfed samples from the room only (23 blood-fed and 49 unfed), a significant association was found between L119F-*GSTe2* mutation and an increased ability to blood feed when comparing the allelic frequencies (OR=4.5; P=0.0001; CI 2.26-9.2). An even stronger association was observed when comparing the genotype frequencies between RR vs SS (OR=12.3; P=0.0004 CI 2.5-60.4) and RS vs SS (OR=8.42; P<0.001; CI 4.37-16.2). This is a clear sign that insecticide resistance can change the performance of LLINs. Moreover, this is crucial since blood feeding is an essential factor in malaria transmission.

6.4 Performance of PBO nets vs pyrethroid only nets against the hybrid strain FUMOZ -FANG

As previously reported, reduced efficacy of pyrethroid only nets was observed against hybrid strain. This study showed a very high mortality of the hybrid FUMOZ/FANG strain against PBO-based nets with mortality of 99.1% for Olyset Plus and 98.8% for PermaNet 3.0. In contrast, lower mortality was observed for the pyrethroid-only nets with mortality rates of only 56.7% for Olyset and 31.4% for PermaNet 2.0. (Table 5.2). Overall, the PBO-based nets were found to be more effective with mortality closed to 100 % when compared to conventional nets. This could be due to the fact that in the hybrid strain, the resistance is driven by the P450 genes. These results suggest that PBO based nets are still a solution in an area where resistance is medisted by P450. As a perspective, such studies need to be carried out using a natural population from Mozambique, since in this study a laboratory strain was used. The advantage of using the wild mosquitoes is that we have a realistic view of the vector population. However the challenge with the wild mosquitoes in is achieving a sufficient sample size. Alternatively, we can release large numbers in order to have enough samples for the genotyping in the lab.

6.5 *CYP6P9a/b* linked with reduced performance of bed nets.

This study, through the experimental hut, reveals that insecticide resistance driven by *CYP6P9a/b* is reducing the efficiency of standard nets widely used for a vector control program in Africa since it cost less than synergist nets. The wide sprays of the Insecticide resistance and the way it changes the efficacy of nets is a threat for vector control and an urgent call to put out a new product for vector control interventions. Despite the high performance of PermaNet 3.0, the *CYP6P9a/b* resistant mosquitoes were able to blood feed before dying. This increased ability to blood fed of resistant mosquitoes is critical because blood feeding is a critical factor for malaria transmission. The greater reduction of efficacy of insecticide-treated nets observed in double resistant mosquitoes is a major concern for the sustainability of insecticide-based interventions relying on pyrethroid notably LLINs and where such resistance is present. The high mortality observed with with PermaNet 3.0 and Olyset Plus against Fumoz Fang suggest that PBO nets are a good solution in area where resistance is mediated by P450.

PBO-nets should be preferably deployed for greater impact. For resistant mosquitoes where P 450 are not the main mechanisims, novel insecticide-treated nets not relying on pyrethroids should be envisaged.

The major limitationof this study is thatis that it was conducted in controlled conditions using experimental hut with nets holed on each side. This may be not an exact representation of what is happening in the real situation. The protocol used for this study is a standardized protocol from WHO. This protocol suggests that the sleeping time, the type of hut may differ from the social habit and the reality of the community.

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8 Appendices

Appendix 1: Data entry of sample collected during the experimental hut trial. Raw data showing sample collected in hut treated with PermaNet 3.0.

AG13

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM
1	Observations Immediate (morning)							PermaNet 3.0								PermaNet 3.0											PermaNet 3.0										PermaNet 3.0		
2				week 1 hut 4						week 2 hut 3								week 3 hut 2							week 4 hut 1										week 5 hut 6				
3			Capture n'	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
4			Date	10/7	11/7	12/7	13/7	14/7	15/7	16/7	17/7	18/7	19/7	20/7	21/7	22/7	23/7	24/7	25/7	26/7	27/7	28/7	29/7	30/7	31/7	1/8	2/8	3/8	4/8	5/8	6/8	7/8	8/8	9/8	10/8	11/8	12/8	13/8	14/8
5	Verandah & window trap	No. Live	Unfed	0	0	0	1	0	0		0	0	2	0	0	0		0	1	3	0	0	0		0	0	2	1	0	0					0	0	0	0	0
6			Bloodfed	0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0					0	1	0	0	0
7		No. Dead	Unfed	0	0	0	1	0	0		0	0	0	0	0	1		0	0	0	1	0	0		0	0	0	0	0	0					0	21	0	0	0
8			Bloodfed	0	0	0	0	0	0		0	0	0	0	0	0		0	2	0	0	0	0		0	0	0	0	0	0					0	0	0	0	0
9	Room	No. Live	Unfed	5	0	0	4	0	0		0	1	0	0	0	0		3	1	2	0	0	6		1	0	9	3	0	1					0	1	0	1	0
10			Bloodfed	0	0	0	0	0	1		0	0	0	0	0	0		0	1	0	1	0	1		0	0	1	0	0	0					0	0	0	1	0
11		No. Dead	Unfed	0	0	4	1	0	2		0	0	2	0	0	0		0	0	0	0	0	0		0	0	0	1	0	0					0	0	0	0	0
12			Bloodfed	0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0					0	0	0	0	0
13	Net	No. Live	Unfed	0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0					0	0	0	0	0
14			Bloodfed	0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0					0	0	0	0	0
15		No. Dead	Unfed	0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0					0	2	0	0	0
16			Bloodfed	0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0					0	0	0	0	0
17																																							
18																																							
19	Additional mortality after 24h																																						
20																																							
21			Capture n'	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
22			Date	11/7	12/7	13/7	14/7	15/7	16/7	17/7	18/7	19/7	20/7	21/7	22/7	23/7	24/7	25/7	26/7	27/7	28/7	29/7	30/7	31/7	1/8	2/8	3/8	4/8	5/8	6/8	7/8	8/8	9/8	10/8	11/8	12/8	13/8	14/8	15/8
23	Verandah & window trap	No. Dead	Unfed	0	0	0	1	0	0		0	0	0	0	0	0		0	0	0	0	0	0		0	0	2	0	0	0					0	0	0	0	0
24			Bloodfed	0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0					0	0	0	0	0
25	Room	No. Dead	Unfed	0	0	0	1	0	0		0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0					0	0	0	0	0
26			Bloodfed	0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0					0	0	0	1	0
27	Net	No. Dead	Unfed	0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0					0	0	0	0	0
28			Bloodfed	0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0					0	0	0	0	0
29																																							
30																																							
31	Total counts at 24h			N Females																																			
32	Verandah & window trap	No. Live	Unfed	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	1	3	0	0	0	0	0	0	0	1	0	0					0	0	0	0	0
33			Bloodfed	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0					0	1	0	0	0
34		No. Dead	Unfed	0	0	0	2	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	2	0	0	0					0	21	0	0	0
35			Bloodfed	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0					0	0	0	0	0
36		No. Live	Unfed	5	0	0	3	0	0	0	0	1	0	0	0	0	0	3	1	2	0	0	6	0	1	0	9	3	0	1					0	1	0	1	0
37			Bloodfed	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	1	0	0	0					0	0	0	0	0

Effet Case / summary analysis / Contol-1 / Yorkol / PermaNet 2.0 / **PermaNet 3.0** / Olyset / Olyset plus / Treatment 6 / T

Appendix 2: LIVAK DNA extraction Protocol

LIVAK DNA Extraction Protocol
LIVAK Grind Buffer :1.6ml 5M NaCl, 5.48g Sucrose,
1.57g Tris, 10.16 ml 0.5M EDTA,
2.5ml 20% SDS Bring volume to 100ml,
filter sterilize.
Store 5ml aliquots at -20oC.
Heat in Water bath & mix before each use
to re-dissolve precipitate.
1. Grind 1 mosquito in 100ul preheated grind buffer in 1.5ml eppendorf. Or, to maximise total yield, grind in 50ul, then rinse pestle with a further 50ul (100ul total in tube). Transfer immediately to 65oC.
2. Incubate at 65oC approx 30 min. Microfuge briefly to collect condensation.
3. Add 14ul 8M K-acetate (to final concentration of 1M). Mix.
4. Incubate on ice approx 30 min.
5. Centrifuge 20 min at 4oC. Transfer supernatant to new 1.5ml eppendorf, be careful not to transfer any debris. If desired re-spin 20 min and transfer supernatant to new tube.
6. Add 200ul 100% ethanol. Mix, e.g. vortex. Spin 15 min at 4oC.
7. Remove and discard supernatant, rinse pellet in approx 100ul icecold 70% ethanol, being careful not to dislodge pellet.
8. Dry pellet: leave open tubes on benchtop approx 1 hour. Suspend pellet in 100ul dH20, incubate at 65oC for 10 mins.

Appendix 3: Cocktail PCR species ID Protocola

Species ID *An. Funestus* PCR

	X1
Buffer A -	2.5 µl
25 mM dNTPs	0.2 µl
Kapa Taq	0.2 µl
$MgCl_2$ 25mM	1.5 µl
UV (F, 10mMl) [TGT GAA CTG CAG GAC ACA T]	
FUN (R, 10mM) [GCA TCG ATG GGT TAA TCA TG]	
VAN (R, 10mM) [TGT CGA CTT GGT AGC CGA AC]	
RIV (R, 10mM) [CAA GCC GTT CGA CCC TGA TT]	0.85µl
PAR (R, 10mM) [TGC GGT CCC AAG CTA GGT TC]	
RIVLIKE (R10mM) [CCG CCT CCC GTG GAG TGG GGG]	
LEES (R, 10mM) [TAC ACG GGC GCC ATG TAG TT]	
ddH_20	17.4 µl
DNA	1.5 µl
Total =	**25 µl**

Conditions

94 °C – 2 min	
94 °C – 30 s	X 35 cycles
45 °C – 30 s	
72 °C – 40 s	
72 °C – 5 min	
10 °C ~ Hold/forever	

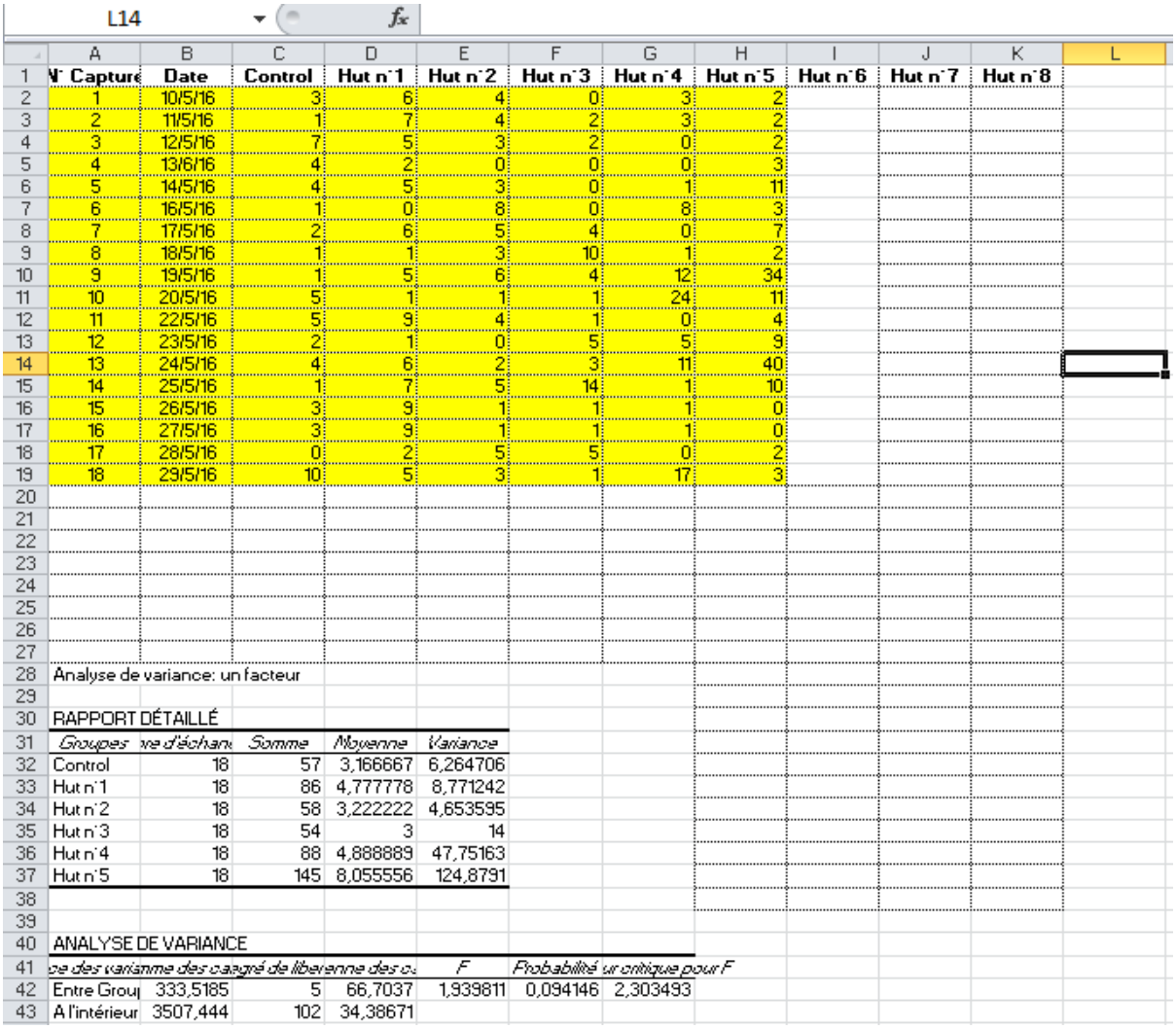
Appendix 4: Data base presenting the distribution of treatments in different huts during the first week of the study. Similar data following the Latin square rotation are available for the others 11 weeks of the study. This data based is the one used for R analysis. 0= control, 1=Yorkool; 2=PermaNet 2.0; 3=PermaNet 2.0, 4=Olyset, 5=Olyset Plus. S1-6= Sleepers 1-6.

S24

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	day	Week	hut	treat	sleeper	%bl_fed	%dead	%exophil	%net				
2	1	Week1	Hut1	0	S1	-1	-1	-1	-1				
3	2	Week1	Hut1	0	S2	100	0	25	75				
4	3	Week1	Hut1	0	S3	50	0	0	10				
5	4	Week1	Hut1	0	S4	62.5	0	12.5	37.5				
6	5	Week1	Hut1	0	S5	11.111111111111	22.2222223	0	11.111111111111				
7	6	Week1	Hut1	0	S6	0	0	0	0				
8	1	Week1	Hut2	1	S1	-1	-1	-1	-1				
9	2	Week1	Hut2	1	S2	0	12.5	12.5	0				
10	3	Week1	Hut2	1	S3	40	0	40	0				
11	4	Week1	Hut2	1	S4	33.3333333	66.6666666	66.6666666	0				
12	5	Week1	Hut2	1	S5	0	0	0	0				
13	6	Week1	Hut2	1	S6	9.09090909	27.2727272	18.181818181	0				
14	1	Week1	Hut3	2	S1	0	0	0	0				
15	2	Week1	Hut3	2	S2	66.6666666	0	0	0				
16	3	Week1	Hut3	2	S3	-1	-1	-1	-1				
17	4	Week1	Hut3	2	S4	33.3333333	0	0	0				
18	5	Week1	Hut3	2	S5	-1	-1	-1	-1				
19	6	Week1	Hut3	2	S6	-1	-1	-1	-1				
20	1	Week1	Hut4	3	S1	0	0	0	0				
21	2	Week1	Hut4	3	S2	-1	-1	-1	-1				
22	3	Week1	Hut4	3	S3	0	100	0	0				
23	4	Week1	Hut4	3	S4	0	40	40	0				
24	5	Week1	Hut4	3	S5	-1	-1	-1	-1				
25	6	Week1	Hut4	3	S6	33.3333333	66.6666666	0	0				
26	1	Week1	Hut5	4	S1	0	0	0	0				
27	2	Week1	Hut5	4	S2	-1	-1	-1	-1				
28	3	Week1	Hut5	4	S3	0	0	0	0				
29	4	Week1	Hut5	4	S4	0	0	83.333333	0				
30	5	Week1	Hut5	4	S5	0	0	0	0				
31	6	Week1	Hut5	4	S6	0	0	33.333333	0				
32	1	Week1	Hut6	5	S1	0	0	25	0				
33	2	Week1	Hut6	5	S2	-1	-1	-1	-1				
34	3	Week1	Hut6	5	S3	40	40	0	0				
35	4	Week1	Hut6	5	S4	50	50	50	0				
36	5	Week1	Hut6	5	S5	20	20	20	0				
37	6	Week1	Hut6	5	S6	0	0	20	0				
38	79	Week10	Hut1	3	S4	0	0	0	0				
39	80	Week10	Hut1	3	S5	-1	-1	-1	-1				
40	81	Week10	Hut1	3	S6	-1	-1	-1	-1				
41	82	Week10	Hut1	3	S1	42.8571428	28.5714285	14.285714	0				
42	83	Week10	Hut1	3	S2	-1	-1	-1	-1				
43	84	Week10	Hut1	3	S3	0	0	0	0				

Sorted Feuil2

Appendix 5: Data entry of samples collected during the Hut effect.



N° Capture	Date	Control	Hut n°1	Hut n°2	Hut n°3	Hut n°4	Hut n°5	Hut n°6	Hut n°7	Hut n°8
1	10/5/16	3	6	4	0	3	2			
2	11/5/16	1	7	4	2	3	2			
3	12/5/16	7	5	3	2	0	2			
4	13/6/16	4	2	0	0	0	3			
5	14/5/16	4	5	3	0	1	11			
6	16/5/16	1	0	8	0	8	3			
7	17/5/16	2	6	5	4	0	7			
8	18/5/16	1	1	3	10	1	2			
9	19/5/16	1	5	6	4	12	34			
10	20/5/16	5	1	1	1	24	11			
11	22/5/16	5	9	4	1	0	4			
12	23/5/16	2	1	0	5	5	9			
13	24/5/16	4	6	2	3	11	40			
14	25/5/16	1	7	5	14	1	10			
15	26/5/16	3	9	1	1	1	0			
16	27/5/16	3	3	1	1	1	0			
17	28/5/16	0	2	5	5	0	2			
18	29/5/16	10	5	3	1	17	3			

Analyse de variance: un facteur

RAPPORT DÉTAILLÉ

Groupes	*Nombre d'échantillons*	*Somme*	*Moyenne*	*Variance*
Control	18	57	3,166667	6,264706
Hut n°1	18	86	4,777778	8,771242
Hut n°2	18	58	3,222222	4,653595
Hut n°3	18	54	3	14
Hut n°4	18	88	4,888889	47,75163
Hut n°5	18	145	8,055556	124,8791

ANALYSE DE VARIANCE

Source des variations	*Somme des carrés*	*Degré de liberté*	*Moyenne des carrés*	*F*	*Probabilité*	*Valeur critique pour F*
Entre Groupes	333,5185	5	66,7037	1,939811	0,094146	2,303493
A l'intérieur	3507,444	102	34,38671			

Appendix 6: Ethical clearance

COMITE NATIONAL D'ETHIQUE DE LA RECHERCHE POUR LA SANTE HUMAINE

Arrêté N° 0977/A/MINSANTE/SESP/SG/DROS/ du 18 avril 2012 portant création, organisation et fonctionnement des comités d'éthique de la recherche pour la santé humaine au sein des structures relevant du Ministère en charge de la santé publique

N° 2016/03/735/CE/CNERSH/SP Yaoundé, le 15 mars 2016

Cnethique_minsante@yahoo.fr

CLAIRANCE ETHIQUE

Le Comité National d'Ethique de la Recherche pour la Santé Humaine (CNERSH), en sa session ordinaire du 04 mars 2016, a examiné le projet de recherche intitulé : **«Etude de l'impact de la résistance aux insecticides chez *Anopheles funestus*, un vecteur majeur du paludisme, sur l'efficacité des outils de lutte antivectorielle au Cameroun»** soumis par le **Docteur Charles WONDJI**, Investigateur Principal, Organisation de Coordination pour la Lutte contre les Endémies en Afrique Centrale (OCEAC).

Le projet est d'un grand intérêt scientifique et social. L'objectif de cette étude est d'évaluer l'impact de la résistance *d'Anopheles funestus* aux insecticides sur les actions de lutte antivectorielle. La procédure de l'étude est bien documentée et claire. Les risques liés à l'étude sont précisés ainsi que les mesures pour les éviter et les minimiser. Les mesures prises pour garantir la confidentialité des données collectées sont présentes dans le document. Cette étude ne prévoit aucun transfert de matériel biologique. Les CVs des Investigateurs les décrivent comme des personnes compétentes, capables de mener à bien cette étude. Pour toutes ces raisons, le Comité National d'Ethique approuve pour une durée d'un an, la mise en œuvre de la présente version du protocole.

Les Investigateurs sont responsables du respect scrupuleux du protocole approuvé et ne devraient y apporter aucun amendement aussi mineur soit-il, sans avis favorable du CNERSH. Les investigateurs sont appelés à collaborer pour toute descente du CNERSH pour le suivi de la mise en œuvre du protocole approuvé. Le rapport final du projet devra être soumis au CNERSH et aux autorités sanitaires du Cameroun.

La présente clairance peut être retirée en cas de non respect de la réglementation en vigueur et des recommandations susmentionnées.

En foi de quoi, la présente clairance éthique est délivrée pour servir et valoir ce que de droit.

Ampliations

- MINSANTE

Le Président

Pr Lazare KAPTUE

N.B : cette clairance éthique ne vous dispense pas de l'autorisation administrative de recherche (AAR), exigée pour mener cette étude sur le territoire camerounais. Cette dernière vous sera délivrée par le Ministère de la Santé Publique.

Appendix 7: Linear mixed effect model analysis showing different sources of variations that may influenced the exophily.

	Value	Std.Error	p-value
(Intercept)	16.249764	7.725620	0.0364
sleeper2	-4.932549	5.953246	0.4081
sleeper3	-0.300115	6.150593	0.9611
sleeper4	7.972452	5.568319	0.1534
sleeper5	4.356004	5.718595	0.4469
sleeper6	2.547911	5.839805	0.6630
Hut2	2.962492	5.533332	0.5928
Hut3	-5.224913	5.754090	0.3647
Hut4	4.504248	5.614402	0.4231
Hut5	-6.310800	5.844721	0.2813
Hut6	-6.974816	5.522378	0.2077
Week2	19.753810	7.828069	0.0122
Week3	8.457334	7.436382	0.2565
Week4	-6.289393	8.023420	0.4338
Week5	4.201448	7.381160	0.5697
Week6	-5.579260	7.661576	0.4671
Week7	-3.276825	8.130004	0.6872
Week8	9.517598	7.753166	0.2207
Week9	17.027261	8.521754	0.0468
Week10	-5.011175	7.652644	0.5132
Week11	-21.324423	15.600047	0.1728
Week12	-6.491758	7.158812	0.3653

Appendix 8: Linear mixed effect model analysis showing different sources of variations that may influenced the mortality

	Value	Std.Error	p-value
(Intercept)	13.432237	8.932373	0.1339
sleeper2	2.261362	6.402623	0.7242
sleeper3	-6.058720	6.611833	0.3603
sleeper4	2.695107	5.986638	0.6530
sleeper5	4.570812	6.153108	0.4582
sleeper6	-0.733462	6.278762	0.9071
Hut2	5.200011	5.953404	0.3832
Hut3	2.809049	6.189717	0.6503
Hut4	5.747833	6.040292	0.3422
Hut5	5.557012	6.289644	0.3778
Hut6	0.028410	5.947106	0.9962
Week2	8.142864	8.423385	0.3346
Week3	-6.702629	7.995142	0.4026
Week4	2.918904	8.626829	0.7354
Week5	5.451842	7.936171	0.4927
Week6	-4.389710	8.239292	0.5946
Week7	-8.807101	8.743382	0.3147
Week8	0.442853	8.344017	0.9577
Week9	4.040681	9.164440	0.6596
Week10	-8.851122	8.229572	0.2831
Week11	18.293036	16.88172	0.2795
Week12	-10.562736	7.696463	0.1711

Appendix 9: Linear mixed model effect analysis showing different sources of variations that may influenced the Blood feeding.

	Value	Std.Error	p-value
(Intercept)	22.223943	9.227391	0.0167
Sleeper 2	3.777416	6.544700	0.5643
Sleeper 3	6.917576	6.758355	0.3070
Sleeper 4	4.361940	6.119352	0.4766
Sleeper 5	1.186007	6.289840	0.8506
Sleeper 6	0.869160	6.417971	0.8924
Hut2	-4.436482	6.085664	0.4667
Hut3	-6.593280	6.327146	0.2984
Hut4	-13.011677	6.174456	0.0360
Hut5	-4.729087	6.429454	0.4627
Hut6	-2.846225	6.079594	0.6401
Week2	5.612935	8.610579	0.5151
Week3	6.941759	8.172390	0.3964
Week4	1.370215	8.818114	0.8766
Week5	2.453404	8.112139	0.7626
Week6	-9.002432	8.422083	0.2861
Week7	-2.650813	8.937378	0.7670
Week8	7.169607	8.529541	0.4014
Week9	15.988995	9.367762	0.0891
Week10	11.157829	8.412140	0.1859
Week11	11.782806	17.263001	0.4955
Week12	15.895848	7.867071	0.0444

Appendix 10: Publications

1. **B.D. Menze**, M.J. Wondji, W. Tchapga, M. Tchoupo, J.M. Riveron and C.S. Wondji. 2018. Bionomics and insecticides resistance profiling of malaria vectors at a selected site for experimental hut trials in central Cameroon. *Malaria Journal*. 17, 317 (2018) https://doi.org/10.1186/s12936-018-2467-2.

2. **B.D. Menze**, M.F. Kouamo, M.J. Wondji, W. Tchapga, M. Tchoupo, M.O. Kusimo, C.S. Mouhamadou, J.M. Riveron and C.S. Wondji. 2020. An Experimental Hut Evaluation of PBO-Based and Pyrethroid-Only Nets against the Malaria Vector *Anopheles funestus* Reveals a Loss of Bed Nets Efficacy Associated with *GSTe2* Metabolic Resistance. *Genes* 11, 143 (2020) doi:10.3390/genes11020143.

3. G.D. Weedall, L.M.J. Mugenzi, **B.D. Menze**, M. Tchouakui, S.S. Ibrahim, N. Amvongo-Adjia, H. Irving, M.J. Wondji, M. Tchoupo, R. Djouaka, J.M. Riveron, C.S. Wondji. 2019. A cytochrome P450 allele confers pyrethroid resistance on a major African malaria vector, reducing insecticide-treated bednet efficacy. *Science Translational Medecine* 11, eaat7386 (2019) doi: 10.1126/scitranslmed.aat7386.

4. L.M.J. Mugenzi, **B.D. Menze**, M. Tchouakui, M.J. Wondji, H. Irving, M. Tchoupo, J. Hearn, G.D. Weedall, J.M. Riveron and C.S. Wondji. 2019. Cis-regulatory CYP6P9b P450 variants associated with loss of insecticide-treated bed net efficacy against *Anopheles funestus*. *Nature Communications* 10, 4652 (2019) https://doi.org/10.1038/s41467-019-12686-5.

1. **B.D. Menze**, M.J. Wondji, W. Tchapga, M. Tchoupo, J.M. Riveron and C.S. Wondji. 2018. Bionomics and insecticides resistance profiling of malaria vectors at a selected site for experimental hut trials in central Cameroon. *Malaria Journal*. 17, 317 (2018) https://doi.org/10.1186/s12936-018-2467-2.

Menze *et al. Malar J (2018) 17:317*
https://doi.org/10.1186/s12936-018-2467-2

Malaria Journal

RESEARCH

Open Access

Bionomics and insecticides resistance profiling of malaria vectors at a selected site for experimental hut trials in central Cameroon

Benjamin D. Menze[1,2*], Murielle J. Wondji[1,2], William Tchapga[2], Micareme Tchoupo[2], Jacob M. Riveron[1,2] and Charles S. Wondji[1,2*]

Abstract

Background: Malaria vectors are increasingly developing resistance to insecticides across Africa. The impact of such resistance on the continued effectiveness of insecticide-based interventions remains unclear due to poor characterization of vector populations. This study reports the characterization of malaria vectors at Mibellon, a selected site in Cameroon for experimental hut study, including species composition, *Plasmodium* infection rate, resistance profiles and mechanisms.

Methods: Indoor resting blood-fed *Anopheles* mosquitoes were collected from houses at Mibellon in 2017 and forced to lay eggs to generate F_1 adult mosquitoes. Insecticides susceptibility bioassays were performed on the F_1 adult mosquitoes following the WHO protocol to assess resistance profile to insecticides. The molecular basis of resistance and *Plasmodium* infection rate were investigated using TaqMan genotyping.

Results: *Anopheles funestus* sensu stricto (s.s.) was predominant in Mibellon (80%) followed by *Anopheles gambiae* s.s. (20%). High levels of resistance to pyrethroids and organochlorides were observed for both species. Moderate resistance was observed against bendiocarb (carbamate) in both species, but relatively higher in *An. gambiae* s.s. In contrast, full susceptibility was recorded for the organophosphate malathion. The PBO synergist assays with permethrin and deltamethrin revealed a significant recovery of the susceptibility in *Anopheles funestus* s.s. population (48.8 to 98.1% mortality and 38.3 to 96.5% mortality, respectively). The DDT/pyrethroid 119F-GSTe2 resistant allele (28.1%) and the dieldrin 296S-RDL resistant (9.7%) were detected in *An. funestus* s.s. The high pyrethroid/DDT resistance in *An. gambiae* correlated with the high frequency of 1014F knockdown resistance allele (63.9%). The 1014S-kdr allele was detected at low frequency (1.97%). The *Plasmodium* infection rate was 20% in *An. gambiae,* whereas *An. funestus* exhibited an oocyst rate of 15 and 5% for the sporozoite rate.

Conclusion: These results highlight the increasing spread of insecticide resistance and the challenges that control programmes face to maintain the continued effectiveness of insecticide-based interventions.

Keywords: Malaria vectors, Insecticide resistance, Characterization, Cameroon

*Correspondence: mbenji2@yahoo.fr; charles.wondji@lstmed.ac.uk
[2] LSTM Research Unit at the Centre for Research in Infectious Diseases (CRID), P.O. Box 13591, Yaoundé, Cameroon
Full list of author information is available at the end of the article

Background

Despite the recent progress made in reducing malaria burden in sub-Sahara Africa through insecticide-based interventions, such as long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS), malaria remains an important health issue [1]. Malaria control in Cameroon has recently witnessed a significant scale-up of vector control intervention through the distribution of LLINs, pushing up the bed net coverage to 70.9% of the population [2, 3]. However, the effectiveness of these insecticide-based control tools is threatened by the emergence of insecticide resistance in major malaria vectors across African countries, including Cameroon [4, 5]. There is, therefore, a need to design and implement suitable resistance management strategies to preserve the efficacy of existing tools. Use of experimental huts is one of the best approaches employed to assess the impact of resistance on the efficacy of control intervention, such as LLINs and IRS [6–8]. However, monitoring the efficacy of these insecticide-based interventions requires a good characterization of the vectors present in the areas under control, importantly, their species composition, insecticides resistance profiles and resistance mechanisms.

In Cameroon, the main malaria vector species are members of the *Anopheles gambiae* sensu lato (s.l.) complex and the *Anopheles funestus* s.l. group [5, 9–13]. However, these species are not evenly distributed [14–16] and their insecticide resistance profiles vary nationwide [5, 14, 17–20]. Therefore, it is important to characterize local populations in order to assess the impact of vector control interventions.

This study provides a thorough characterization of mosquito vector populations in an area selected for experimental hut trials in Cameroon. The site is located in a geographical transition between forested south and savannah north. This study presents a full characterization of the main malaria vectors in Mibellon, including their species diversity, susceptibility profiles and investigates the molecular basis of the resistance and their *Plasmodium* infection rate.

Methods

Study sites

Adult *Anopheles* mosquitoes were collected from Mibellon (6°46′N, 11°70′E), a village in Cameroon located in the Adamawa region, Mayo Banyo Division and Bankim Sub-division. The Adamawa region is in the mountainous area forming a transition between Cameroon's forested south and savanna north. Mibellon is located in close proximity to permanent water bodies, including a lake and swamps which provide suitable breeding sites for mosquitoes. Human activities are mainly fishing, hunting and subsistence farming, including maize, watermelon and coffee plantations. A survey in the area revealed a high usage of insecticides in the coffee and watermelon farms. These insecticides are mainly pyrethroids, neonicotinoids and carbamates.

Mosquito collection and rearing

The blood-fed *Anopheles* mosquitoes were collected every morning between 06:00 and 11:00 in the houses. Verbal consent was given by the household owners before starting mosquito collection. Mosquitoes were collected in January 2017 using the Prokopack electrical aspirator (John W Hook, Gainesville, FL, USA) and kept in netted paper cups that were stored in a cool box. Samples were later transported to the insectary of the LSTM Research Unit in Yaoundé (Cameroon).

Mosquitoes were kept 4–5 days until they became fully gravid and then induced to lay eggs using the forced eggs-laying method [21]. The eggs were put in paper cup containing water for them to hatch. After hatching the larvae were placed in trays and reared to adult mosquitoes [21, 22].

Species identification

The field-caught females were sorted according to morphological keys as previously described [23]. After genomic DNA extraction using the Livak method [24], PCR species identification was performed as described [25] to identify *An. funestus* group members. Whereas the SINE PCR [26] was used to differentiate members of the *An. gambiae* s.l. complex.

Plasmodium falciparum infection rate

Sixty collected *An. funestus* females were cut in two parts: the head and thorax together and the abdomen separately. Genomic DNA was extracted separately from head/thorax and abdomen and the oocyst and the sporozoite rate evaluated using the TaqMan assay [27] using MX 3005 (Agilent, Santa Clara, CA, USA). For *An. gambiae*, due to low number of mosquitoes, the whole mosquito was used and only the overall *Plasmodium* infection rate estimated. One µl of DNA sample was used as template in a 3-step PCR programme with a denaturation at 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The primers (Falcip+: TCT-GAA-TAC-GAA-TGT-C, OVM+: CTG-AAT-ACA-AAT-GCC, Plas-F: GCT-TAG-TTA-CGA-TTA-ATA-GGA-GTA-GCT-TG, Plas r: GAA-AAT-CTA-AGA-ATT-TCA-CCT-CTG-ACA) were used together with two probes labelled with fluorophores: FAM to detect *Plasmodium falciparum*, and HEX to detect *Plasmodium ovale*, *Plasmodium vivax* and *Plasmodium malariae*. *Plasmodium falciparum* samples and a mix of *P. ovale*, *P. vivax* and *P. malariae* were used as positive controls.

Insecticide susceptibility assays

Following WHO protocol [28], F_1 *An. funestus* sensu stricto (s.s.) and *An. gambiae* s.s. mosquitoes aged between 2 and 5 days were exposed to pyrethroids: permethrin (0.75%), deltamethrin (0.05%) and etofenprox (0.05%), the organochlorine: DDT (4%), the organophosphate: malathion (5%) and the carbamates: bendiocarb (0.1%) and propoxur (0.1%). For each test, mosquitoes exposed to untreated papers were used as control. The assay was carried out at temperatures of 25 °C ± 2 °C and 80% ± 10% relative humidity.

PBO synergist assays

To investigate the possible involvement of cytochrome P450 s in the observed resistance, female *An. funestus* s.s. were pre-exposed to 4% piperonyl butoxide (PBO) for 1 h and immediately exposed to DDT (4%), permethrin (0.75%) and deltamethrin (0.05%) for 60 min. The mortality was assessed after 24 h and compared with mortality obtained for mosquitoes not pre-exposed to PBO.

Cone assays

Cone assays were performed using five types of LLINs (Olyset Plus, Olyset Net, Yorkool, PermaNet 2.0 and PermaNet 3.0 (side). These tests were carried out using pieces of LLINs (30 cm × 30 cm). Ten unfed mosquitoes (*An. funestus* s.s. and *An. gambiae* s.s.) females aged 2–5 days were introduced into each cone placed on the LLIN for 3 min. After exposure, the mosquitoes were removed from the cones using a mouth aspirator and then transferred into paper cups and fed with 10% sugar solution. Numbers of mosquitoes knocked-down were recorded after 60 min. A negative control (untreated net) was included in each of the LLIN cone test. Post-exposure mortality was recorded after 24 h of observation. The assay was carried out at temperature of 25 °C ± 2 °C and 80% ± 10% relative humidity.

Genotyping of L119F-GSTe2: metabolic resistance marker in *Anopheles funestus* s.s.

The L119F-GSTe2 mutation was genotyped to assess the role played by the glutathione S-transferase gene in the DDT and pyrethroid resistance observed at Mibellon as described previously [29]. An allele specific PCR (Tchouakui et al., unpublished) was used to detect the 3 genotypes of the L119F-GSTe2 mutation. Amplification was carried out using PCR parameters of 95 °C for 5 min; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s, final extension at 72 °C for 10 min. The primers employed for the genotyping are: L119F-Fwd: ATG ACC AAG CTA GTT CTG TAC ACG CT; L119F-Rev: TTC CTC CTT TTT ACG ATT TCG AAC T; L119F-Res1: CGG GAA TGT CCG ATT TTC CGT AGA A**t**AA; L119-F-Sus1: CAT TTC TTA TTC TCA TTT ACA GGA GCG TAaT**C.**

Genotyping of A296S-RDL in *Anopheles funestus* s.s.

TaqMan genotyping assays were performed in 10 µl volume containing 1 × Sensimix (Bioline), 80 × primer/probe mix and 1 µl genomic DNA. The probes were labelled with two distinct fluorophores FAM and HEX, FAM to detect the resistant allele and HEX to detect the susceptible allele. The assay was performed on an Agilent MX3005 real-time PCR machine with cycling conditions of 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min as described previously [30].

L1014F and L1014S *kdr* genotyping in *Anopheles gambiae*

The L1014F-kdr mutation and the L1014S-kdr responsible for DDT and pyrethroid resistance in *An. gambiae* s.l. were genotyped in Mibellon mosquitoes. The reaction mixture of 10 µl final volume containing 1 × Sensimix (Bioline), 80 × primer/probe mix and 1 µl template DNA was used for this assay. The probes were labelled with two distinct fluorophores: FAM to detect the resistant allele and HEX to detect the susceptible allele. The assay was performed on an Agilent MX3005 real-time PCR machine with cycling conditions of 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min as previously described [31].

G119S *ace-1* genotyping in *Anopheles gambiae*

The G119S *ace-1* responsible to organophosphate and carbamate resistance in *An. gambiae* s.l. was genotyped in Mibellon mosquitoes. Ten µl volume containing 1 × Sensimix (Bioline), 80 × primer/probe mix and 1 µl template DNA. Probes were labelled with two specific fluorophores FAM and HEX, FAM to detect the resistant allele, HEX to detect the susceptible allele. The assay was performed on an Agilent MX3005 real-time PCR machine with cycling conditions of 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

Results

Field collection

A total of 722 adult female *Anopheles* mosquitoes were collected indoor at Mibellon out of which 584 (80.1%) were morphologically identified as *An. funestus* group and the remaining 138 (19.9%) identified to be *An. gambiae* complex.

Species diversity among *Anopheles funestus* group and *Anopheles gambiae* complex

Out of the 120 *An. funestus* s.l. mosquitoes randomly selected and tested, *An. funestus* s.s. was found to be the

only member of the group present at Mibellon. For *An. gambiae* s.l., out of 89 samples analysed all of them were found to be *An. gambiae* s.s. (S form).

Insecticide susceptibility bioassays with samples from Mibellon

Insecticide susceptibility bioassays in An. funestus s.s.

A total of 2700 F_1 mosquitoes were tested to assess the resistance profile to seven insecticides (Fig. 1). *Anopheles funestus* s.s. (females and males) were resistant to type I and type II pyrethroids. For permethrin (Type I), mortality was 48.88 ± 5.76% for females and 90.72 ± 3.77% for males. For deltamethrin (Type II), mortality was 38.34 ± 5.79% for females and 53.96 ± 11.37% for males. However, mortality was higher for the pseudo-pyrethroid etofenprox with mortality rate of 82.9 ± 8.7% for females and 97.83 ± 2.17% for males (Fig. 1a). For the organochlorine DDT, resistance was observed with mortality rate of 55.28 ± 8.28% for females and 83.78 ± 3.13% for males (Fig. 1a). For the carbamates, a possible resistance was observed against bendiocarb with 90.67 ± 4.3% mortality for females and 95.06 ± 1.97% for males (Fig. 1a) whereas a susceptibility was observed for propoxur with 98.41 ± 1.59% mortality for females and 100% mortality for males (Fig. 1a). Full susceptibility was observed for the organophosphate malathion (Fig. 1a).

Insecticide susceptibility bioassays in An. gambiae s.s.

A total of 971 *An. gambiae* s.s. were tested to assess the resistance profile to seven insecticides. The *An. gambiae* s.s. population was highly resistant to type I and type II pyrethroids (Fig. 2). For permethrin, mortality was 0 ± 0% for females and 4.44 ± 2.42% for males (Fig. 2). Very high level of resistance was also observed with deltamethrin with no mortality for females and 1.52 ± 1.52% for males (Fig. 2). Resistance was also observed for the pseudo-pyrethroid etofenprox with mortality rate of 9.92 ± 7.66% for females and 13.46 ± 3.93% for males (Fig. 2). High level of resistance to the organochlorine DDT was observed with mortality rate of 1.39 ± 1.39% for females and 1.85 ± 1.85% for males (Fig. 2). For the carbamate bendiocarb, mortality rates of 66.23 ± 2.6% for females and 59.2 ± 3.73% for males were recorded, however, full susceptibility was observed for propoxur (Fig. 2). For the organophosphate malathion, a near full susceptibility was observed with mortality rate of 98.25 ± 1.75% for females and 98.48 ± 1.52% for males (Fig. 2).

PBO synergist assays with An. funestus s.s.

Pre-exposure of *An. funestus* s.s. to PBO led to recovery of the susceptibility to both pyrethroids type I and II with an increased mortality observed after PBO exposure from 48.88 ± 5.76% to 98.81 ± 3.77% for permethrin and from 38.34 ± 5.79% to 96.54 ± 1.16% mortality for deltamethrin (Fig. 1b). Partial recovery was observed for DDT with increased susceptibility from 55.28 ± 8.28% to 84.16 ± 5.37% mortality after exposure to PBO (Fig. 1b).

Susceptibility profile against bed nets by cone assays

Insecticide susceptibility with cone assays in An. funestus s.s.

Low mortality rates were recorded against most of the nets tested. The mortality rates were 2.78 ± 2.78%, and 2.5 ± 2.5%, for Olyset Net and Olyset Plus, respectively; 0 ± 0% and 48.33 ± 6.49% for PermaNet 2.0 and

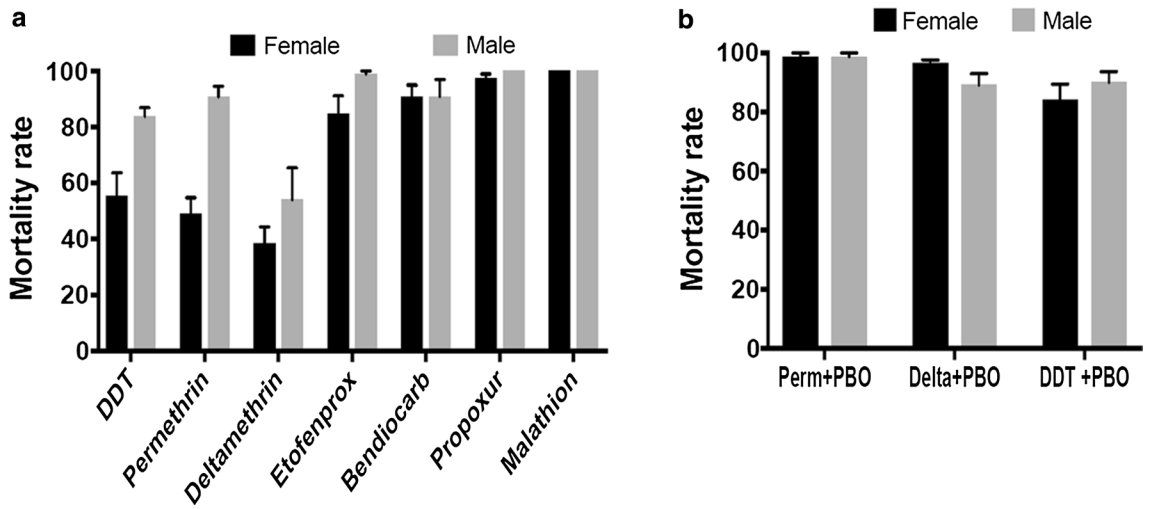


Fig. 1 Susceptibility profile of *Anopheles funestus* to insecticides. **a** Recorded mortalities following 60-min exposure of *An. funestus* s.s. from Mibellon to different insecticides. Data are shown as mean ± SEM. **b** Activities of PBO combined to permethrin, deltamethrin and DDT on *An. funestus* s.s. from Mibellon. Data are shown as mean ± SEM

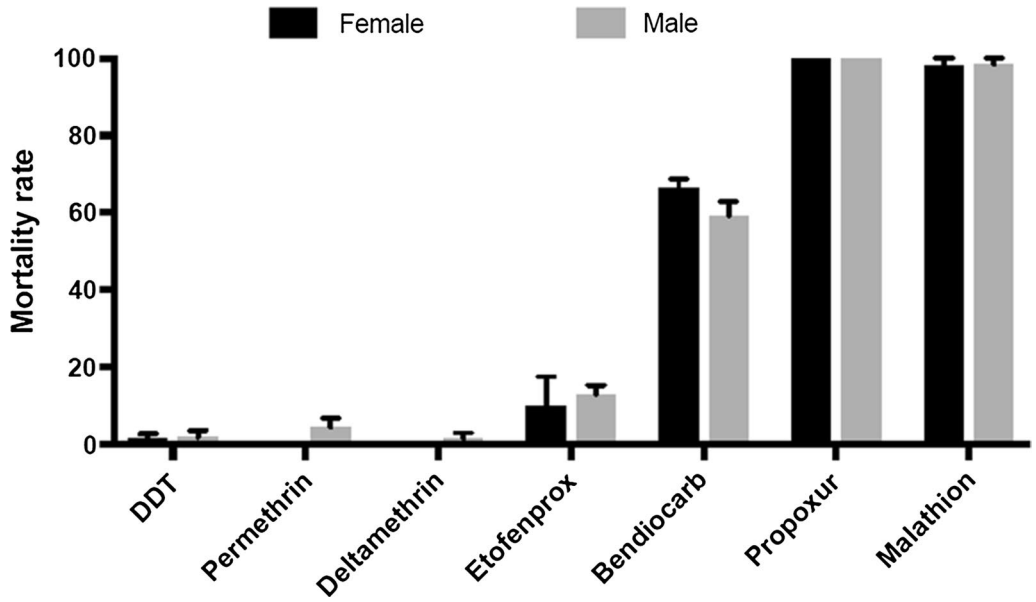


Fig. 2 Susceptibility profile of *Anopheles gambiae* to insecticides. Recorded mortalities following 60-min exposure of *An. gambiae* s.s. Mibellon to different families of insecticide. Data are shown as mean ± SEM

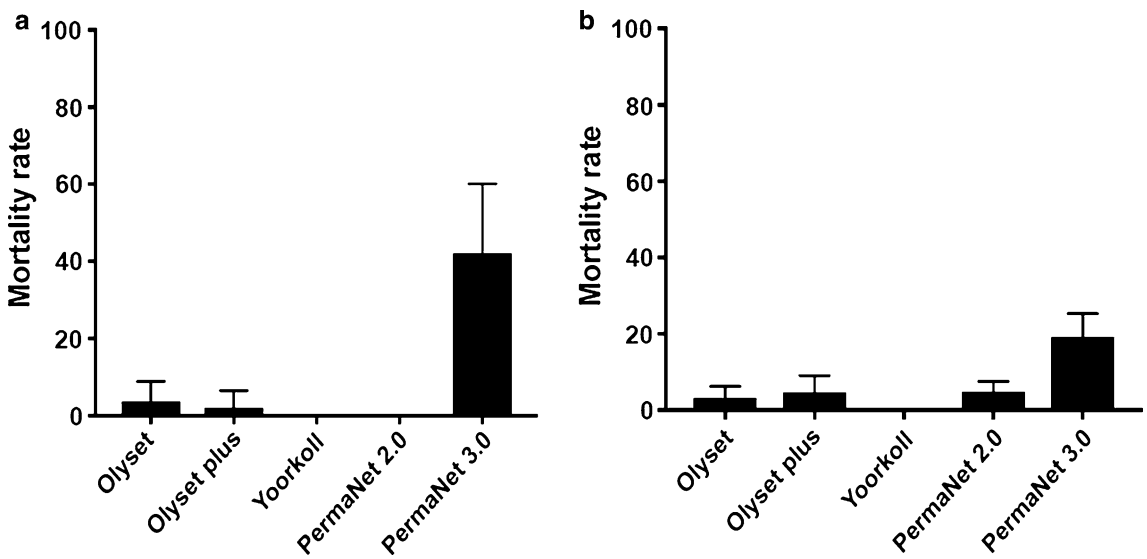


Fig. 3 Cone assays with various nets for *Anopheles funestus* and *Anopheles gambiae*. **a** Recorded mortalities following 3-min exposure by cone assay of *An. funestus* s.s. **b** *Anopheles gambiae* s.s. from Mibellon to Olyset, Olyset Plus, Yorkool, PermaNet 2.0 and PermaNet 3.0 (side). Data are shown as mean ± SEM

PermaNet 3.0, respectively; and, 0 ± 0% for Yorkool (Fig. 3a).

Insecticide susceptibility cone assays in An. gambiae s.s.

Similarly, *An. gambiae s.s* showed low mortality rates with the bed nets tested. The mortality rates were 3.13 ± 3.13% and 4.55 ± 4.55% for Olyset Net and Olyset Plus, respectively; 4.77 ± 2.76% and 19.09 ± 6.22% for PermaNet 2.0 and PermaNet 3.0, respectively; and, 0 ± 0% mortality for Yorkool (Fig. 3b).

Molecular basis of the insecticide resistance in *An. funestus* s.s. population

L119F-GSTe2 detection in An. funestus s.s.

From 110 F_0 females collected from the field 7 were homozygous resistant (RR) (6.3%), 48 were heterozygous (RS) (43.2%) and 55 were homozygous susceptible (SS) (49.5%). Overall, the frequency of the 119F resistant allele was 28 and 72% for the L119 susceptible allele.

RDL-A296S detection in An. funestus s.s.

Out of the 92 samples genotyped, 3 were RR (3.2%), 12 were RS (13.1%) and 75 were SS (83.7%) with frequency of resistant allele of only 9.7% and susceptible allele of 90.3%.

Molecular basis of insecticide resistance in *An. gambiae* s.s.

L1014F/L1014S kdr detection in An. gambiae s.s.

Out of 72 samples genotyped, 22 were RR (30.5%) 48 were RS (66.6%) and 2 were SS (2.7%) with frequency of the resistant allele 1014F of 63.9% and the susceptible L1014 allele of 36.1%. Out of the 76 samples genotyped for the L1014S marker, 3 were RS (3.9%) and 73 were SS (96.1%), thus a very low frequency of 1.97% was recorded for the resistance allele.

G119S ace-1 detection in An. gambiae s.s.

Out of 50 samples genotyped, neither RR nor RS were detected as all the samples were found to be SS.

Plasmodium infection rate in the *An. s funestus* s.s. population in Mibellon

A total of 60 *An. funestus* from Mibellon were tested for *Plasmodium* infection using TaqMan from the head-thorax and from the abdomen separately. The analysis of the head and thorax revealed 3 (5%) mosquitoes infected, which included 2 (3.3%) *P. falciparum* and 1 (1.7%) infection which is either *P. ovale*, *P. vivax* or *P. malariae*. However, 8 (15%) mosquitoes were detected infected when the abdomen was examined, including 5 (8.3%) *P. falciparum*, 3 (5%) infections which are either

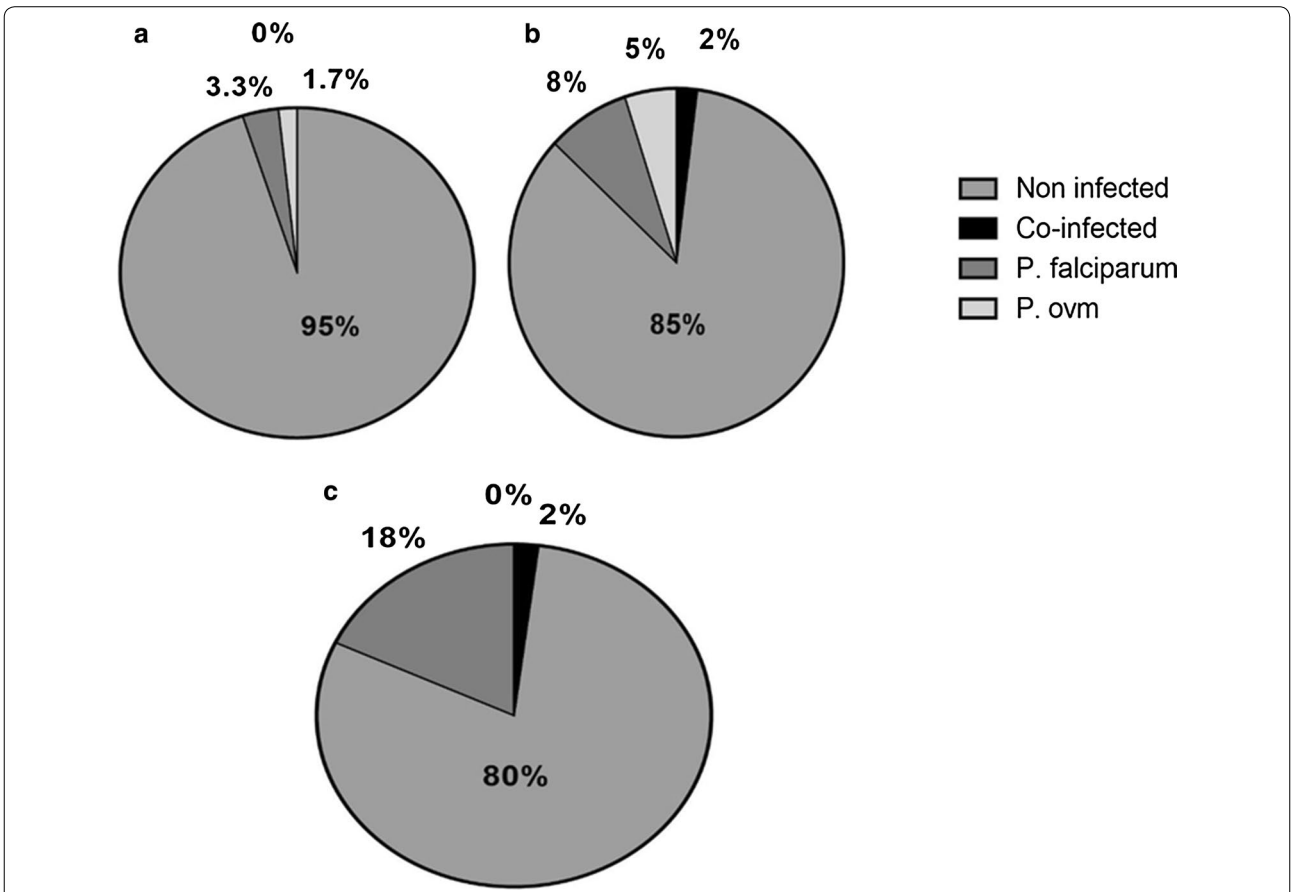


Fig. 4 *Plasmodium* infection rate in malaria vectors from Mibellon in 2017. **a** *Plasmodium* infection rate in *An. funestus* s.s. with head and thorax. **b** *Plasmodium* infection rate in *An. funestus* s.s. with abdomen only. (C) *Plasmodium* infection rate in *An. gambiae* s.s. for whole mosquitoes

P. ovale, *P. vivax* or *P. malariae* and 1 (1.7%) mixed infections (Fig. 4).

Plasmodium infection rate in the *An. gambiae* s.s. population in Mibellon

A total of 60 *An. gambiae* were tested for *Plasmodium* infection from the whole body. The analysis revealed 12 (20%) mosquitoes infected, which included 11 (18.3%) *P. falciparum* and 1 (1.7%) mixed infection (Fig. 4).

Discussion

Characterization of malaria vector populations is a prerequisite for the implementation of successful vector control interventions and for assessing the impact of insecticides resistance. This study comprehensively characterized the main malaria vectors in Mibellon, a location in Cameroon chosen for the implementation of experimental huts to assess the efficacy of insecticide-based interventions.

Species composition

Out of the 9 species of *An. funestus* group described, only *An. funestus* s.s. was detected in Mibellon. This result is similar to the one observed in the northern part of Cameroon, in Gounougou, where *An. funestus* s.s. was 99.5% of the total collection and *Anopheles leesoni* was 0.5% [5]. The predominance of *An. funestus* s.s. in the *An. funestus* group is also mentioned in West Africa as observed at Kpome in Benin [30]. The focus on indoor blood-fed mosquito collection in this study may have prevented to collect the outdoor resting members of the group. Nevertheless, this is in contrast with the distribution of members of this group observed in eastern and southern parts of Africa where several members are collected indoors. For instance, in Uganda *Anopheles parensis* was found predominantly indoors [32, 33]. Similarly, many other species of the *An. funestus* group have been collected indoors in southern Africa, including *An. parensis, An. leesoni* and *Anopheles rivulorum* [34–36]. For the *An. gambiae* complex, *An. gambiae* s.s., previously known as S form, was the only species found. This result is similar to previous observations showing that *An. gambiae* s.s. was the main species in localities south of the Adamawa mountains, characterized by humid savannah and forested areas [10, 37].

The multiple insecticide resistance in both major vectors is a challenge for vector control

The multiple resistance pattern observed in this *An.s funestus* population is similar to the pattern observed in the northern part of the country, with the exception of bendiocarb resistance which was higher in the north [5]. This result suggests that resistance in *An. funestus* is widespread in Cameroon and could be a concern for effective insecticide-based control tools against this vector. *Anopheles funestus* is highly resistant to both type I and II pyrethroids. The resistance observed in Mibellon seems higher than in Gounougou (2012). This difference could be due to the length of time between the two studies as resistance could have increased since 2012 when the study at Gounougou was carried out. It could also be evidence that the pyrethroid resistance level in *An. funestus* in Cameroon is increasing. This increase could be due to the massive distribution of LLINs implemented by Cameroonian Government in the past 5 years. Mibellon is also located in an area where agriculture is the main activity, and agricultural use of pesticides could be another factor that is driving the increase in resistance level. A similar level of increase of pyrethroid resistance was observed in southern Malawi [35] and also reported in Uganda for *An. funestus* [32]. The high recovery of susceptibility observed after PBO exposure for deltamethrin, permethrin and DDT suggests that cytochrome P450 genes are playing a significant role in these resistance patterns. Altogether, this resistance to pyrethroids should be of great concern for malaria control programmes in Cameroon. If no strong action is taken to manage it, there is a risk that the massive distribution of pyrethroid-impregnated LLINs taking place in Cameroon could be jeopardized. The possible resistance observed to the carbamate bendiocarb in *An. funestus* population shows that such insecticide should be ruled out as an alternative to pyrethroid for IRS. The full susceptibility to the organophosphate malathion, as also demonstrated across the continent so far, suggests that this insecticide class is the most suitable for IRS against this species.

Very high level of resistance to several classes, including organochlorine, pyrethroid and carbamate, was also observed in *An. gambiae* s.s. population. This high resistance in *An. gambiae* is in line with the increased resistance reported in this species in several sites across Cameroon [14, 17–20]. The resistance in *An. gambiae* was higher than in *An. funestus* for most insecticides (e.g., no mortality against permethrin in *An. gambiae vs* 40.9% in *An. funestus*) suggesting a greater selection operating in *An. gambiae*. This could be explained by a selection of resistance from breeding sites contaminated with insecticides used for the protection of the crops. As *An. gambiae* breeding sites are often located at the vicinity of crops, the selection could be greater in this species compared to *An. funestus* because of mosquito-breeding sites in Mibellon, where there is a large lake that insecticides from farms drain into.

Bio-efficacy of LLINs in cone assays

The low mortality rates observed with permethrin and deltamethrin for both species in Mibellon correlated with the reduced bio-efficacy of most LLINs observed with cone assays including PBO-based nets such as Olyset Plus. This loss of efficacy may be due to selection pressure induced by the massive distribution of bed nets by the government [2, 3] in addition to the use of pesticide for farming in the area. For both species, the mortality for all the nets is very low except for PermaNet 3.0 (side). For *An. funestus*, the mortality rates for all LLINs are lower than has been recently reported from other countries such, as DR Congo [38]. However, because cone assays could underestimate the efficacy of LLINs, as they do not assess their additional excito-repellent effect [39], future studies with experimental huts are needed to establish the impact of resistance on LLINs in this region.

Predominance of metabolic resistance in *An. funestus* contrasts with high frequency of knockdown resistance in *An. gambiae*

The near full recovery observed for pyrethroids in *An. funestus* after pre-exposure to PBO indicates that resistance is mainly conferred by metabolic resistance particularly by cytochrome P450s [40]. This is in line with previous reports of the absence of *kdr* in this species in Cameroon [5] and across Africa [41]. The frequency of the 119F-GSTe2-resistant allele in Mibellon field population (28%) is lower than in the northern part of Cameroon in Gounougou (52%) [5] or in Ghana (44.2%) [42] and Benin (56.25%) [30]. The frequencies in Mibellon are closer to that observed in the eastern part of Africa in Uganda (20.4%) [21, 32]. GSTe2 has been shown to confer cross-resistance between DDT and pyrethroids [42]. The partial recovery of DDT susceptibility after PBO assays suggests that GSTe2 is probably playing a role in the resistance in this *An. funestus* s.s. population.

The frequency of the 296S-RDL-resistant allele is only 9.7%, which is lower than was observed in the northern region at Gounougou. This low frequency could be as a result of recovery to susceptibility for dieldrin after this insecticide was removed from public and agricultural sectors in Cameroon, as observed in Gounougou where the frequency of this mutation went down from 80% in 2006 to 40% in 2012 and 14.6% in 2015 [5, 43]. Such reversal of resistance is encouraging for the implementation of resistance management strategies.

The very high resistance levels to pyrethroids in *An. gambiae* s.s. (no mortality to permethrin), correlates with the high frequency of the 1014F *kdr* allele (63.9%). This is in line with other reports from Africa where high pyrethroid resistance in *An. gambiae* s.l. has been associated with nearly fixed *kdr* allele in the population, as observed recently in DR Congo [38], or previously in Côte d'Ivoire [44]. However, this high level of *kdr* is in contrast to frequencies observed in other locations across Cameroon as highlighted by a recent review [17]. The very low frequency of the 1014S *kdr* allele in Mibellon is similar to previous reports across Cameroon showing that this marker, originally present in East Africa, has now migrated to Central and West Africa although still at very low frequencies [45]. Overall, the fact that 1014F *kdr* frequency is not fixed in Mibellon in the presence of such high pyrethroid and DDT resistance suggests that other mechanisms are playing an important role, probably metabolic resistance as shown for other *An. gambiae* s.l. populations in Cameroon [18, 19, 46]. Further investigation of the resistance mechanisms will help elucidate the molecular basis driving resistance in this population. The total absence of the 119S *ace-1* mutation is in line with the susceptibility of this population to organophosphate and carbamate as this mutation is responsible for organophosphate and carbamate resistance [47, 48].

Malaria transmission roles of both vectors

This study further confirms the role of *An. funestus* in malaria transmission with sporozoite infection rate of 5%. This is lower compared to that observed [49] in Nkoteng where *P. falciparum* infection rate was found to be 8.6%. In Benin (Kpome), *An. funestus* population was found with high *Plasmodium* infection during the dry season (infection rate of 18.2%), although from the whole mosquitoes [30]. The higher infection rate found in abdomen (15%) compared to head and thorax (5%) in this study further supports the significant barriers that the midgut plays in preventing oocyst migration to salivary gland [50]. The number of *An. gambiae* s.s. infected with *Plasmodium* (20%) is higher compared to *An. funestus* s.s. (15%). This rate is very high compared to previous results in Cameron (6.5–8.1%) [12]. This could be linked to the higher level of insecticide resistance in the *An. gambiae* population, because they are resistant and live longer, which could increase their ability to be infected.

Conclusion

Multiple resistance observed in both *An. funestus* s.s. and *An. gambiae* s.s. at Mibellon in central Cameroon is a concern for ongoing insecticide-based interventions although the full susceptibility to organophosphate offers an alternative to IRS. However, the impact of such multiple resistance on the effectiveness of insecticide-based control interventions needs to be evaluated, notably through experimental huts. The presence of resistance in both major vectors makes this area suitable for such studies.

Abbreviations
ADN: deoxyribonucleic acid; DDT: dichlorodiphenyltrichloroethane; PCR: polymerase chain reaction; LLINs: long-lasting insecticidal nets; IRS: indoor residual spraying; Kdr: knockdown resistance; *s.s*: sensu *stricto* (in the strict sense); s.l.: sensu lato; PBO: piperonyl butoxide; WHO: World Health Organization.

Authors' contributions
CSW conceived, designed and coordinated the research. BDM and WT carried out the sample collection and bioassays. BDM, MJW and MT performed the PCR and TaqMan. CSW and BDM contributed toward data analysis. BDM, JMR and CSW wrote the manuscript with contribution from all the authors. All authors read and approved the final manuscript.

Author details
[1] Vector Biology Department, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK. [2] LSTM Research Unit at the Centre for Research in Infectious Diseases (CRID), P.O. Box 13591, Yaoundé, Cameroon.

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An Experimental Hut Evaluation of PBO-Based and Pyrethroid-Only Nets against the Malaria Vector *Anopheles funestus* Reveals a Loss of Bed Nets Efficacy Associated with *GSTe2* Metabolic Resistance

Benjamin D. Menze [1,2,*], Mersimine F. Kouamo [2,3], Murielle J. Wondji [1,2], Williams Tchapga [2], Micareme Tchoupo [2], Michael O. Kusimo [2], Chouaibou S. Mouhamadou [4], Jacob M. Riveron [5] and Charles S. Wondji [1,2,*]

1 Vector Biology Department, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK; Murielle.Wondji@lstmed.ac.uk
2 Centre for Research in Infectious Diseases (CRID), LSTM Research Unit, Yaoundé 13591, Cameroon; mersimine.kouamo@crid-cam.net (M.F.K.); William.tchapga@crid-cam.net (W.T.); micareme.tchoupo@crid-cam.net (M.T.); gkusimo@gmail.com (M.O.K.)
3 Faculty of Sciences, University of Yaoundé I, Yaoundé 337, Cameroon
4 Centre Suisse de Recherches scientifiques (CSRS), Yopougon 1303, Abidjan, Cote d'ivoire; mouhamadou.chouaibou@csrs.ci
5 Syngenta UK Limited, CPC4 Capital Park, Fulbourn, Cambridgeshire CB21 5XE, UK; Jacob.Riveron_Miranda@syngenta.com
* Correspondence: Benjamin.Menze@lstmed.ac.uk (B.D.M.); charles.wondji@lstmed.ac.uk (C.S.W.)

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Abstract: Growing insecticide resistance in malaria vectors is threatening the effectiveness of insecticide-based interventions, including Long Lasting Insecticidal Nets (LLINs). However, the impact of metabolic resistance on the effectiveness of these tools remains poorly characterized. Using experimental hut trials and genotyping of a glutathione S-transferase resistance marker (L119F-*GSTe2*), we established that GST-mediated resistance is reducing the efficacy of LLINs against *Anopheles funestus*. Hut trials performed in Cameroon revealed that Piperonyl butoxide (PBO)-based nets induced a significantly higher mortality against pyrethroid resistant *An. funestus* than pyrethroid-only nets. Blood feeding rate and deterrence were significantly higher in all LLINs than control. Genotyping the L119F-*GSTe2* mutation revealed that, for permethrin-based nets, 119F-*GSTe2* resistant mosquitoes have a greater ability to blood feed than susceptible while the opposite effect is observed for deltamethrin-based nets. For Olyset Plus, a significant association with exophily was observed in resistant mosquitoes (OR = 11.7; $p < 0.01$). Furthermore, *GSTe2*-resistant mosquitoes (cone assays) significantly survived with PermaNet 2.0 (OR = 2.1; $p < 0.01$) and PermaNet 3.0 (side) (OR = 30.1; $p < 0.001$) but not for Olyset Plus. This study shows that the efficacy of PBO-based nets (e.g., blood feeding inhibition) against pyrethroid resistant malaria vectors could be impacted by other mechanisms including GST-mediated metabolic resistance not affected by the synergistic action of PBO. Mosaic LLINs incorporating a GST inhibitor (diethyl maleate) could help improve their efficacy in areas of GST-mediated resistance.

Keywords: malaria; Long Lasting Insecticidal Nets; insecticide resistance; metabolic resistance; glutathione S-transferase; *Anopheles funestus*; piperonyl butoxide

1. Introduction

The scale-up of insecticide-based interventions including Long Lasting Insecticidal Nets (LLINs) and Indoor Residual Spraying (IRS) has significantly contributed to the considerable reduction of malaria burden in the past decade [1]. Unfortunately, growing insecticide resistance in malaria vectors beside drugs resistance, lack of vaccine and reduced donor funding are threatening these successes. However, the actual impact of resistance, notably metabolic resistance, on the effectiveness of vector control tools against pyrethroid resistant mosquito populations remains a topic of debate especially when using entomological outcomes. Indeed, some studies have suggested that pyrethroid resistance does not yet impact the effectiveness of LLINs [2] whereas others have revealed a negative impact [3]. Furthermore, to help manage resistance, novel LLINs with the piperonyl butoxide (PBO), an insecticide synergist, have been designed by various manufacturers [4–6]. A randomized control trial comparing PBO-based nets to pyrethroid-only nets [3] led WHO to recommend in 2017 that national malaria control programmes and their partners should consider the deployment of pyrethroid-PBO nets in areas where the main malaria vector(s) have pyrethroid resistance that is: (a) confirmed, (b) of intermediate level, and (c) conferred (at least in part) by a monooxygenase-based resistance mechanism [7]. However, it remains unclear how these pyrethroid-PBO nets will performed when other mechanisms such as GST-based metabolic resistance are driving the resistance. One of the key challenges of assessing the impact of such metabolic resistance on the effectiveness of insecticide-based interventions such as LLINs has been the lack of molecular markers for resistance, as this phenotype is not easily associated with the outcome of the interventions. Recent efforts have detected a key genetic marker in the glutathione S-transferase epsilon 2 gene (*GSTe2*). This marker confer metabolic-mediated resistance to pyrethroids and dichlorodiphenyl-trichloroethane (DDT) in the major malaria vector *Anopheles funestus* in West and Central Africa [8]. Besides cytochrome P450s and esterases, GSTs are, one of the main enzyme families conferring metabolic resistance to insecticides [9] either through a direct metabolism or by catalyzing the secondary metabolism of substrates oxidized by cytochrome P450s [9]. Over-expression of GST epsilon 2 (*GSTe2*) has been associated with DDT and/or pyrethroids resistance in several mosquito species including *An. gambiae* [10], *An. funestus* [8] and *Aedes aegypti* [11]. In *An. funestus*, genomic and structural analyses revealed that a leucine to phenylalanine amino acid change (L119F) in *GSTe2*, has enlarged the substrate-binding pocket of the enzyme, conferring DDT/pyrethroid resistance in West and Central African populations [8]. This single amino acid change was used to design a simple field applicable DNA-based diagnostic tool [12] providing the opportunity to address questions regarding the direct impact of GST-mediated metabolic resistance on insecticide-based interventions such as LLINs.

The pyrethroid resistance in *An. funestus* across Africa is driven by metabolic resistance [13–15] as no knockdown resistance *(kdr)* has been reported so far for this species [16]. This predominance of the metabolic resistance mechanism in *An. funestus* through over-expression of detoxification genes such as GSTs or P450s makes this vector suitable to assess the impact of metabolic resistance on control interventions. The presence of the GST-mediated metabolic resistance in *An. funestus* populations such as in Cameroon [8,17,18] provides the opportunity to assess how the effectiveness of PBO-based nets is impacted when malaria vectors exhibit other type of metabolic resistance than cytochrome P450-based resistance which have been so far the only focus of synergists.

Experimental hut studies are the method of choice to evaluate the efficacy of LLINs against mosquito populations using entomological indices as a proxy for potential epidemiological impact [19,20]. It also provides relevant samples to assess how metabolic resistance impacts the effectiveness of LLINs as done previously to assess impact of target site mutations such as *kdr* [21].

Here, we assessed the performance of conventional pyrethroid-only nets versus PBO-based against a pyrethroid resistant *An. funestus* population from Cameroon using experimental huts. Furthermore, we took advantage of the L119F-*GSTe2* DNA-based diagnostic assay [8], to assess the impact of GST-mediated metabolic resistance on the performance of these five LLINs.

2. Materials and Methods

2.1. Study Area

The experimental hut station was located in Mibellon (6°4′60′′ N, 11°30′0′′ E), a village in Cameroon located in the Adamawa Region; Mayo Banyo Division and Bankim Sub-division. The Adamawa region is in the mountainous zone forming a transition between Cameroon's forested south and savanna north. Malaria transmission is perennial with a high transmission as shown by very high infection rate of Plasmodium infection caused by *Plasmodium falciparum* but also *P. malariae* [12]. The village was located in close proximity to permanent water bodies including a lake and swamps which provide suitable breeding sites for *An. funestus* s.l. Human activities are mainly fishing, hunting and subsistence farming including maize, watermelon and coffee plantations. At the experimental station, 12 huts built following the World Health Organisation (WHO) standard [22], were available for a wide range of experimental hut trials. *An. funestus* s.s. was the main malaria vector in the area [18]. *Mansonia* sp., and *Culex* sp. were also present in the area. *Anopheles* mosquitoes in the area were highly resistant to pyrethroid and DDT [18]. The trial was carried out for 10 weeks during the rainy season between 10 July and 16 October 2016.

2.2. Experimental Hut Design

The huts are built following the prototype recommended by WHO for the West African region [22]. The hut is constructed on a concrete base surrounded by a drain channel to trap ants. The walls are made from concrete bricks and plastered inside and outside with a plaster made from a mixture of cement and sand. The roof is made from corrugated iron and the ceiling is made from plywood. The 4 windows located on three side of the hut are designed to create an angle with a 2 cm gap, which will facilitate the entry of mosquitoes flying upward and prevent the mosquitoes from escaping once they have entered the hut. A veranda trap is built at the back of the hut according to WHO protocol [23]. A curtain is used as a separation between the veranda and the rest of the hut. Before bedtime, each sleeper is required to raise the curtain to give mosquitoes the opportunity to take refuge in the veranda. In the morning, it is recommended that the sleeper lowers the curtain before starting the collection, to allow a separate collection of mosquitoes in the veranda and in the hut.

2.3. Net Treatment/Arm Comparison

During the experimental hut trial, four LLINs and one untreated net as negative control were compared (Table 1). These included two pyrethroid-only LLINs (PermaNet 2.0 and Olyset) and two PBO-based nets (PermaNet 3.0 and Olyset Plus) (Table 1). Each net was holed according to WHO protocol [23]. Six holes were generated (4 cm × 4 cm) per net, two on each of the long sides and one on each of the short sides.

Table 1. Description of the long-lasting insecticidal nets used.

Treatment Arm	Description	Manufacturer
Untreated	100% polyester with no insecticide	Local market
Olyset	8.6×10^{-4} kg/m^2 (2%) of permethrin incorporated into polyethylene	Sumitomo Chemical
Olyset Plus	8.6×10^{-4} kg/m^2 (2%) of permethrin and 4.3×10^{-4} kg/m^2 (1%) of Piperonyl butoxide (PBO) incorporated into polyethylene	Sumitomo Chemical
PermaNet 2.0	100% polyester coated with 1.8 g/kg of deltamethrin	Vestergaard Frandsen
PermaNet 3.0	Combination of 2.8 g/kg of deltamethrin coated on polyester with strengthened border (side panels) and deltamethrin (4.0 g/kg) and PBO (25 g/kg)	Vestergaard Frandsen

2.3.1. Hut Effect

Prior to the study, the hut effect was assessed to evaluate any specific attractiveness of huts. Untreated nets were hung in the six huts used for the study, and during 3 weeks, between the 10 and 29 May 2016, volunteers sleeping underneath collected mosquitoes each morning.

2.3.2. Bioassays and Cone Assays

Cone bioassays were performed at the beginning of the study using the *An. coluzzii* susceptible Ngousso laboratory strain. This was done to confirm the quality of the five bed nets used in the study. Cone bioassays were also done in the insectary using F1 progeny from field collected *Anopheles funestus* from Mibellon. For PermaNet 2.0, PermaNet 3.0 (side and top), Olyset and Olyset Plus beside an untreated control net, five batches of 10 unfed females, 2–5 days old, were exposed to each bed nets using WHO cone assays for three minutes [22]. They were then transferred into a holding paper cup containers and the knock down was checked after 60 min and the mortality after 24 h post-exposure during which mosquitoes were provided sugar solution. Bioassays were also performed to generate highly resistant (alive after 90 min exposure) and highly susceptible (dead after 30 min) mosquitoes against 0.75% permethrin, 0.05% deltamethrin and 4% DDT WHO papers. The 30 and 90 min exposure tests were performed in separate tubes then scored 24 h post-exposure. For each test 4 replicates of 25 mosquitoes were used. The samples were then used to assess the association between L119F-*GSTe2* genotypes and resistance to these insecticides.

2.4. Experimental Hut Trial

The experimental hut trial was carried out during 60 night following the protocol described in the guidelines for laboratory and field-testing of long-lasting insecticidal nets [23]. To correct any specific attractiveness observed during the hut effect assessment, bed nets were rotated according to the Latin design square rotation [23] so that at the end of the study each net would have spent six days in each hut. The huts were cleaned weekly before the rotation of the nets. Six volunteer males were selected to sleep in the room from 20:00 GMT in the evening to 5:00 GMT in the morning. The sleepers were also rotated every day so that at the end of the week each sleeper would have spent one night in each hut. The rotation of the sleepers was done to correct any bias due to any specific attractiveness from the sleepers. In addition, the sleepers were blinded to the treatments.

2.4.1. Mosquito Collection

Mosquitoes were collected every morning, using hemolysis tubes from: (i) inside the nets, (ii) in the room: floor, walls and roof, and (iii) in the veranda exit trap. Mosquitoes collected from each compartment were kept separately in a bag to avoid any mixing between samples from different compartments. Samples were then classified as dead, alive, blood fed or unfed. The 'alive' samples were kept in the paper cup and provided with sugar solution for 24 h and mortalities monitored.

The field-caught females were sorted according to morphological keys as previously described [24]. Mosquitoes belonging to the *An. funestus* group were species identified to species specific level using a cocktail PCR as previously described [25].

2.4.2. Bed Nets Performance Assessment

The performance of the bed nets were expressed relative to control (untreated nets) in term of:

i. Deterrence/entry rate: the reduction in hut entry relative to control. Deterrence (%) = 100 × (Du − Dt)/Du, where Du is the total number of mosquitoes found in untreated hut (control) and Dt is the total number of mosquitoes in the treated hut.
ii. Entry rate (%) = 100 × (Ht/Hn) where Ht is the total number of mosquitoes found in the hut and Hn is the total number of mosquitoes collected in all the 5 huts.
iii. Exophily (Excito-repellency): the proportion of mosquitoes found exited in the veranda trap Exophily (%) = 100 × (Ev/Et) where Ev is the total number of mosquitoes fund in veranda and Et is the total number of both inside the hut and veranda.
iv. Blood feeding rate (BFR). This rate was calculated as follows: Blood feeding rate = (N mosquitoes fed) × 100/total N mosquitoes. Where "N mosquitoes fed" was the number of mosquitoes fed, and "total N mosquitoes" was the total number of mosquitoes collected.

v. Blood-feeding inhibition (BFI): the reduction in blood-feeding in comparison with the control hut. Blood feeding inhibition is an indicator of personal protection (PP). More precisely, the personal protection effect of each bed net is the reduction of blood feeding percentage induced by the net when compared to control. The protective effect of each bed net can be calculated as follows:

vi. Personal protection (%) = 100 × (Bu − Bt)/Bu, where Bu is the total number of blood-fed mosquitoes in the huts with untreated nets and Bt is the total number of blood-fed mosquitoes in the huts with treated nets [23].

vii. Immediate and delay mortality: the proportion of mosquitoes entering the hut that are found dead in the morning (immediate mortality) or after being caught alive and held for 24 h with access to sugar solution (delay mortality) [23]. In this study we focused on the overall mortality calculated as follows: Mortality (%) = 100 × (Mt/MT) where Mt is the total number of mosquitoes found dead in the hut and MT is the total number of mosquitoes collected in the hut.

2.4.3. Ethical Clearance

The National Ethics Committee for Health Research of Cameroon approved the protocol of the study (ID:2016/03/725/CE/CNERSH/SP). Written, informed and signed consent was obtained from sleepers before starting the trials. The consent form provided all the information and the evaluation process about the study. Information was translated in local language when needed. All the volunteers involved in the study were followed-up and treated when showing malaria symptoms. All methods were performed in accordance with the relevant guidelines and regulations.

2.5. Impact of the L119F-GSTe2 Mutation on Insecticide-Treated Nets

The samples collected during the investigation on the performance of nets were grouped in several categories: dead, alive, blood fed, unfed; room and veranda and inside nets. The L119F-*GSTe2* mutation was genotyped in each group using an Allele Specific-PCR. This allows a direct measure of the relative survival and feeding success of resistant and susceptible insects in the presence of the different bed nets.

2.6. Genotyping

Samples classified as dead, alive, blood fed, unfed, room and veranda were used for DNA extraction using the Livak protocol [26]. The L119F-*GSTe2* mutation was genotyped to assess how the glutathione S-transferase gene, *GSTe2*, impacts the performance of the bed nets. An allele specific PCR [27,28] was used to detect the three genotypes of the L119F-*GSTe2* mutation (homozygote resistant:RR, heterozygote resistant: RS and homozygote susceptible: SS). The PCR was carried out using 10 mM of each primer and 1 µL of gDNA as template in 15 µL reaction containing 10× Kapa Taq buffer A, 0.2 mM dNTPs, 1.5 mM MgCl2, 1 U Kapa Taq (Kapa Biosystems, Wilmington, MA, USA). Amplification was carried out using thermocyclic parameters: 95 °C for 5 min; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s, and final extension at 72 °C for 10 min. The following primers were used: L119F-Fwd: ATG ACC AAG CTA GTT CTG TAC ACG CT; L119F-Rev: TTC CTC CTT TTT ACG ATT TCG AAC T; L119F-Res1: CGG GAA TGT CCG ATT TTC CGT AGA AtAA; L119-F-Sus1: CAT TTC TTA TTC TCA TTT ACA GGA GCG TAaTC. PCR products were separated on 2% agarose gel by electrophoresis. The bands corresponding to different genotypes were interpreted as previously described [12,28].

2.7. Data Analysis

2.7.1. Experimental Hut Trial

To calculate the proportion of each entomological outcomes and the level of significance between the treatments and between the control for each entomological outcomes, the XLSTAT software (Addinsoft, Berkeley, CA, USA) was used, as done previously [29,30]. The numbers of mosquitoes collected in the huts with different treatments were analysed by negative binomial regression. The effects of the treatments on each of the main proportional entomological outcomes (exophily, blood feeding and mortality) were assessed using binomial generalized linear mixed models (GLMMs) with a logit link function, fitted using the 'lme4' package for R 3.6. (R Development Core Team, 2019). A separate model was fitted for each outcome and for each mosquito species. In addition to the fixed effect of each treatment, each model included random effects to account for the following sources of variation: between the five huts used in the studies; between the five sleepers who slept in the huts; between the ten weeks of the trial.

2.7.2. Test of Association between L119F-Mutation and the Entomological Outcomes

To investigate the association between the GSTe2 mutation and the ability of the mosquitoes to survive, blood feed or escape, Vassar stats was used to estimate the Odds ratio (OR) based on a fisher exact probability test with a 2 × 2 contingency table.

2.7.3. Hut Effect Analysis

The one-way analysis of variance (ANOVA) using Prism 7.0 (GraphPad, San Diego, CA, USA) was used to determine whether there were any statistically significant differences between the means of the mosquitoes collected from the six huts. In this study, for all the analyses, an alpha of 0.05 was used as the cut off for significance.

3. Results

3.1. Cone Assays Using the An. gambiae Susceptible Lab Strain Ngousso

At the beginning of the study each bed net was exposed to the *An. coluzzii* susceptible lab strain Ngousso using WHO cone assays. Olyset plus and PermaNet 3.0 showed a mortality of 100 ± 00%, PermaNet 2.0 showed 93.3 ± 3.33% mortality and Olyset gave mortality rate of 96.97 ± 3.33% whereas no mortality was recorded for the untreated net (Figure 1A).

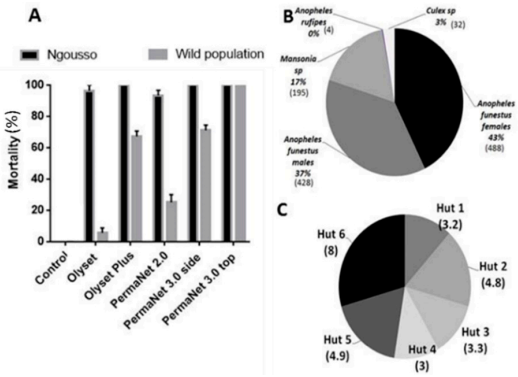

Figure 1. Quality control before the experimental hut trial in Mibellon: Species composition in Mibellon: (**A**) Quality control of the efficacy of all the four nets checked against the susceptible laboratory strain of *Anopheles gambiae* Ngousso and LLINs efficacy testing using cone assays against the pyrethroid resistant *An. funestus* population from Mibellon, Cameroon; (**B**) Number of mosquitoes collected during the hut effect assessment; (**C**) Average of *Anopheles funestus.* ss collected by hut during the 18 days of the hut effect investigation.

3.2. Cone Assays with An. Funestus from the Field (Mibellon)

Olyset and Olyset plus showed mortality of 5.63 ± 3.2%, and 67.23 ± 3.4% respectively when exposed to the F_1 population of *An. funestus* from Mibellon using WHO cone assays. PermaNet 2.0 showed 25.06 ± 5.06% mortality and PermaNet 3.0 side and PermaNet 3.0 top gave 71.04 ± 3.33% and 100 ± 0% respectively whereas no mortality was recorded for the Yorkool and the untreated net (Figure 1A).

3.3. Hut Effect

After 18 days collections, a total of 1147 mosquitoes were collected in the huts with untreated net. Out of the 1147, 488 were females *An. funestus*, 428 *An. funestus* males, 195 *Mansonia* spp., 32 *Culex* sp., and four *An. rufipes*. The average of *An. funestus* females collected per room after 18 days ranged from 3 to 8 per day (Figure 1C). No significant difference ($p = 0.09$; DF = 5) between the numbers of mosquitoes collected in the different huts was observed (Figure 1C).

3.4. Mosquito Abundance

A total of 4656 mosquitoes were collected in five huts by human volunteers sleeping in the huts for 10 weeks corresponding to 360 man-nights. Out of the mosquitoes collected 1155 (25%) were *An. funestus.* s.s. females, 2421 (52%) were *An. funestus* males, 1004 (22%) were *Mansonia* spp., 03 (0.06%) were *An. gambiae* ss., 66 (1%) were *Culex* sp. and 04 (0.08%) were *Aedes* sp. (Figure 2A). However, only female *An. funestus* and *Mansonia* sp., due to its significant nuisance in this area, were considered for analysis (Tables 2 and 3).

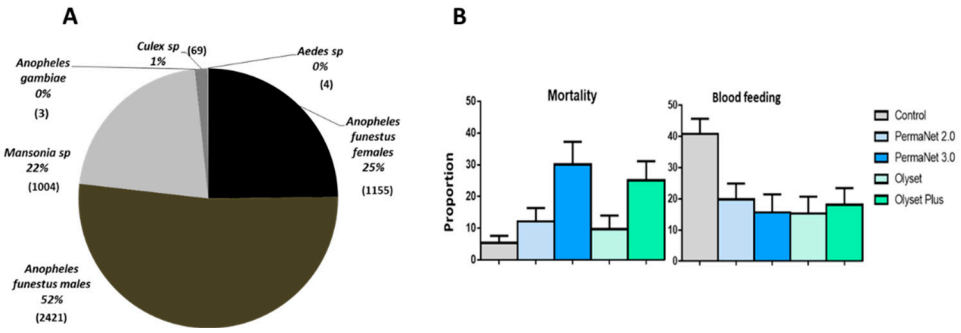

Figure 2. Performance of the four LLINs in experimental hut trials against pyrethroid resistant *An. funestus* in Cameroon. (**A**) Mosquito species composition during the experimental hut study. (**B**) Proportion of mortality and blood feeding rate for the four LLINs against *An. funestus.*

Table 2. Results of the performance of the five brands of LLINs against wild *An. funestus* females in experimental huts.

	Treatments				
	Control	**PermaNet 2.0**	**PermaNet 3.0**	**Olyset**	**Olyset Plus**
Females caught	390	237	153	176	199
Exophily%	6.9	17.7 ***	17.0 ***	29.0 ***	22.1 ***
95% Confidence limits	4.40–9.44)	(12.85–22.58)	(11.04–22.94)	(22.27–35.68)	(16.34–27.88)
Blood fed (%)	40.8	19.8 ***	15.7 ***	15.3 ***	17.2 ***
95% Confidence limits	(35.89–45.65)	(14.75–24.91)	9.92–21.45)	10.02–20.67)	(12.74–23.44)
Blood feed inh. (%)	0.0	51.36	61.52	62.37	55.63
Personal protection (%)	0.0	70.44	84.90	83.01	77.35
Overall mortality (%)	5.4	12.2 **	30.1 ***	9.7 *	25.1 ***
95% Confidence limits	(31.14–7.62)	(8.06–16.41)	(22.80–37.33)	(5.29–14.02)	(19.10–31.15)
Immediate mortality	2.6	5.4	27.6 ***	6.1 *	18.3 ***
entry rate	33.7	20.5 *	13.2 ***	15.2 ***	13.6 ***
Deterrence (%)	0.0	39.2	60.8	54.9	49.0

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$.

Table 3. Results of the performance of the four brands of LLINs against wild *Mansonia* spp. in experimental huts.

	Control	PermaNet 2.0	PermaNet 3.0	Olyset	Olyset Plus
Total	342	142	216	141	163
Exophily%	42.4	37.3 *	31.9 **	28.4 **	31.3 **
95% Confidence limits	(37.16–47.64)	(29.37–45.28)	(25.73–38.16)	(20.93–35.81)	(24.17–38.41)
Blood feeding (%)	30.1	16.2 **	9.3 ***	15.6 ***	20.2 **
95% Confidence limits	(25.25–34.98)	(10.14–22.26)	(5.39–13.12)	(9.61–21.59)	(14.08–26.41)
Blood feeding inh. (%)	0.0	46.22	69.26	48.19	32.78
Personal protection (%)	0.0	77.66	80.53	78.64	67.96
Overall mortality (%)	37.4	51.4 **	65.5 ***	40.4 **	68.1 ***
Immediate mortality	36.8	46.5	62.0 ***	39.0 ***	65.0 ***
Entry rate	34.0	14.1 ***	21.5 **	14.0 ***	16.2 ***
Deterrence (%)	0.0	58.5	36.8	58.8	52.3

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$.

3.5. Performance of the Nets against An. Funestus s.s. Population

3.5.1. Deterrent Effect/Entry Rate

In comparison to control, the entry rate was significantly reduced in all the huts for the five LLINs with the deterrence rates ranging from 39.2% for PermaNet 2.0 to 60.8% for PermaNet 3.0. PermaNet 3.0 had a significantly higher deterrence compared to PermaNet 2.0 but this was not the case for Olyset Plus (49%) over Olyset (54.9%) (Table 2).

3.5.2. Induced Exophily Rate

A low exophily rate was recorded in the control hut for *An. funestus* s.s. (6.9%). However, the exophily rate was significantly higher in the hut with Olyset (29.0%; $p < 0.001$), Olyset Plus (22.1%; $p < 0.001$), PermaNet 2.0 (17.7%; $p < 0.001$) and PermaNet 3.0 (17.0%; $p < 0.001$) compared to the control hut (Figure 2A and Table 2). No significant difference is observed between the five tested LLINs. A significant high exophily activity was observed during week 2 and week 9 ($p < 0.05$) (Table S1).

3.5.3. Mortality (Overall Mortality)

Low mortality rates were recorded in the control hut for *An. funestus* s.s. (5.4%). However, the mortality rate was significantly higher in the hut with Olyset Plus (25.1%; $p < 0.01$), PermaNet 2.0 (12.2%; $p < 0.01$) and PermaNet 3.0 (30.1%; $p < 0.001$) compared to the control hut. The mortality rate was significantly higher with the two PBO-based nets than all the pyrethroid-only nets ($p < 0.001$). However, no significant variation was observed between the mortality in the huts with Olyset (9.7 %; $p > 0.05$) compared to control (Figure 2B and Table 2). It is also clear from our analysis that mortalities obtained were not influenced by the sleepers ($p > 0.05$), by the huts ($p > 0.05$) and by the weeks ($p > 0.05$) (Table S2).

3.5.4. Blood Feeding Inhibition (BFI)

High blood feeding rates were recorded in the control hut (40.8%). However, the blood feeding rate (BFR) was significantly lower in the hut with Olyset (BFR = 15.3; BFI = 62.3%; $p < 0.001$), Olyset Plus (BFR = 18.1%; BFI = 55.6%; $p < 0.001$), PermaNet 2.0 (BFR = 19.8%; BFI = 51.3%; $p < 0.001$) and PermaNet 3.0 (BFR = 15.7%; BFI = 61.5%; $p < 0.001$) compared to the control hut (Figure 2B and Table 2). PermaNet 3.0 had a higher BFI than PermaNet 2.0 but this was not the case with Olyset Plus compared to Olyset although the differences were not significant. We noticed a significant reduction of the blood feeding in hut 4 ($p < 0.05$) (Table S3).

3.5.5. Personal Protection (PP)

The lowest performance in terms of PP was recorded with PermaNet 2.0 (70.44%). PermaNet 3.0 provided a higher PP when compared to PermaNet 2.0 but not significant (84.9% vs. 70.4%; $p > 0.05$).

No significant difference was observed when comparing the PP provided by Olyset and Olyset Plus (83 vs. 77.3%; $p > 0.05$) (Table 2).

3.6. Performance of the Nets against Mansonia spp. Population

3.6.1. Deterrent Effect

In comparison to control, the entry rate was significantly reduced for the four LLINs with deterrence rates ranging from 36.8% for PermaNet 3.0 to 58.8% for Olyset. Contrary to *An. funestus*, PermaNet 2.0 had a higher deterrence rate (58.5%) compared to PermaNet 3.0 (36.8%) and similar for Olyset (58.8%) compared to Olyset Plus (52.3%) (Table 3).

3.6.2. Induced Exophily Rate

A higher exophily rate was recorded in the control hut for *Mansonia* spp. (42.4%) than *An. funestus* (6.9%). However, the exophily rate of *Mansonia* spp., was significantly lower in the hut with Olyset (28.4%; $p < 0.01$), Olyset Plus (31.3%; $p < 0.01$) and PermaNet 3.0 (31.9%; $p < 0.01$) compared to the control hut. For PermaNet 2.0 no significant difference was observed when compared to control (37.3%; $p > 0.05$) (Table 3).

3.6.3. Mortality

The overall mortality rate recorded in the control hut for *Mansonia* spp. was 37.4%. However, this rate was significantly higher in the hut with Olyset Plus (68.1%; $p < 0.001$), PermaNet 2.0 (51.4%; $p > 0.05$) and PermaNet 3.0 (65.3%; $p < 0.001$) compared to the control. No significant variation was observed between the mortality in the huts with Olyset (40.4%; $p > 0.05$) compared to control (Table 3). The two PBO nets PermaNet 3.0 and Olyset Plus had a significantly higher mortality rate than the pyrethroid-only nets PermaNet 2.0 and Olyset (Table 3).

3.6.4. Blood Feeding Inhibition (BFI)

The blood feeding rate recorded in the control hut for *Mansonia* spp. was 30.1%. However, this rate was significantly lower in the hut with Olyset (15.6%; BFI = 48.1%; $p < 0.01$), Olyset Plus (20.2%; 32.7%; $p < 0.001$), PermaNet 2.0 (16.2%; BFI = 46.2%; $p < 0.01$) and PermaNet 3.0 (9.3%; BFI = 69.26%; $p < 0.001$) compared to the control hut. PermaNet 3.0 had higher BFI than all the other nets but this was not significant when compared to PermaNet 2.0 and Olyset Plus.

3.6.5. Personal Protection (PP)

The lowest PP was recorded with Olyset Plus (67.96%) whereas PermaNet 3.0 provided a higher PP but not significant when compared to PermaNet 2.0 (80.5% vs. 77.6%; $p > 0.05$) but higher than the Olyset nets. No significant difference was observed when comparing the PP provided by Olyset and Olyset Plus (78.6% vs. 67.9%; $p > 0.05$) (Table 3).

3.7. Comparative Analysis of the Impact of L119F-GSTe2 Mutation on the Efficacy of Conventional and PBO-Based Nets

Impact on Mortality

Due to the low number of dead mosquitoes (5) the impact on mortality could not be assessed for Olyset net. No correlation was observed between the L119F-*GSTe2* mutation and the ability to survive exposure to Olyset Plus (26 dead and 110 alive) when comparing the allelic frequency (OR = 0.85; $p > 0.05$; CI 0.45–1.59). A similar result was observed for the genotypic frequencies including between RR vs. SS (OR = 0.61; $p > 0.05$; CI 0.22–1.66), RS vs. SS (OR = 1.02; $p = 1$; CI 0.56–1.8) and RR vs. RS (OR = 0.59; $p > 0.05$; CI 0.21–1.68) (Figure 3A and Table 4).

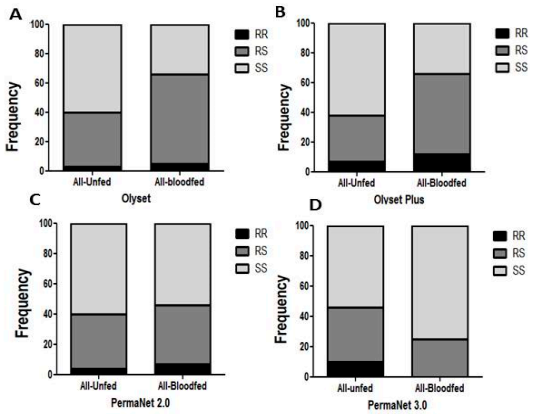

Figure 3. Impact of the L119F-*GSTe2* mediated metabolic resistance on bednet efficacy for blood feeding ability: (**A**) Genotype distribution of L119F-*GSTe2* between blood fed and unfed mosquitoes after exposure to Olyset showing a significant increased ability to blood feed for resistant mosquitoes; (**B**) Genotype distribution of L119F-*GSTe2* between blood fed and unfed mosquitoes after exposure to Olyset Plus showing a significant increased ability to blood feed for resistant; (**C**) Genotype distribution of L119F-*GSTe2* between blood fed and unfed mosquitoes after exposure to PermaNet 2.0 showing an inverse marginal increased ability to blood feed for homozygote susceptible SS compared to homozygote resistant RR ($p < 0.05$); (**D**) Genotype distribution of L119F-*GSTe2* between blood fed and unfed mosquitoes after exposure to PermaNet 3.0 showing an increased ability to blood feed of susceptible mosquitoes compared to resistant mosquitoes (R vs. S: OR = 0.29 $p < 0.05$).

Table 4. Impact of L119F-GSTe2 mutation on the ability of various insecticide-treated nets to kill mosquitoes.

			Mortality	
	Genotype	**OR**	**PV**	**CI**
Olyset Plus	RR vs. SS	0.61	>0.05	0.22–1.66
	RR vs. RS	0.59	>0.05	0.21–1.68
	RS vs. SS	1.02	1	0.56–1.8
	R vs. S	0.85	>0.05	0.45–1.59
PermaNet 3.0	RR vs. SS	3.47	>0.05	1–11.9
	RR vs. RS	3.56	>0.05	1–12.7
	RS vs. SS	0.97	1	0.53–1.76
	R vs. S	1.42	>0.05	0.74–2.7
PermaNet 2.0	RR vs. SS			
	RR vs. RS			
	RS vs. SS	1.1	1	0.6
	R vs. S	1.18	>0.05	0.6–2.2

Similarly, no significant association was observed for PermaNet 2.0 between the mutation and the ability to survive (24 dead and 89 alive mosquitoes) when comparing the allelic frequency (OR = 1.18; $p > 0.05$; CI 0.6–2.2) (Table 4) as well as the genotypic frequency of RS vs. SS (OR = 1.1; $p = 1$; CI 0.6–6.6) (Figure S1B and Table 4).

For PermaNet 3.0, no significant association was observed between the mutation and the ability to survive when comparing the allelic frequency (OR = 1.42; $p > 0.05$; CI 0.74–2.7) (Table 4) but comparing the genotypic frequency of RR vs. SS provided a higher Odds ratio close to significance (OR = 3.47; $p > 0.05$; CI 1–11.9) (Figure S1C).

3.8. Impact on Blood Feeding

For Olyset net, a significant association was observed between the 119F_*GSTe2* resistance allele and an increased ability to blood feed (21 blood fed and 92 unfed) when comparing the allelic frequencies (OR = 2; $p < 0.05$; CI 1.06–3.7) and the genotypic frequency of RS v SS (OR = 2.97; $p < 0.001$; CI 1.6–5.3) (Figure 3A and Table 5).

Table 5. Impact of L119F-*GSTe2* mutation on the efficacy of various bed nets to prevent blood feeding.

	Genotype	OR	PV	CI
Olyset Plus	RR vs. SS	3.3	<0.05	1.19–9.2
	RR vs. RS	1.07	>0.05	0.38–3.02
	RS vs. SS	3.08	<0.01	1.67–5.66
	R vs. S	2.23	<0.05	1.2–4.1
	Mosquitoes in Room			
	RR vs. SS	12.3	<0.001	2.5–60.4
	RR vs. RS	1.46	>0.05	0.29–7.2
	RS vs. SS	8.42	<0.001	4.37–16.2
	R vs. S	4.56	<0.001	2.26–9.2
Olyset	RR vs. SS	2.6	>0.05	0.6–11.7
	RR vs. RS	0.89	>0.05	0.2–3.9
	RS vs. SS	2.97	<0.001	1.65–5.35
	R vs. S	2	<0.05	1.06–3.7
	Blood feeding in Room			
	RS vs. SS	3	<0.001	1.67–5.4
	R vs. S	1.71	>0.05	0.89–3.3
PermaNet 3.0	RR vs. SS	inf		
	RR vs. RS	inf		
	RS vs. SS	0.5	<0.05	0.26–0.92
	R vs. S	0.35	<0.05	0.17–0.73
	Blood feeding in Room			
	RS vs. SS	0.41	>0.05	0.21–0.77
	R vs. S	0.29	>0.05	0.14–0.63
PermaNet 2.0	RR vs. SS	0.4	>0.05	0.1–1.56
	RR vs. RS	0.48	>0.05	0.12–1.9
	RS vs. SS	0.8	>0.05	0.47–1.5
	R vs. S	1.31	>0.05	0.6–2.5

For Olyset Plus net, when considering the blood feed and unfed samples from the room only (23 blood fed and 49 unfed) a significant association was found between L119F-GSTe2 and an increased ability to blood feed when comparing the allelic frequencies (OR = 4.5; $p < 0.001$; CI 2.26–9.2). An even stronger association was observed when comparing the genotype frequencies between RR vs. SS (OR = 12.3; $p < 0.001$; CI 2.5–60.4) and RS vs. SS (OR = 8.42; $p < 0.001$; CI 4.37–16.2). But no association was established when comparing RR v RS (OR = 1.46; $p > 0.05$; CI 0.29–7.2) (Figure 3B and Table 5). No association was also obtained when comparing RR v RS (OR = 1.07; $p > 0.05$; CI 0.38–3.02) when genotyping all the mosquitoes fed and unfed collected in in the hut treated with Olyset (33 blood fed and 103 unfed).

For PermaNet 2.0, no association was observed when comparing the allelic frequency R vs. S (OR = 1.31; $p > 0.05$; CI 0.55–2.01) (Figure 3C and Table 5). But an association was observed when comparing genotypic frequency RS vs. SS (OR = 1.4; $p < 0.001$; CI 0.77–2.53).

For PermaNet 3.0, a negative association was observed between the mutation and the ability to blood feed as resistant mosquitoes significantly blood fed less than susceptible when comparing the

allelic frequency (OR = 0.35; $p < 0.05$; CI 0.17–0.73) and the genotypic frequency of RS vs. SS (OR = 0.5; $p < 0.05$; CI 0.26–0.92) (Figure 3D and Table 5).

3.9. Impact on Exophily

For Olyset, no association was observed between the L119F-*GSTe2* mutation and exophily (67 in room and 48 in veranda) when comparing the allelic frequency (OR = 1.17; $p > 0.05$; CI 0.6–2.2) (Table 4) and the genotypic frequency RS vs. SS (OR = 1.18; $p > 0.05$; CI 0.6–2.1) (Figure 4A and Table 6).

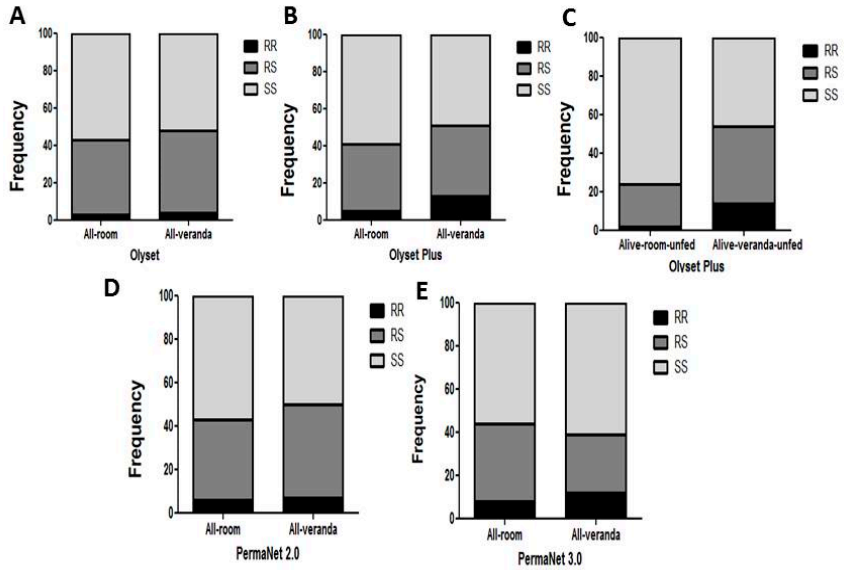

Figure 4. Impact of the *L119F-GSTe2* mediated metabolic resistance on bednet efficacy -exophily: (**A**) Genotype distribution of L119F-*GSTe2* between indoor (Room) and outdoor (verandah) mosquitoes after exposure to Olyset showing no association; (**B**) Genotype distribution of L119F-*GSTe2* between indoor (Room) and outdoor (verandah) mosquitoes after exposure to Olyset Plus showing a significant increased ability to exit the room when considering all mosquitoes; (**C**) A greater association is observed with exophily with Olyset Plus when only analyzing unfed mosquitoes; (**D**) Genotype distribution of L119F-*GSTe2* between indoor (Room) and outdoor (verandah) mosquitoes after exposure to PermaNet 2.0 showing a significant association (RS vs. SS: OR = 1.35; $p < 0.01$); (**E**) Genotype distribution of L119F-*GSTe2* between indoor (Room) and outdoor (verandah) mosquitoes after exposure to PermaNet 3.0 showing no association.

For Olyset Plus, an association was observed between the mutation and the ability to escape to the veranda when considering the allelic frequency (OR = 3.4; $p < 0.001$; CI 1.67–6.9) (Table 6). An even stronger association was observed between the mutation and a preference for the veranda when comparing RR vs. SS (OR = 11.76; $p < 0.01$; CI 2.59–53.4), RS vs. SS (OR = 2.99; $p < 0.01$; CI 1.58–5.65). But no significant association was observed between RR vs. RS (OR = 3.9; $p = 1$; CI 0.8–18.59) (Figure 4B,C and Table 6). For PermaNet 2.0, an association was observed between the L119F-*GSTe2* mutation and the ability to exit the room when comparing the genotypic frequency RS vs. SS (OR = 1.35; $p < 0.01$; CI 0.75–2.3). A stronger association was observed when assessing the impact only among the unfed mosquitoes for RS vs. SS (OR = 3.37; $p < 0.001$; CI 01.84–6.17) but not at the allelic level (Figure 4D and Table 6). For PermaNet 3.0, no association was observed between the L119F-*GSTe2* mutation and a preference for the room or the veranda when comparing the allelic frequency (OR = 0.94; $p = 1$; CI 0.5–1.8) (Table 6). The same trend was observed when comparing the genotypic frequency RR vs. SS (OR = 1.22; $p = 1$; CI 0.46–3.2), RS vs. SS (OR = 0.59; $p > 0.05$; CI 0.31–1.12) RR vs. RS (OR = 1.68; $p > 0.05$; CI 0.6–4.7) (Figure 4E and Table 6).

Table 6. Impact of L119F-GSTe2 mutation on the efficacy of various bed nets in repellency.

	Exophily			
	Genotype	OR	PV	CI
Olyset Plus	RR vs. SS	3.1	>0.05	1.1–9
	RR vs. RS	2.48	>0.05	0.83–7.42
	RS vs. SS	1.25	>0.05	0.67–2.26
	R vs. S	1.1	>0.05	0.6–2.2
	Unfed Alive			
	RR vs. SS	11.76	<0.01	2.59–53.4
	RR vs. RS	3.9	1	0.8–18.59
	RS vs. SS	2.99	<0.01	1.58–5.6
	R vs. S	3.4	<0.001	1.67–6.9
	RS vs. SS	1.18	>0.05	0.6–2.1
	R vs. S	1.17	>0.05	0.62–2.2
PermaNet 3.0	RR vs. SS	1.22	1	0.46–3.2
	RR vs. RS	1.68	>0.05	0.6–4.7
	RS vs. SS	0.59	>0.05	0.31–1.12
	R vs. S	0.94	1	0.5–1.8
PermaNet 2.0	RS vs. SS	1.35	<0.01	0.75–2.43
	R vs. S	1.22	>0.05	0.65–2.3
	Unfed Alive			
	RS vs. SS	3.37	<0.001	1.84–6.17
	R vs. S	1.8	>0.05	0.99–3.38

3.10. Correlation between L119F-GSTe2 and Mortality from Cone Assays

Due to the low number of dead mosquitoes obtained from the experimental huts, the impact of the L119F-*GSTe2* on the ability of mosquitoes to survive exposure to various nets was assessed using samples from cone assays. Only a few dead were obtained for Yorkool and Olyset, preventing the assessment of these nets.

3.10.1. Olyset Plus

No association was observed between the mutation and the mortality (40 dead vs. 40 alive mosquitoes) at both allelic level (OR = 1.04; $p = 1$; CI 0.5–1.8) and genotypic for RR vs. SS (OR = 1.1; $p > 0.05$; CI 0.4–2.7) (Figure 5A and Table 7).

3.10.2. PermaNet 2.0

Resistant 119F-*GSTe2* mosquitoes exhibited a greater ability to survive than susceptible mosquitoes (34 dead and 46 alive) at both allelic level (OR = 1.8; $p < 0.01$; CI 0.9–3.5) and genotypic frequency RR v SS (OR = 2.09; $p < 0.01$; CI 1.1–4.2) (Figure 5B and Table 7).

3.10.3. PermaNet 3.0

Resistant 119F-*GSTe2* mosquitoes when exposed to PermaNet 3.0 showed a greater ability to survive than susceptible mosquitoes even than with PermaNet 2.0 at allelic level (OR = 3.8; $p < 0.001$; CI 1.8–7.7). A higher correlation was further observed when comparing the genotypic frequencies for RR vs. SS (OR = 30.1; $p < 0.001$; CI 3.8–234), RS vs. SS (OR = 2.4; $p < 0.01$; CI 1.3–4.5) and RR vs. RS (OR = 12.3; $p < 0.001$; CI 1.8–7.7) (Figure 5C and Table 7). This result applies only to PermaNet 3.0 side net as no mosquito survived exposure to PermaNet top containing PBO.

3.11. Validation of the Association between L119F-GSTe2 and Resistance to Pyrethroids and DDT in Mibellon

To further validate the impact of L119F-*GSTe2* on LLINs, we established the association between this marker and the ability to survive exposure to the pyrethroids used for Olyset (permethrin) and PermaNet (deltamethrin) nets besides DDT. Correlation analysis between L119F genotypes and deltamethrin revealed that homozygote resistant mosquitoes were significantly more likely to survive exposure to deltamethrin than both homozygote susceptible SS (OR = 4.6; $p < 0.001$) and heterozygotes (OR = 3.75; $p < 0.001$). However, there was no significant difference between RS and SS (Figure 5D). A similar pattern was observed for permethrin (Figure 5E and Table 5). Analysis of the correlation with DDT revealed a much stronger association between L119F and DDT resistance as RR exhibited a greater ability to survive exposure to DDT than SS (OR = 66.7; $p < 0.001$) and also more than RS (OR = 4.8; $p < 0.001$). Contrary to both pyrethroids heterozygote RS mosquitoes are also more able to survive exposure to DDT than homozygote susceptible (OR = 14.0; $p < 0.001$) (Figure 5F and Table 7).

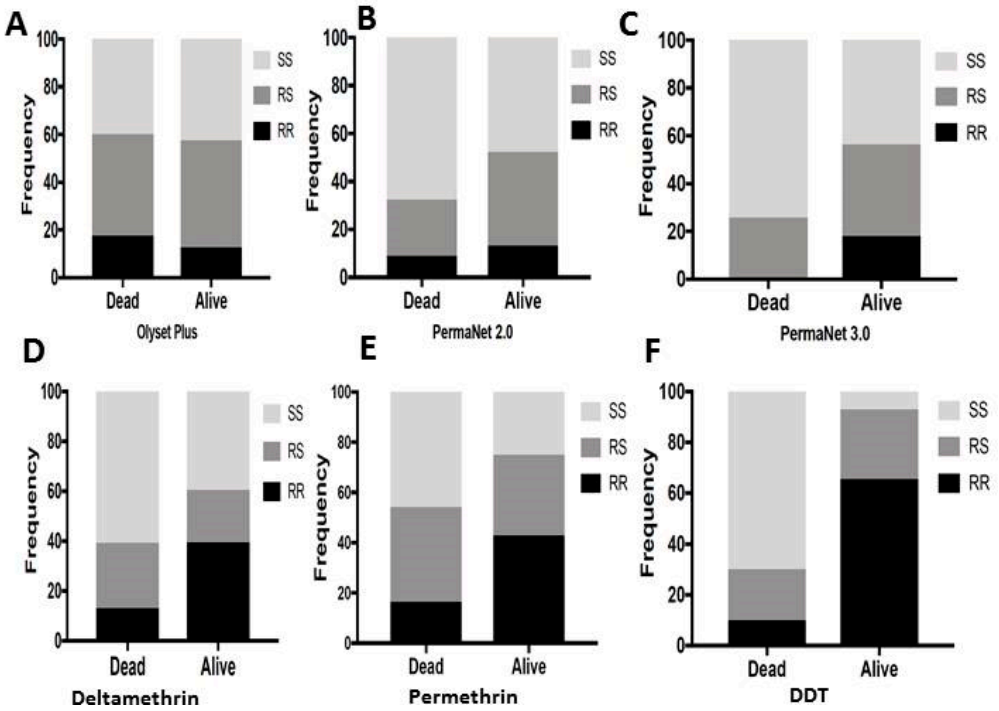

Figure 5. Association between L119F-*GSTe2* mutation and ability to survive exposure to LLINs (cone assays) and WHO papers (bioassays): (**A**) Genotype distribution of *L119F*-GSTe2 between alive and dead mosquitoes after exposure to Olyset Plus showing no association; (**B**) Genotype distribution of L119F-*GSTe2* between alive and dead mosquitoes after exposure to PermaNet 2.0 showing a significantly increased ability of resistant mosquitoes to survive (R vs. S: $p < 0.01$); (**C**) Genotype distribution of L119F-*GSTe2* between alive and dead mosquitoes after exposure to PermaNet 3.0 showing a significantly greater ability of resistant mosquitoes to survive than that seen for PermaNet 2.0 (RR vs. SS: OR = 30.1; $p < 0.001$); (**D**) Genotype distribution of L119F-*GSTe2* between alive (90 min) and dead (30 min) mosquitoes after exposure to deltamethrin showing a significantly greater ability of homozygote resistant mosquitoes to survive (RR vs. SS: OR = 4.6; $p < 0.001$); (**E**) Similarly for permethrin, homozygote resistant mosquitoes exhibit a significantly greater ability of to survive (RR vs. SS: OR = 4.8; $p < 0.001$); (**F**) For DDT, a much greater ability of mosquitoes with the resistance allele to survive exposure to DDT papers (RR vs. SS: OR = 66.7; $p < 0.001$).

Table 7. Impact of GSTe2 on the ability of field population *An. funestus* to survive using samples from cone assays.

Cone Assays				
		OR	**PV**	**CI**
Olyset Plus 40 alive vs. 40 dead	RR vs. SS	1.1	>0.05	0.4–2.7
	RS vs. SS	0.9	1.0	0.5–1.8
	RR vs. RS	1.1	>0.05	0.4–2.7
	R vs. S	1.04	1.0	0.5–1.8
PermaNet 2.0 46 alive vs. 34 dead	RR vs. SS	2.09	<0.01	1.1–4.2
	RS vs. SS	2.3	<0.001	1.2–4.4
	RR vs. RS	0.8	>0.05	0.3–2.4
	R vs. S	1.8	<0.01	0.9–3.5
PermaNet 3.0 39 alive vs. 31 dead	RR vs. SS	30.1	<0.001	0.8–5.3
	RS vs. SS	2.4	<0.01	1.3–4.5
	RR vs. RS	12.3	<0.001	1.5–98.6
	R vs. S	3.8	<0.001	1.8–7.7
WHO Bioassays				
		OR	**PV**	**CI**
Deltamethrin 38 alive vs. 38 dead	RR vs. SS	4.6	<0.001	2.5–8.4
	RS vs. SS	1.22	>0.05	0.69–2.15
	RR vs. RS	3.75	<0.001	2.1–6.8
	R vs. S	2.80	<0.01	1.54–5.1
Permethrin 28 alive vs. 37 dead	RR vs. SS	4.8	<0.001	2.66–8.8
	RS vs. SS	1.6	>0.05	0.86–2.8
	RR vs. RS	3.1	<0.001	1.7–5.5
	R vs. S	2.99	<0.001	1.7–5.3
DDT 29 alive vs. 30 dead	RR vs. SS	66.7	<0.001	27.1–164
	RS vs. SS	14.0	<0.001	6.3–31.2
	RR vs. RS	4.8	<0.001	2.4–9.5
	R v S	15.3	<0.001	7.7–30.5

OR, Odds ratio; PV, *p* value; CI, Confidence interval.

4. Discussion

This study has taken advantage of the predominant presence of metabolic resistance in *An. funestus* and the recent availability of a simple DNA-based diagnostic tool for *GSTe2*, to establish the impact of GST-mediated metabolic resistance on the effectiveness of both pyrethroid-only and PBO-based LLINs against *An. funestus*.

4.1. PBO plus pyrethroid-Based Nets are More Effective than Pyrethroid-Only Nets against Pyrethroid Resistant An. funestus

4.1.1. Mortality

The mortality rates obtained in this study with experimental huts were generally low (7.3 to 30.1%), but such mortality levels are similar to those observed in other experimental hut studies including in Benin [5], or recently in Burkina Faso where the mortality rates ranged from 9.5% to 46.1% in two locations of high pyrethroid resistance in *An. gambiae* [31]. However, higher mortality has been observed for another highly resistant pyrethroid resistant population of *An. gambiae* in Ivory Coast (Yaokoffikro) for PermaNet 3.0 (54%) and PermaNet 2.0 (34.7%) [32] than seen here for the *An. funestus* population in Mibellon, which could potentially be associated with differences in resistance mechanisms. The low level of mortality observed is likely due to the level of pyrethroid resistance in *An. funestus* in Mibellon with permethrin mortality around 48.88 ± 5.76% and deltamethrin

mortality around 38.34 ± 5.79% [18]. Nevertheless, despite the ongoing resistance both PBO-based nets induced a significantly higher mortality than the pyrethroid-only LLINs against both *An. funestus* and *Mansonia* spp. This higher mortality of PermaNet 3.0 and Olyset Plus with *An. funestus* is also in line with the cone assay results although PermaNet 3.0 (Top) presented a higher mortality for both huts and cone assays than Olyset Plus as also seen for *An. gambiae* in Burkina Faso [31]. Overall, the higher mortality with both PBO-nets suggests that, in areas of pyrethroid resistance, these new generation LLINs (PermaNet 3.0 and Olyset Plus) provide a better protection against both malaria vectors and nuisance mosquitoes such as *Mansonia* spp.

4.1.2. Blood Feeding

The blood feeding rate of all the five nets, an indicator of the personal protection, provided by the net was significantly lower (≤23.8%; $p < 0.001$) compared with the untreated control (40.8%). However, the blood feeding inhibition rate was higher for PermaNet 3.0 (61.5%) than PermaNet 2.0 (51.4%) this was not the case between Olyset Plus (55.6%) and Olyset (62.4%). Similar results have been obtained for *An. gambiae* in Benin [6] where comparable blood feeding inhibition rates were observed for Olyset Plus (85%) and Olyset (82%) despite the greater mortality rate with Olyset Plus (81%) vs. 42% for Olyset. The same pattern of blood feeding inhibition was observed here for *An. funestus* and *Mansonia* spp. with PermaNet 3.0 providing the highest inhibition level in *Mansonia* spp. whereas Olyset Plus was even lower than Olyset.

4.1.3. Exophily

The excito-repellency effect of all the nets was at least twice higher compared to control showing that *An. funestus* mosquitoes were affected by the repellent effect of pyrethroids. These results are in line with previous results demonstrating the repellent effect of pyrethroid nets against *Anopheles* mosquitoes [19,32]. No significant difference was observed in the level of exophily between the conventional nets compared to synergist suggesting that PBO did not impact the repellent effect of pyrethroids. Comparing *An. funestus* to *Mansonia* spp., revealed a significant difference in term of exophily with only 6.9% of *An. funestus* exiting the hut with untreated net whereas 42.4% *Mansonia* spp. exited the room with control net. This is different from the previous observation in Ivory Coast where a similar exophily (~35%) was observed for both *An. gambiae* and *Mansonia* spp. in experimental huts [32]. Furthermore, while all the 5 LLINs significantly increased the exophily rate for *An. funestus*, this was not the case in *Mansonia* spp. with no significant change observed probably as *Mansonia* spp. are more exophilic [33].

4.2. GSTe2 Mediated Metabolic Resistance Is Reducing Efficacy of LLINs

With the design of the L119F-*GSTe2* diagnostic tool [8], this study has investigated for the first time the impact of GST-mediated metabolic resistance on the efficacy of LLINs.

4.2.1. Experimental Hut Study

A significant association was observed between the blood feeding ability of *An. funestus* and the L119F-*GSTe2* mutation as mosquitoes with the 119F resistance allele have significantly higher blood feeding rate compared to those with L119 susceptible allele against Olyset Net ($p < 0.001$) and Olyset plus ($p < 0.001$). This is similar with observations for the cytochrome P450s *CYP6P9a* and *CYP6P9b* for which the resistant alleles *CYP6P9a_R* and *CYP6P9b_R* were recently shown, in a release-recapture experimental hut study, to also provide a greater ability to blood feed and to survive exposure to pyrethroid-only LLIN (PermaNet 2.0) [14,34]. This suggests that L119F-*GSTe2* mutation likely contributes to an increased malaria transmission as every additional bite increases the chance of sporozoite to be passed to the populations. This is particularly a concern as 119F-RR resistant mosquitoes have also been shown to live longer [27,28] and to have a greater vectorial capacity to transmit *Plasmodium* [12]. However, it is noticeable that the impact of L119F-*GSTe2* on blood feeding

ability is mainly seen for Olyset Plus and Olyset LLINs impregnated with permethrin but present an opposite effect for PermaNet 3.0 and 2.0 both impregnated with deltamethrin. The cause of such difference between permethrin- and deltamethrin-based nets is unclear at this point since the greater blood feeding in Olyset net is not associated with greater survivorship. Functional analyses with transgenic Drosophila, In vitro metabolism assays with recombinant *GSTe2* enzyme combined with genotype/phenotypes analyses had shown that *GSTe2* was able to confer resistance to both permethrin and deltamethrin but more so to permethrin [35,36]. This could partly explain the differences observed between permethrin- and deltamethrin-based nets although further studies will be needed to fully establish the underlying reason. Similar genotype/phenotype studies performed to assess the impact of the knockdown target-site resistance (*kdr*) on resistance did not reveal such a significant association with blood feeding potentially due to the fact that *kdr* frequency was already very high in tested *An. gambiae* population [21]. However, indirect comparison of *kdr* effect in two separate populations of *An. gambiae* in Benin; one with high *kdr* frequency (Ladji) and another with low frequency (Malanville) did suggest that *kdr* could also impact the efficacy of LLINs [19].

Interestingly, the L119F-GSTe2 mutation was also associated with an increased exophily for Olyset Plus and PermaNet 2.0 suggesting that the increased expression of GSTe2 could impact mosquito's behavior helping them avoids exposure to insecticides. Similarly, over-expression of carboxylesterase in *Culex pipiens* [37] has been previously associated with a behavioral change as resistant mosquitoes were found to have a reduced mobility [37] supporting that metabolic resistance could impact mosquitoes' behavior in the presence of insecticide-based interventions.

No significant association was observed between L119F-*GSTe2* and mortality in the experimental hut's samples for all LLINs potentially due to the low number of dead mosquitoes which did not provide statistical power coupled with lower frequency of RR in this location. However, a trend was observed for PermaNet 3.0 with an increased proportion of 119F homozygote resistant mosquitoes able to survive exposure to this LLIN than both homozygote susceptible L119-SS (OR = 3.47; $p > 0.05$) and heterozygotes (OR = 3.46; $p > 0.05$).

However, other mechanisms than GSTs are also at play in this location of Mibellon, as shown recently [14], revealing the over-expression of cytochrome P450 genes such as CYP6P5 and CYP325a. Unfortunately, there is not yet a molecular marker to help assess the impact of these genes with the different samples collected after experimental huts. Generating more DNA-based markers as done for the CYP6P9a/b [14] and for L119F-GSTe2 [8] will further help to gain a more complete picture of the impact of resistance as a whole. No kdr target-site resistance has yet been detected in An. funestus (Irving et Wondji 2017) including in Mibellon [18].

4.2.2. Impact from Cone Assays

The trend for a correlation between *GSTe2* and reduced mortality with LLINs was supported by results from cone assays where a very strong association was observed between L119F-*GSTe2* and ability to survive exposure to PermaNet 3.0 (side) (OR = 30.1) and PermaNet 2.0 although at lower extent (OR = 2.09). The lack of association observed with Olyset Plus could be explained by the effect of PBO which for this net is incorporated on the entire net whereas for PermaNet 3.0 PBO is only on the top. The PermaNet 3.0 (side) used here does not contain PBO. One reason why a greater association is found with PermaNet 3.0 (side) could be the fact that with higher deltamethrin concentration than PermaNet 2.0, mosquitoes surviving exposure to PermaNet 3.0 are those likely to be much more resistant because of a greater expression of GSTe2 and other cytochrome P450s. So the higher dose of deltamethrin in PermaNet 3.0 has further selected mosquitoes with 119F-*GSTe2* resistant allele than with the lower dose in PermaNet 2.0. This is also the likely reason why a trend of a greater ability to survive was seen in the experimental hut for PermaNet 3.0.This suggests that mosquitoes displaying several resistance mechanisms than just P450-based resistance could avoid even PBO-based nets as when cytochrome P450 enzymes are inhibited by PBO, mosquitoes bearing other mechanisms, as in this case the 119F resistance allele, are able to better survive exposure to PBO-based nets. However,

this cannot explain why such results were not observed with Olyset Plus the other PBO-net. However, the spread of a *GSTe2*-mediated metabolic resistance conferring the ability of mosquitoes to survive exposure to synergist LLINs will constitute a concern at a time when such nets are gradually being introduced to fight pyrethroid resistant mosquitoes. GST resistance will also need to be taken into consideration than just cytochrome P450 resistance. Nevertheless the greater efficacy seen with the roof of PermaNet 3.0 in cone bioassays shows that P450 role remains important. Future studies will need to also perform synergist assays with DEM and PBO to establish the contribution of both GSTs and cytochrome P450s to better inform of the likely efficacy of LLINs.

5. Conclusions

This study has revealed a loss of efficacy of LLINs against pyrethroid resistant populations of *An. funestus* in Cameroon using experimental huts. However, PBO based nets (PermaNet 3.0 and Olyset Plus) are more efficient than conventional pyrethroid-only nets. It is the reason why as suggested by WHO 2017 report we recommend the deployment of PBO nets in area where pyrethroid resistance is confirmed and at least partly conferred by P450. Noticeably, the GST-mediated metabolic resistance not impacted by PBO could threaten the continued effectiveness of the PBO-based LLINs and the impact of this mechanism needs also to be considered to maximize the effectiveness of LLINs. One option could be to generate new mosaic LLINs also incorporating the Diethyl Maleate (DEM) beside PBO to inhibit GST metabolic activity and further increase the efficacy of these nets. However, further studies will need to be performed before including establishing the extent of synergism provided by DEM in various mosquito populations and the most suitable position on the net (either on top as PBO or on the entire net).

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/11/2/143/s1, Figure S1: Impact of the *L119F-GSTe2* mediated metabolic resistance on bednet efficacy looking at the mortality rate after exposure to LLINs, Table S1: Linear mixed effect model analysis showing different sources of variations that may influenced the exophily, Table S2: Linear mixed effect model analysis showing different sources of variations that may influenced the mortality, Table S3: Linear mixed model effect analysis showing different sources of variations that may influenced the Blood feeding.

Author Contributions: C.S.W. conceived, designed and coordinated the research. B.D.M., M.F.K., J.M.R. and W.T. carried out the sample collection and bioassays. B.D.M., M.F.K., M.J.W. and M.T. performed the PCR and TaqMan. C.S.M. contributed in the design and building of experimental huts; B.D.M. and C.S.W. performed data analysis; B.D.M., J.M.R., M.O.K. and C.S.W. wrote the manuscript with contribution from all the authors. All authors have read and agreed to the published version of the manuscript.

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Cis-regulatory *CYP6P9b* P450 variants associated with loss of insecticide-treated bed net efficacy against *Anopheles funestus*

Leon M.J. Mugenzi[1,2,3], Benjamin D. Menze[1,2], Magellan Tchouakui[2], Murielle J. Wondji[1,2], Helen Irving[1], Micareme Tchoupo[2], Jack Hearn[1], Gareth D. Weedall[1,4], Jacob M. Riveron[1,2] & Charles S. Wondji[1,2*]

Elucidating the genetic basis of metabolic resistance to insecticides in malaria vectors is crucial to prolonging the effectiveness of insecticide-based control tools including long lasting insecticidal nets (LLINs). Here, we show that *cis*-regulatory variants of the cytochrome P450 gene, *CYP6P9b*, are associated with pyrethroid resistance in the African malaria vector *Anopheles funestus*. A DNA-based assay is designed to track this resistance that occurs near fixation in southern Africa but not in West/Central Africa. Applying this assay we demonstrate, using semi-field experimental huts, that CYP6P9b-mediated resistance associates with reduced effectiveness of LLINs. Furthermore, we establish that *CYP6P9b* combines with another P450, *CYP6P9a*, to additively exacerbate the reduced efficacy of insecticide-treated nets. Double homozygote resistant mosquitoes (RR/RR) significantly survive exposure to insecticide-treated nets and successfully blood feed more than other genotypes. This study provides tools to track and assess the impact of multi-gene driven metabolic resistance to pyrethroids, helping improve resistance management.

[1] Vector Biology Department, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK. [2] Centre for Research in Infectious Diseases (CRID), P.O. Box 13501, Yaoundé, Cameroon. [3] Department of Biochemistry and Molecular Biology, Faculty of Science University of Buea, P.O. Box 63, Buea, Cameroon. [4] School of Natural Sciences and Psychology, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, UK. *email: charles.wondji@lstmed.ac.uk

Malaria remains a major public health burden in Africa. Control strategies rely predominantly on insecticide-based interventions such as indoor residual spraying and long-lasting insecticide nets (LLINs). These tools have been estimated to be responsible for more than 68% of the decrease in malaria mortality in the past 15 years having helped prevent more than 663 million clinical cases of malaria[1]. Increasing insecticide resistance in malaria vector species presents a major challenge to the continued success of public health interventions. The elucidation of the molecular basis of insecticide resistance in these vectors and its evolution across Africa is a crucial step to design resistance management strategies to prevent potentially devastating public health consequences. Detailed information on resistance mechanisms is a prerequisite to detect resistance markers facilitating the design of field-applicable diagnostic tools. Unlike current WHO bioassays that only detect resistance once it is well established in the population[2], these molecular diagnostic tools can detect and track resistance at an early stage, which is an essential requirement of resistance management efforts.

Resistance to insecticides is primarily caused by two major mechanisms: target-site resistance (e.g., knockdown resistance, *kdr*) and metabolic resistance through elevated expression of detoxification genes, especially the cytochrome P450s[3,4]. Target-site resistance through knockdown resistance (*kdr*) mutations in the voltage-gated sodium channel gene is well characterised with DNA-based diagnostic tools designed since the late 1990s[5,6].

In contrast, metabolic resistance, considered to be more likely to cause control failure[7], remains—despite recent progress[8–11]—poorly characterised due to the complexity of this resistance mechanism[12]. This is the reason why although several cytochrome P450 genes associated with pyrethroid resistance have been reported, only a single DNA-based marker has been detected to date[13] limiting the design of field applicable diagnostic tools to detect and track this resistance. Indeed, progress was recently made with the detection of the first DNA-based P450 resistance marker in mosquitoes in the *CYP6P9a* gene on the *rp1* (resistance to pyrethroid 1) quantitative trait locus (QTL)[14,15] allowing assessment of the impact of metabolic resistance on the effectiveness of insecticide-treated nets[13].

However, the *CYP6P9a* marker does not explain all of the genetic variance in pyrethroid resistance and is currently only present in southern Africa[13,16,17]. Thus, there is a need to characterise the molecular basis of pyrethroid resistance Africa-wide and detect related genetic variants so that DNA-based diagnostic assays can be designed to enhance our ability to detect resistance and rigorously characterise the impact on insecticide-based interventions.

To tackle the molecular complexity of metabolic resistance to pyrethroids and detect resistance markers and design reliable diagnostic assays, we carry out large scale transcriptional and genomic profiling of *Anopheles funestus* mosquitoes across Africa. Our results reveal the complex and diverse molecular changes underlying insecticide resistance across the species' range characterised by differential gene expression patterns and genomic signatures, notably transcription factor binding sites (*cis*-acting regulatory elements) regulating expression of detoxification genes. This thorough elucidation of metabolic resistance leads to the detection of a P450 DNA-based marker in the *CYP6P9b* gene from which a simple field-applicable polymerase chain reaction (PCR) diagnostic assay is designed. Field assessment of the impact of pyrethroid resistance using this marker in semi-field conditions in experimental huts revealed that this metabolic resistance marker in combination with the other P450 marker (*CYP6P9a*) is associated with exacerbated loss of efficacy of insecticide-treated nets.

Results

Transcription analysis of resistant *An. funestus* Africa-wide. Previous efforts to detect genes associated with pyrethroid resistance Africa-wide have compared mosquitoes alive after permethrin exposure to the lab-susceptible FANG strain only[13]. A limitation of this approach is that the over-expressed genes might not be necessarily linked to resistance but rather other differences in genetic background. Therefore, to detect potential genes associated with pyrethroid resistance across Africa [Southern (Malawi), East (Uganda), Central (Cameroon) and West (Ghana)], we used here two additional approaches by first comparing permethrin-resistant mosquitoes from one country to permethrin-resistant mosquitoes from another country to detect the genes specific for each region of Africa. Second, we performed in Malawi a triangular comparison of the expression patterns of mosquitoes alive after exposure (R) to those not exposed (Control; C) as well as the lab-susceptible strain FANG (S).

Comparisons of the transcription profiles between the four African regions were performed to detect or confirm genes differentially expressed according to their resistance background. When the southern Africa population (Malawi) is compared to all other regions directly, the striking difference is the high up-regulation of two P450 genes *CYP6P9a* and *CYP6P9b* in Malawi with fold change (FC) of 28.3, 22.7 and 9.5 greater than in Cameroon, Uganda and Ghana for *CYP6P9a* (Supplementary Data 1). Both genes result from a recent duplication event[14]. A similar but lower difference is observed for *CYP6P9b* with FC values of 5.5, 3.4 and 3.5, respectively. In contrast to *CYP6P9b, CYP6P9a* is also significantly more over-expressed in Ghana compared to Cameroon and Uganda. The southern Africa mosquito population has more genes significantly up-regulated than other regions including cytochrome P450s, *CYP4H25, CYP4H28, CYP9J11* (except Ghana) and *CYP6P2* (except Cameroon) although the FC is much lower than for the duplicated genes. Two cuticular protein genes, potentially involved in cuticular resistance (AFUN005672 and AFUN009940) were also significantly over-expressed in Malawi compared to the three other regions (Supplementary Data 1).

The biggest difference between the West African sample of Ghana and others was the duplicated *CYP6P4a* and *CYP6P4b* that are significantly more up-regulated in Ghana than other regions with FC values of 25.8, 7.6 and 21.2 for *CYP6P4a*, respectively, against Cameroon, Malawi and Uganda or FC values of 22.7, 6.5 and 9 for *CYP6P4b*. These genes are also significantly more expressed in mosquitoes from southern Africa than in Central and East Africa.

The Central African population of Cameroon exhibited a greater difference than other regions for the cytochrome P450 *CYP325A* gene with FC values of 5.3, 12.1 and 4.5 against Malawi, Uganda and Ghana, respectively, although the overall abundance of reads for this gene is lower (<500). The carboxylesterase gene AFUN002514 was also significantly overexpressed in Cameroon compared to the south (FC 3.8) and the east (FC 4.7) but not against Ghana where it is also over-expressed compared to Malawi (FC 2.5) and Uganda (FC 3.3). This suggests that the over-expression of the carboxylesterase AFUN002514 is specific to West-Central Africa.

The East African population from Uganda exhibited the greatest difference in expression to all other regions for the cytochrome P450 *CYP9K1* with FC values of 4.6, 2.6 and 1.8 against Cameroon, Malawi and Ghana suggesting that this gene is potentially specific to pyrethroid resistance in the East Africa region. The P450 *CYP307A1* is also up-regulated in Uganda relative to other regions (Supplementary Data 1). Further differences between regions are highlighted in Supplementary Note 1.

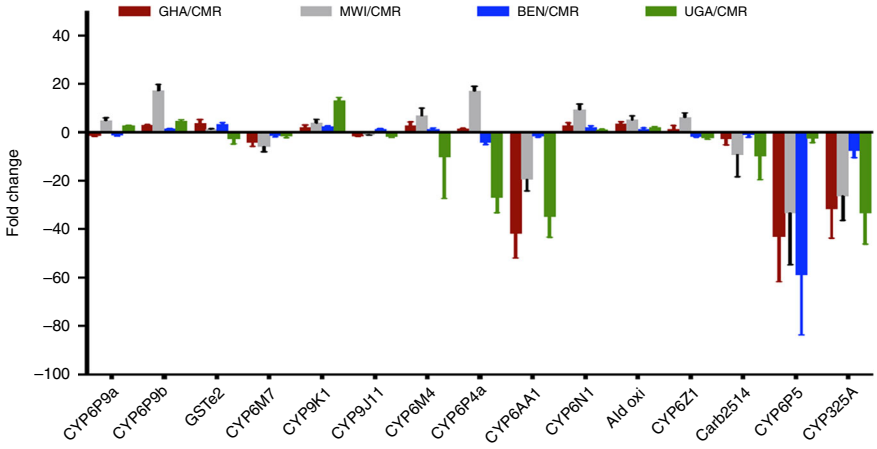

Fig. 1 Comparative transcription profiles of *An. funestus* between countries. qRT-PCR comparison of the expression profile of major detoxification genes associated with pyrethroid resistance between populations of *An. funestus* from different African regions supporting a shift in the role of these genes between regions. The data shown are mean + SEM ($n = 3$). Source data are provided as a Source Data file

In addition, a triangular analysis of transcriptional profiles between resistant (R), unexposed control (C) and susceptible (S) mosquito samples in Malawi confirmed the major role potentially played by *CYP6P9a* and *CYP6P9b* in these countries with a slightly higher level in R–S compared to C–S (FC 60.1 vs. 57.1 for *CYP6P9a*; FC 23.7 vs. 18.5 for *CYP6P9b*) (Supplementary Table 1; Supplementary Note 2). Moreover, the quantitative reverse transcription (qRT) PCR expression patterns broadly supported the differences observed with RNAseq in the main genes driving pyrethroid resistance in different African regions (Fig. 1; Supplementary Note 3).

Genetic polymorphisms of CYP6P9b-mediated resistance. To detect potential genetic variants associated with pyrethroid resistance, we focused our attention on the *CYP6P9b* P450 because this gene was highly over-expressed in resistant mosquitoes especially in southern Africa and after the recent characterisation of *CYP6P9a*, the other over-expressed gene in southern Africa[13].

To have a full view of the potential regulatory elements driving the over-expression of *CYP6P9b*, we amplified and sequenced the full 1 kb intergenic region (Supplementary Fig. 1; Supplementary Note 4) between *CYP6P9b* and *CYP6P5* in individual resistant (FUMOZ_R) and susceptible (FANG) mosquitoes. An indel of 3 bp (AAC) was consistently observed with deletion in the resistant FUMOZ and presence in susceptible FANG. The core promoter elements detected using GPminer include TATA boxes (8 in FUMOZ and 7 in FANG and 1 GC box in each strain), 1 GC box and 1 CCAAT box. The *CYP6P9b* promoter also exhibits two sites for the putative arthropod initiator (Inr) contrary to one for the 800 bp promoter fragment of *CYP6P9a* previously studied[13]. Furthermore, several transcription factor binding sites were detected including six sites for the CncC nrf2/MAF (in both strains) and several sites for GATA, MYB or AHR transcription factors.

The polymorphism pattern of the promoter region of *CYP6P9b* was also assessed Africa-wide by sequencing the 1 kb intergenic region in mosquitoes from West, Central, East and Southern Africa. All populations from southern Africa (Mozambique, Malawi and Zambia) exhibited a low genetic diversity with Mozambique presenting the least diverse set with a number of haplotype (h) = 2 and a haplotype diversity (hd) = 0.189. However, the population of Benin (West Africa) exhibited no segregating sites with only a single haplotype observed. This absence of polymorphism in Benin (similar for *CYP6P9a*) contrasts highly with neighbouring populations notably that of Nigeria which exhibits the highest diversity (40 segregating sites, h = 13; hd = 0.95) (Supplementary Table 2).

Overall, southern Africa populations consistently exhibited a different polymorphism pattern to other regions notably with the presence of an AAC deletion 50 bp upstream of nrf2/MAF binding sites and 72 bp from a CCAAT box. The AAC deletion located 703 bp from start codon is tightly associated with other polymorphisms in a haplotype (STH13) (Supplementary Fig. 2). This haplotype predominates in southern Africa (68/82) reflecting the marked selective sweep observed around this gene in this region[18]. A maximum likelihood phylogenetic tree of all haplotypes (Fig. 2a) revealed three broad clusters corresponding to southern Africa, West and Central Africa; the unique Benin haplotype is different from other West Africa haplotypes and is closer to the Central Africa Cluster (Fig. 2a).

The difference in genetic diversity between southern Africa and other regions was further supported by the higher genetic differentiation between these populations as shown by the neighbour-joining tree of the genetic distance (N_{ST} estimates) (Fig. 2b). A haplotype network built with the Templeton, Crandall and Sing (TCS) programme, further highlights the reduced diversity in southern Africa with the predominant STH13 haplotype in all populations from this region (Fig. 2c); greater diversity is seen in other samples with a high number of mutational steps between haplotypes observed particularly in Nigeria. Nevertheless, other countries such as Benin and Ghana also have a predominant haplotype suggesting possible selection also acting on this gene or in nearby genes in these locations.

To assess whether the differences observed in the 5'UTR region of *CYP6P9b* between southern Africa and other regions could have resulted from selection due to insecticide pressure, we assessed the polymorphism of the 1 kb fragment of *CYP6P9b* in samples collected before the scale-up of LLINs in Mozambique (2002) and samples collected after scale-up (2016). This 1 kb fragment was polymorphic before scale-up with many segregating sites (18) and haplotypes (h = 6; hd = 0.797) as well as high nucleotide diversity ($\pi = 0.011$) (Supplementary Table 2; Fig. 2d). In contrast, the post-intervention samples exhibited very low diversity with low haplotypes number (h = 2; hd = 0.189) and reduced nucleotide diversity ($\pi = 0.0053$). Construction of the maximum likelihood tree further highlights the contrast between both sets of samples as the pre-intervention mosquitoes cluster together and become more diverse (Fig. 2d). In contrast, the post-intervention samples cluster noticeably away from the

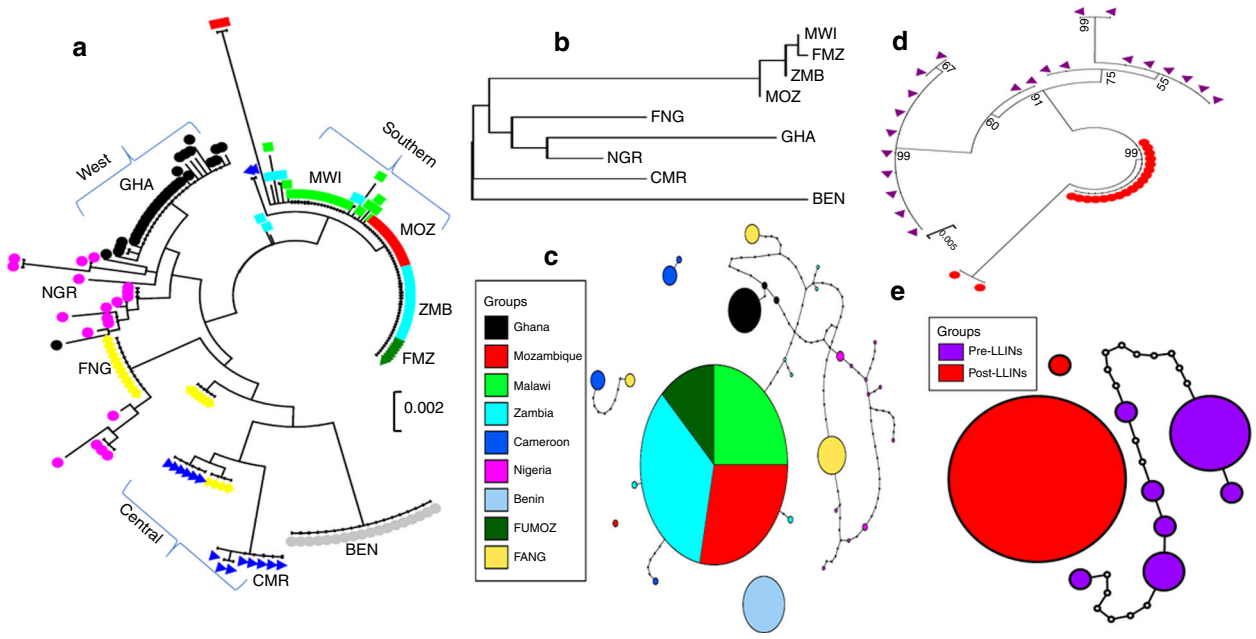

Fig. 2 Genetic diversity patterns of the *cis*-regulatory region of *CYP6P9b*. **a** Maximum likelihood phylogenetic tree of *CYP6P9a* promoter haplotypes across Africa. **b** Neighbour-joining phylogenetic tree of CYP6P9b-based genetic distance between nine African populations (N_{ST} estimates). **c** Africa-wide TCS network for the *CYP6P9b* haplotypes showing predominant haplotypes in southern Africa. A fixed haplotype is observed in Benin (sky blue) and a nearly fixed haplotype is seen in Ghana (black). Other locations show a greater diversity. Lines connecting haplotypes and each node represent a single mutation event. **d** A maximum likelihood phylogenetic tree of *CYP6P9b* showing a cluster of highly diverse haplotypes before scale up of bed nets (pre-intervention) but a nearly fixed haplotype post-intervention. **e** TCS haplotype network in Mozambique pre- and post-intervention revealing a major resistant haplotype post-intervention but a more diverse set of haplotypes before pre-bed nets

pre-intervention with a drastically reduced haplotype number. The TCS haplotype network further supported the drastic difference between pre- and post-intervention samples with more mutational steps observed between pre-intervention haplotypes whereas a highly predominant haplotype is observed in post-intervention samples (18/20). This is not observed in the pre-intervention samples, which is probably an indication of its low frequency before bed nets (Fig. 2e). Furthermore, the AAC motif now undetectable in the southern populations and located 50 bp upstream of a nrf2/MAF binding site was present in all pre-intervention samples suggesting that this deletion plays a role in the regulation of *CYP6P9b*. These major differences between pre- and post-intervention samples show that scale up of insecticide-treated nets is likely a major factor that has driven this evolution in *An. funestus* populations in southern Africa.

***CYP6P9b* promoter analysis detects resistance variants**. The 1 kb fragment upstream of the translation start site of *CYP6P9b* was assessed for its promoter activity using a luciferase assay in the *An. gambiae* 4a-2 cell line. To narrow down the regions containing the potential regulatory motifs, six different sized fragments of the 1 kb, 600 base pairs (bp), 400 bp, 300 bp, 100 bp and 80 bp immediately upstream of the translation start codon from both the resistant FUMOZ and the susceptible FANG strain were cloned upstream of a reporter gene in a pGL3 vector. These constructs were used in luciferase reporter gene assays but did not show a significant difference in the relative luciferase activity of the 1 kb insert between the two strains. Similarly, the shorter versions of the putative promoter did not present a significantly different activity between these two strains. Nevertheless, the progressive truncation of the 1 kb insert helped identify the major regulatory elements in either strain. Shortening the promoter fragment to 600, 400 and 300 bp reduced their activity in FUMOZ by 49.9 ($P < 0.0001$; unpaired t test), 21.1 ($P = 0.0074$; unpaired t test) and 39.0% ($P = 0.0043$; unpaired t test) respectively, and similarly in FANG with reductions of 54.4 ($P = 0.0014$; unpaired t test), 57.6 ($P < 0.0001$; unpaired t test) and 57.7 ($P < 0.0001$; unpaired t test), respectively (Fig. 3a). However, for both strains, a much higher reduction in luciferase activity is seen when the promoter fragment is further truncated to just 100 (>91%) and 80 bp (>93%) ($P < 0.0001$; unpaired t test) with deletion of all regulatory elements including CncC nrf2/MAF and CCAAT boxes (Fig. 3a).

A DNA-based diagnostic tool to detect *CYP6P9b* resistance. To design a DNA-based diagnostic assay to detect *CYP6P9b*-mediated resistance, we screened the 1 kb for suitable restriction sites in complete linkage with the AAC deletion in the resistant mosquitoes across southern Africa including in the FUMOZ strain (Supplementary Fig. 3a). This allowed the design of a simple PCR restriction fragment length polymorphism (RFLP). We found a restriction site for the *Nmu*CI (*Tsp*45I) (cut site 5′-GTSAC-3′) spanning a C/T mutation located 11 bp downstream of the AAC deletion and designed a PCR-RFLP that, in mosquitoes carrying the *CYP6P9b* variants associated with susceptibility to insecticides, cuts the 550 bp amplicon in two fragments of 400 and 150 bp, whereas the amplicon carrying the resistance-associated allele remains uncut (Fig. 3b; Supplementary Fig. 3b). To assess the efficacy of this diagnostic assay, we genotyped the FUMOZ-R and FANG strains for the CYP6P9b-R marker and establish that all 50 tested FUMOZ-R mosquitoes were homozygote resistant RR whereas all the FANG were homozygote susceptible SS.

To further validate the ability of CYP6P9b_R marker to predict pyrethroid resistance phenotype in *An. funestus*, mosquitoes were crossed between FUMOZ and FANG at the F_8 generation. Genotyping of the same mosquitoes previously used to validate the *CYP6P9a*[13] revealed that those surviving exposure to

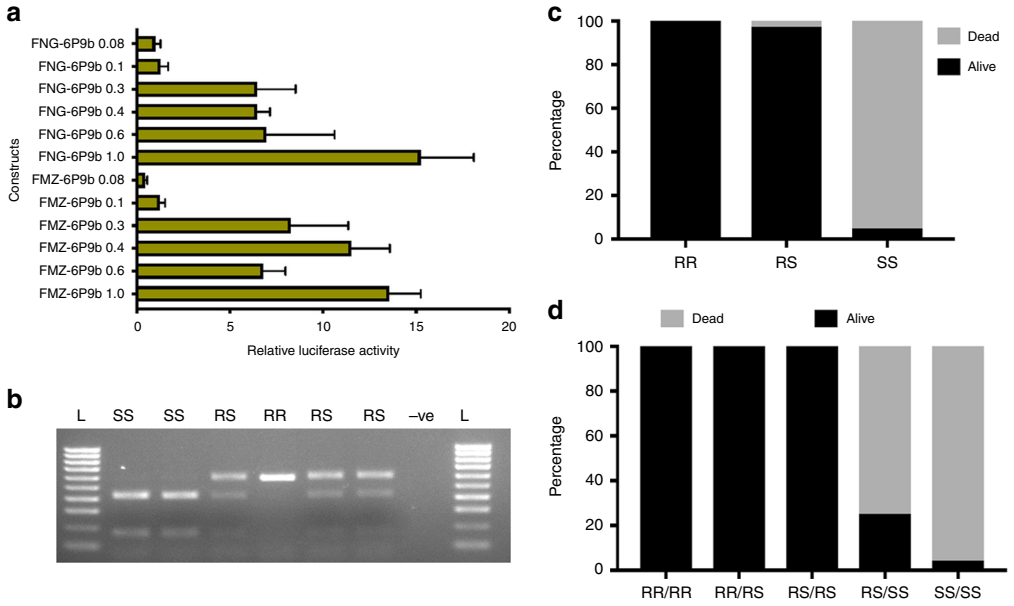

Fig. 3 DNA-based diagnostic assay for CYP6P9b-mediated metabolic resistance to pyrethroids. **a** Comparative luciferase assay between promoter fragment from the highly resistant FUMOZ and highly susceptible (FANG) lab strains with progressive serial deletions of *CYP6P9b* 5′ flanking region to detect the causative variants. Bars represent the mean ± S.D. of four independent transfections of three replicates ($n = 6$). **b** Agarose gel of PCR-RFLP with *Nmu*CI of *CYP6P9b* showing the RR, RS, and SS genotypes. **c** Distribution of the *CYP6P9b* genotypes between susceptible and resistant mosquitoes showing a very strong correlation between *CYP6P9b* and resistance phenotype. **d** Distribution of the combined genotypes of both *CYP6P9a* and *CYP6P9b* showing that both genotypes combined to increase the pyrethroid resistance. Source data are provided as a Source Data file

permethrin for 180 min are predominantly homozygote resistant (10/48) and heterozygotes (36/48) with two bearing the homozygote susceptibility genotype. From the 47 dead mosquitoes after 30 min exposure to permethrin (highly susceptible), 46/47 were homozygote susceptible and one heterozygote (Supplementary Fig. 3c). A significant association was observed between permethrin resistance and the *CYP6P9b* genotype with a highly significant odds ratio when comparing RR vs. SS (OR = infinity; $P = 1.1 \times 10^{-50}$, Fisher's exact test) and RS vs. SS (OR = 715; $P = 2.4 \times 10^{-45}$, Fisher's exact test) (Fig. 3c).

The contribution of *CYP6P9b* to pyrethroid resistance was also compared to that of *CYP6P9a* revealing that both genes combine additively to confer higher level of resistance. Indeed, all mosquitoes with the double homozygote resistant genotypes (RR/RR) were alive after 180 min exposure. However, only half of the homozygous resistance-genotype carriers for *CYP6P9b* also had the homozygous resistant genotype for *CYP6P9a* with the other half being only heterozygous for *CYP6P9a* (Supplementary Fig. 3d). 66.7% of mosquitoes that survived were double heterozygous (RS/RS), whereas only 10.4% were RR/RR carriers and 16.7% RR/RS carriers (Fig. 3d: Supplementary Fig. 3d) suggesting possible fitness cost associated with possessing double resistance alleles for both genes. None of the dead mosquitoes had RR/RR, RR/RS or RS/RS genotypes; this shows a strong correlation with permethrin resistance with high OR (infinity; $P < 0.0001$, Fisher's exact test).

Distribution of the resistant *CYP6P9b* allele across Africa. Genotyping of the *CYP6P9b* was successful in most countries, but some samples failed to amplify in Kenya, Uganda, Cameroon and DR Congo (western region only). It is possible that a structural variant is present in these countries preventing the amplification of the 550 bp fragment. The *CYP6P9b* resistant allele (CYP6P9b_R) is detected throughout southern Africa at a very high frequency (>90%) with fixation observed in Mozambique (Fig. 4a), consistent with previous results from *CYP6P9a*[19]. An intermediate frequency is observed in Tanzania (63.4% for CYP6P9b_R) and in Eastern Democratic Republic of Congo (66.6%). CYP6P9b_R is absent from other parts of Africa including East (Kenya, Uganda), Central (Democratic Republic of Congo (DRC)-West, Cameroon) and West Africa (Fig. 4b). Furthermore, the potential association of CYP6P9b_R with CYP6P9a_R in field populations was assessed in Tanzania and DRC where there is segregation of these genotypes. This implies that there is a greater linkage of *CYP6P9a* and *CYP6P9b* genotypes in Tanzania with 71.8% of identical genotypes; this is much lower in DRC where only 51% identical genotypes are observed between the two genes in assessed mosquitoes (Fig. 4c). However, the RS/RS genotype is predominant (>36%) in both countries with double homozygote resistant RR/RR being higher in Tanzania (28.5%) than DRC (16.7%).

CYP6P9b_R associated with reduced efficacy of insecticide-treated nets. The impact of the *CYP6P9b* resistance allele on the effectiveness of conventional (PermaNet 2.0) and PBO-based (PermaNet 3.0) nets was assessed using a release-recapture of mosquitoes from the hybrid strain between highly resistant FUMOZ-R and fully susceptible FANG as described for *CYP6P9a*[13]. Mosquitoes from FANG/FUMOZ crosses (F_4) were moderately resistant to pyrethroids with mortality rates of >70% for both permethrin and deltamethrin after 1 h exposure.

To assess the impact of *CYP6P9b* on the efficacy of LLINs, we first analysed this impact on the mortality of mosquitoes after exposure to PermaNet 2.0 because the number of live mosquitoes was too low with PermaNet 3.0. Both nets are impregnated with deltamethrin. The association between *CYP6P9b* genotypes and the ability to survive exposure to PermaNet 2.0 was first assessed using only mosquitoes collected in the room and unfed to avoid possible bias due to feeding or exophily status. This analysis revealed a significant difference in the distribution of genotypes between dead and live mosquitoes (chi square = 1180; $P < 0.0001$, chi-square) (Fig. 5a). Comparing the proportion of different

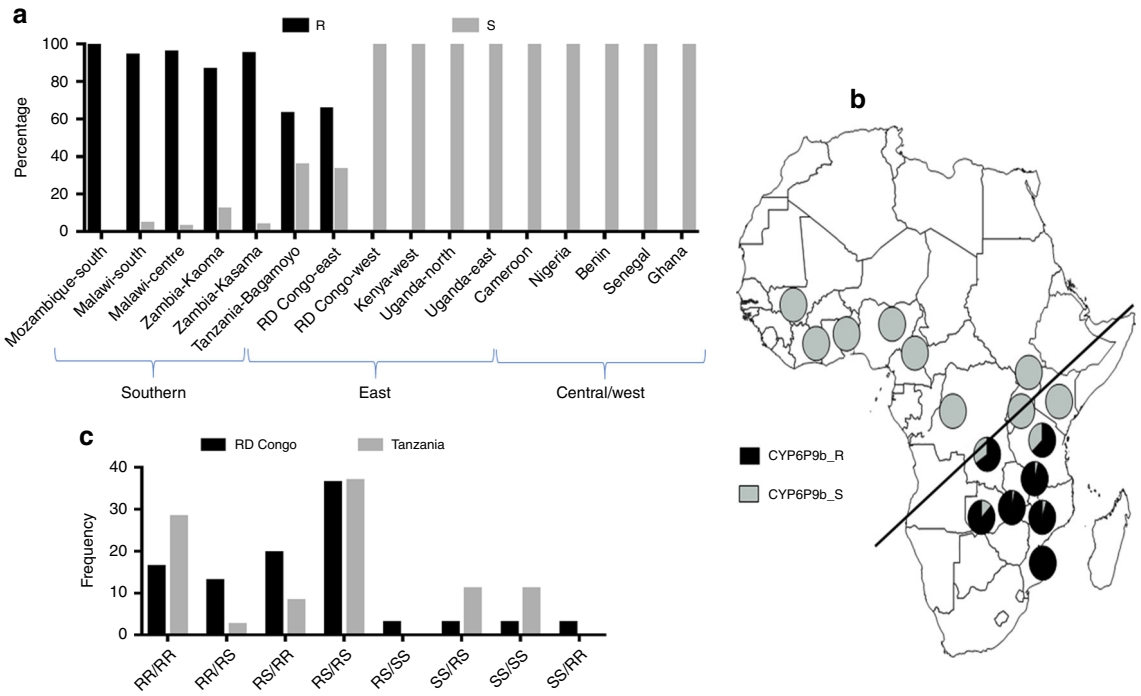

Fig. 4 Geographical distribution of *CYP6P9b* Africa-wide. **a** Frequency of the CYP6P9B_R allele across Africa showing that it is highly predominant in southern Africa, moderately present in East Africa but completely absent elsewhere on the continent. **b** Map of Africa showing the distribution of the CYP6P9b-resistant alleles with a near fixation in southern Africa. The map was generated using a blank map freely available from https://www.rkkerkenschijndel.nl/map-of-africa-drawing.html and pie-charts representing frequency of both alleles in each location were added on the map. **c** Comparative distribution of the combined genotypes of *CYP6P9a* and *CYP6P9b* in Tanzania and RD Congo supporting an independent segregation of genotypes of both genes in the field. Source data are provided as a Source Data file

genotypes according to mortality outcome revealed that *CYP6P9b* homozygote resistant (RR) were significantly more able to survive exposure to PermaNet 2.0 than homozygotes-susceptible mosquitoes (OR = 109.3; CI = 40.7–293.2; $P < 0.0001$, Fisher's exact test) (Table 1). This correlation is even stronger than previously observed for *CYP6P9a* in the same mosquitoes (OR = 34.9; CI = 15.8–77.1; $P < 0.0001$, Fisher's exact test)[13].

Similarly, heterozygote mosquitoes (RS) survived exposure to PermaNet 2.0 significantly better than homozygote susceptible (SS) (OR = 34.7; CI = 14.6–82.5; $P < 0.0001$, Fisher's exact test). The additive resistance conferred by each allele of *CYP6P9b* was shown by the fact that homozygote resistant mosquitoes (RR) could also survive better than heterozygotes (OR = 3.14; CI = 1.4–6.9: $P = 0.0058$, Fisher's exact test). Overall, the strength of this association was further shown by the significant ability to survive exposure when possessing a single *CYP6P9b*-resistant allele (R) compared to the susceptible allele (S) (OR = 7.2; CI = 3.813.4; $P < 0.0001$, Fisher's exact test) (Fig. 5b). A similar pattern of significant correlation between *CYP6P9b* genotypes and mortality was also observed when analysing all the dead and live samples regardless of blood feeding and exophilic status but with a lower OR value (Table 1).

The impact of the CYP6P9b_R was also assessed on the ability to blood feed against PBO-based net (PermaNet 3.0). A significant difference was observed in the distribution of *CYP6P9b* genotypes between blood-fed and unfed mosquitoes when exposed to PermaNet 3.0 (chi square = 28.8; $P < 0.0001$, chi-square). This resulted in a significant association between *CYP6P9b* genotypes and the ability to blood feed with homozygote resistant (RR) mosquitoes feeding significantly more than homozygote susceptible (SS) mosquitoes (OR = 5.04: CI = 1.7–14.6; $P = 0.0001$, Fisher's exact test). This is also true for homozygote resistant (RR) when compared to heterozygotes (OR = 2.4: CI = 1.3–4.39; $P = 0.0085$, Fisher's exact test). No significant difference was observed between heterozygotes and susceptible genotype (Table 1). However, *CYP6P9b* did not impact blood feeding when mosquitoes were exposed to PermaNet 2.0, and no difference was observed for the control net (Fig. 5c; Table 1). No significant association was observed for PermaNet 2.0 (Supplementary Fig. 4a) or in the control untreated net (Supplementary Fig. 4b).

Combined effect of *CYP6P9b* and *6P9a* on LLIN efficacy. As *CYP6P9b* genotypes were shown to be independent from those of *CYP6P9a*, we next assessed how combinations of genotypes at both genes impact the efficacy of LLINs focusing on PermaNet 2.0 for mortality and PermaNet 3.0 for blood feeding. Analysis of the impact of combined genotypes on mortality with PermaNet 2.0 confirmed the independent segregation of genotypes at both genes with several combinations of genotypes observed including RR/RR, RR/RS, RS/RS, RS/SS and SS/SS (Fig. 6a). A comparison of the distribution of both sets of genotypes revealed that double homozygote resistant (RR/RR) mosquitoes at both genes had a far greater ability to survive exposure to PermaNet 2.0 than all other combinations. This shows that both genes act additively to confer a greater resistance to pyrethroids associated with a higher reduction of bed net efficacy when mosquitoes are double resistant. The additive resistance was further supported in that RR/RR mosquitoes had the greatest ability to survive exposure to PermaNet 2.0 when compared to double susceptible (SS/SS) (OR = 76.5; CI = 15.1–387.7) when considering all samples. There was even greater correlation if only considering unfed mosquitoes in the room (Fig. 6b). A significantly increased survival is also observed in RR/RR when compared to other combinations although with a lower odds ratio, such as against RS/SS (OR = 47.7; $P < 0.0001$, Fisher's exact test), against RR/SS (OR = 6.1;

$P = 0.01$, Fisher's exact test) and against RS/RS (OR = 3.2; $P = 0.042$, Fisher's exact test). No significant difference was observed between RR/RR and RR/RS (OR = 1.6; $P = 0.82$, Fisher's exact test) (Fig. 6b). A similar trend is observed when comparing homozygote resistant mosquitoes to one gene and heterozygote to the other (RR/RS) against other combinations although at a lower strength (Fig. 6c). No significant difference is observed between RR/RS and RS/RS suggesting that having one resistance-associated allele of each gene is as good as having homozygote resistance for one gene.

Overall, a gradual decrease of impact is seen for genotypes RR/RR > RR/RS > RS/RS > RR/SS > RS/SS > SS/SS. Analysis of the combined genotype distribution for blood feeding (Fig. 6d) also revealed a significantly increased ability to blood feed for double homozygote-resistant mosquitoes when exposed to PermaNet 3.0 with the highest significance against double homozygote susceptible (OR = 6.5; $P < 0.0001$, Fisher's exact test) (Fig. 6e; Supplementary Table 3). No significant association was observed for PermaNet 2.0 (Supplementary Fig. 4c) and in the control untreated net (Supplementary Fig. 4d).

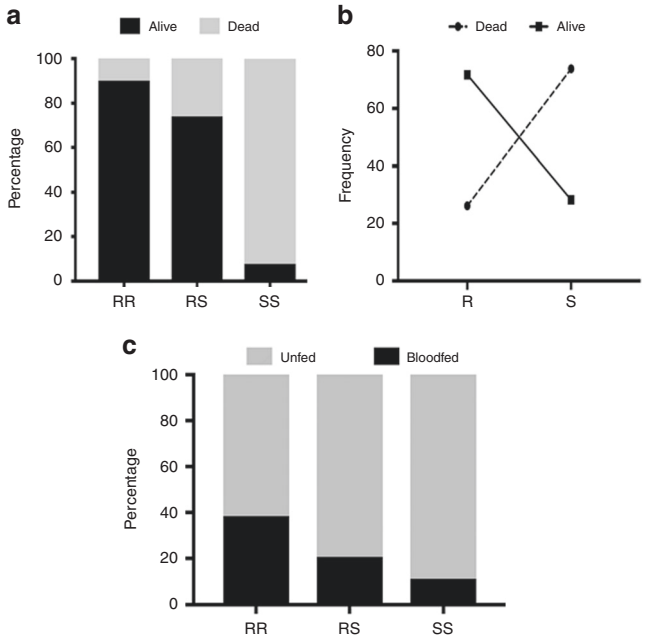

Fig. 5 Impact of the *CYP6P9b*-based metabolic resistance on bed nets' efficacy. **a** Distribution of *CYP6P9b* genotypes between dead and alive mosquitoes after exposure to PermaNet 2.0 net in experimental huts showing that CYP6P9b_R significantly allows mosquitoes to survive exposure to this insecticide-treated net. **b** Association between frequency of CYP6P9b-R and ability to survive exposure to PermaNet 2.0. **c** Distribution of *CYP6P9b* genotypes between blood-fed and unfed mosquitoes after exposure to the PBO-based net PermaNet 3.0 showing that CYP6P9b_R allele increases the ability to take a blood meal. Source data are provided as a Source Data file

Discussion

The detection of the resistance markers associated with *CYP6P9b* over-expression described here provides a tool to monitor pyrethroid resistance in field populations of the major malaria vector *An. funestus*. In addition, it also allows the assessment of the interplay between P450 genes in their overall genetic variance to resistance as seen here between *CYP6P9a* and *CYP6P9b*. Overall, this study established key insights regarding the molecular basis of pyrethroid resistance in malaria vectors in Africa.

Genomic analyses performed in this study suggest differences in the genomic evolution of metabolic resistance to pyrethroids across Africa: mosquitoes from different geographic regions exhibited specific sets of genes and genetic variants associated with resistance. The comparative RNAseq-based transcription profiling between regions revealed key differences in the level of expression of major resistance genes, including *CYP6P9a* and *CYP6P9b* which are highly expressed in southern Africa. This difference in gene expression is in line with previous reports[11,13,17]. Other genes predominate in other regions with *CYP9K1* in Uganda, *CYP6P5* and *CYP325A* in Central Africa (Cameroon) and *CYP6P4a/b* in Ghana (West Africa). The resistance profile does not reflect the relatedness between populations[13] suggesting each population independently evolves resistance mechanisms. This contrasting expression profile also supports previous observations[16] and highlights the challenges in designing diagnostic tools for metabolic resistance in the face of

Table 1 Correlation between genotypes of *CYP6P9b* and mortality and blood feeding after exposure to insecticide-treated nets in experimental huts

		OR	*P* value	CI	OR	*P* value	CI
Mortality							
PermaNet 2.0			*CYP6P9a*			*CYP6P9b*	
Unfed	RR vs. SS	34.9	<0.0001	15.8–77.1	109.3	<0.0001	40.7–293.2
	RS vs. SS	19.9	<0.0001	9.7–40.9	34.7	<0.0001	14.6–82.5
	RR vs. RS	1.75	0.26	0.81–3.8	3.14	0.0058	1.4–6.9
	R vs. S	6.25	<0.0001	3.3–11.7	7.2	<0.0001	3.8–13.4
All samples	RR vs. SS	10.8	<0.0001	5.6–20.8	62.1	<0.0001	23.6–163.6
	RS vs. SS	5.3	<0.0001	2.8–9.8	21.9	<0.0001	8.7–55.1
	RR vs. RS	2.04	0.0002	1.1–3.7	2.8	0.0025	1.48–5.2
	R vs. S	3.17	0.02	1.78–5.65	4.7	<0.0001	2.6–8.7
Blood feeding							
PermaNet 2.0	RR vs. SS	1.75	0.19	0.82–3.7	1.29	0.54	0.58–2.9
	RR vs. RS	2.5	0.052	1.09–5.75	0.92	0.5	0.49–1.7
	RS vs. SS	0.7	0.67	0.28–1.7	1.68	1	0.79–3.5
	R vs. S	1.43	0.26	0.82–2.5	0.94	1	0.5–1.6
PermaNet 3.0	RR vs. SS	4.54	<0.0001	2.3–8.7	5.04	<0.0001	1.7–14.6
	RR vs. RS	2.6	0.0012	1.43–4.7	2.4	0.0085	1.31–4.39
	RS vs. SS	1.74	0.17	0.87–3.47	2.1	0.3	0.73–6.03
	R vs. S	2.14	0.18	1.17–3.19	4.1	<0.0001	2.2–7.5

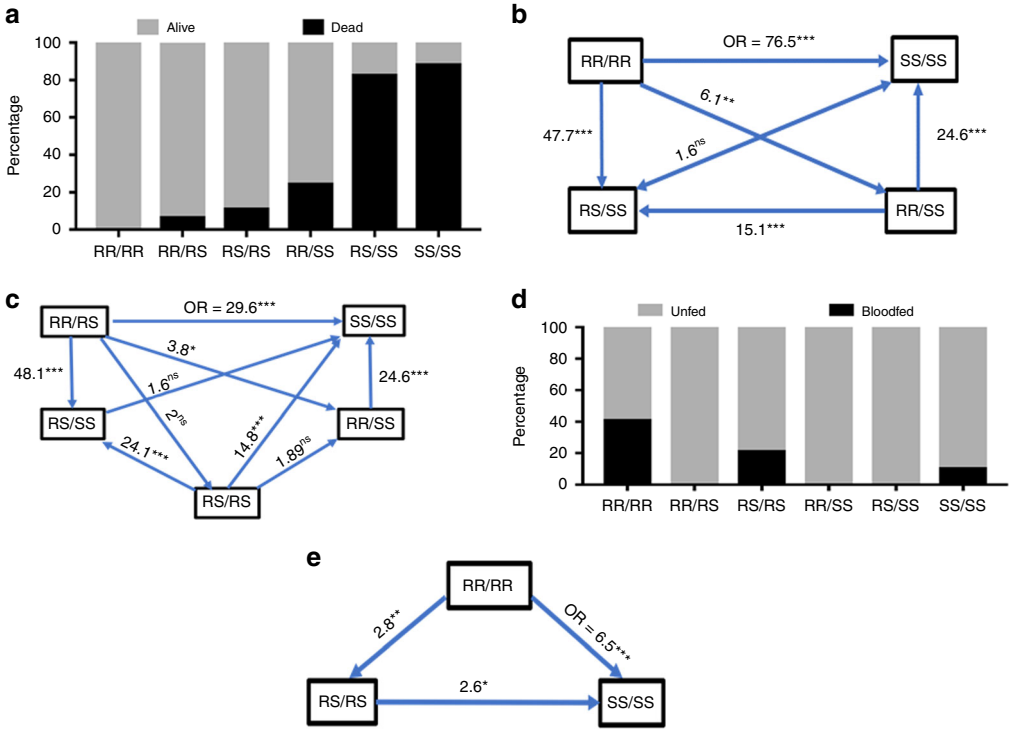

Fig. 6 *CYP6P9b* combines with *CYP6P9a* to further reduce bed nets' efficacy. **a** Distribution of the combined genotypes of both *CYP6P9a* and *CYP6P9b* showing that genotypes at both genotypes combined to additively increase the ability to survive after exposure to PermaNet 2.0. **b** Ability to survive exposure to PermaNet 2.0 (Odds ratio) of the double homozygote-resistant (RR/RR) genotypes of *CYP6P9a* and *CYP6P9b* compared to other genotypes supporting the additive resistance effect of both genes. Significance is shown by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, as estimated using Fisher's exact test. **c** Ability to survive exposure to PermaNet 2.0 (odds ratio) of the combined homozygote resistant and heterozygote (RR/RS) genotypes of *CYP6P9a* and *CYP6P9b* compared to other genotypes. **d** Distribution of the combined genotypes of both *CYP6P9a* and *CYP6P9b* after exposure to PermaNet 3.0 revealing an additive effect of both genes in increasing the ability to blood feed. **e** Comparison of blood feeding ability of combined genotypes of *CYP6P9a* and *CYP6P9b* showing a significantly higher ability (odds ratio) to blood feed for mosquitoes that double homozygote resistant (RR/RR). Source data are provided as a Source Data file

such molecular plasticity of populations of malaria vectors. However, some of the differences observed between geographic regions could be the consequence of differences in genetic background between populations rather than just insecticide resistance. Further functional characterisation of these over-expressed genes both in vivo and in vitro[16] will allow to confirm their role in resistance.

The detection of another P450 DNA-based marker in this study is a great progress in our efforts to detect and track the spread of metabolic resistance to insecticides in malaria vectors. The mutation associated with resistance here is located in the *cis*-regulatory region as established for *CYP6P9a*[13] or for previous observations in the mosquito *Culex quinquefasciatus*[20] and in *Drosophila*[21]. Similarly, the *CYP6P9b* AAC indel resistance mutation is associated with transcription factor binding sites notably to the insect ortholog of the mammalian transcript factor Nrf2, Cap 'n' Collar isoform-C (CncC) with Maf-S; these regulate transcription of detoxification genes in several insects[22]. This suggests that more attention should be paid to *cis*-regulatory regions to detect potential causative mutations that could drive resistance although this is not the unique mechanism as *trans*-acting regulatory loci[12] or allelic variations of resistance genes[9] may also be involved.

Because of the physical proximity of *CYP6P9b* to *CYP6P9a*, one could have assumed that both genes might be in complete linkage disequilibrium and any marker on one of the genes will fully explain resistance associated with the other. However, our data suggest otherwise; while we observed significant co-occurrence of resistance alleles in both genes, the genotypes at both genes can segregate independently and we observe increased pyrethroid resistance in mosquitoes with the combined genotype. The percentage of linkage between both genotypes varies significantly from nearly 100% in southern Africa to 71% in the East (Tanzania) and 51% in DRC. This suggests that both genotypes are not fully physically linked. The higher proportion of linked genotypes in southern Africa and Tanzania is possibly a result of a greater selection acting on this population as seen in southern Africa where proportion of identity is close to 100% and where high level of resistance has been reported[23,24].

The independent segregation between *CYP6P9a* and *CYP6P9b* is also observed in the lab—the hybrid strain between the lab-resistant FUMOZ and lab-susceptible FANG exhibited significant independent genotypes for both genes at the F_4 generation. Therefore, the availability of the CYP6P9b_R diagnostic assay developed here is an important tool that should increase our ability to detect and track the spread of resistance. Moreover, when combined with CYP6P9a_R, it could help to assess the risk of multi-gene driven high-resistance in this malaria vector. The current P450 diagnostic tools should allow control programmes to monitor both P450 alleles to track escalating pyrethroid resistance intensity in the field. This marker only applies to *An. funestus* and not to other malaria vectors including those of the *An. gambiae* complex. However, because *CYP6P3*, an ortholog of *CYP6P9a/b*, has also been shown to play an important role in insecticide resistance in *An. gambiae*, the results described here can serve as a model to detect similar markers in other malaria vectors, specifically of this species complex.

The experimental hut study performed here assessed the impact of *CYP6P9b* and revealed that *CYP6P9b* is associated with reduced efficacy of bed nets, particularly pyrethroid-only nets, independent of *CYP6P9a*[13]. However, the key message from this study is that a greater reduction of bed net efficacy is observed when the *CYP6P9b* resistant genotype combines with the *CYP6P9a* resistant genotype because double homozygote resistant mosquitoes survived exposure to pyrethroid-only nets much better than all other genotypes tested.

This shows the great risk that metabolic resistance poses to insecticide-based interventions as highlighted in the WHO global plan for insecticide-resistance management which states that if nothing is done pyrethroid resistance could lead to an increase burden of malaria in Africa[2]. This concern is further supported by a recent report from the 2018 World Malaria Report indicating that after nearly two decades of continued decline, malaria cases have significantly increased in 2017[25]. The spread of both CYP6P9b_R and CYP6P9a_R alleles poses a significant risk to the effectiveness of control programmes notably in southern Africa where both alleles are nearing fixation. The diagnostic tools designed here should help track their spread and help anticipate the management of such resistance. PBO-based nets (here PermaNet 3.0) are far more effective against such populations than pyrethroid-only nets, as also recently revealed in field trials in Tanzania[26]. Nevertheless, the high blood-feeding ability of resistant mosquitoes even against PBO-nets suggests that metabolic resistance affects the efficacy of such nets and calls for the development of other management options, for example nets that do not rely on pyrethroids. Our experimental hut trial study is based on a test with hybrid strains from two laboratory strains and the impact of the resistance alleles described here on the efficacy of LLINs in natural populations remains to be established, work that must be undertaken urgently.

In conclusion, this study used a comprehensive transcriptomic and targeted genomic approach to detect a P450-based resistance marker and designed a simple PCR assay to detect and track P450-mediated metabolic resistance to pyrethroid in the major malaria vector *An. funestus* in Africa. We show that the *CYP6P9b_R* resistance allele described here can combine with the previously detected *CYP6P9a_R*[19] present on the same *rp1* QTL, conferring greater insecticide resistance in our bed nets test than either resistance genotype in isolation. Out of the tested populations, we detected this allele only in southern and eastern African mosquito populations. The greater reduction of efficacy of insecticide-treated nets observed in double-resistant mosquitoes is a major concern for the sustainability of insecticide-based interventions solely relying on pyrethroids. Therefore, PBO-based nets should be preferably deployed for greater impact while encouraging the development of alternative mosquito management options in malaria-endemic regions.

Methods

Collection and rearing of mosquitoes. The two *An. funestus* laboratory colonies used here are the FANG colony—a completely insecticide-susceptible colony originating from Angola—and the FUMOZ colony derived from southern Mozambique that is highly resistant to pyrethroids and carbamates[27]. Mosquito collection was performed to cover the four main regions in sub-Saharan regions: southern (Malawi, Chikwawa (16°1' S, 34°47' E) in 2014[28]), eastern (Uganda, Tororo (0°45' N, 34°5' E) 2014[29]), central (Cameroon, Mibellon (6°46′ N, 11°70′ E) in 2015[30]), and west (Ghana, Obuasi (5°56′ N, 1°37′ W) in 2014[31]) across the continental range of *An. funestus* as recently described[13]. Overall, blood-fed, gravid females were collected indoor in houses using electric aspirators between 06:00 AM-10.00AM in each location. Females were left to become fully gravid for 4 days and then introduced into 1.5 ml Eppendorf tube to lay eggs following the forced-egg laying method[32]. Larvae were reared to generate F_1 adults and used for WHO bioassays as done by Weedall et al[13]. These populations are all resistant to pyrethroids and as well as to other insecticide classes[28–31]. Morphological and molecular identifications were performed to establish the species following Weedall et al.[13].

RNA extraction with library preparation and sequencing. The total RNA from three pools of ten female mosquitoes (alive after 1 h permethrin exposure) was extracted using the Arcturus PicoPure RNA isolation kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. This included a DNase treatment step. The total RNA was depleted for ribosomal RNA (rRNA) with Ribo-Zero low input kit for human/mouse/rat (Epicentre, Madison, WI, USA) using 100 ng of starting material. The Ribo-Zero mRNA-enriched material was used to prepare RNAseq libraries with the ScriptSeq v2 RNAseq library preparation kit (Epicentre) (15 cycles of PCR amplification). Agencourt AMPure XP beads (Beckman and Coulter, Beverly, MA, USA) were used to purify the libraries followed by a quantification using a Qubit fluorometer (Life Technologies). The size distribution was measured with a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

Pools of libraries (8/lane) were sequenced (2 × 125 bp paired-end sequencing) with v4 chemistry on a HiSeq 2500 (Illumina, San Diego, CA, USA). All sequence library preparation and sequencing were performed by the Centre for Genomic Research (CGR), University of Liverpool.

Analysis of RNAseq data. RNAseq data were analysed as recently described when analysing the contribution of the *CYP6P9a* gene[13], including the initial processing and quality assessment of the sequence data. The Subread aligner version 1.4.6[33] was used to align the RNAseq R1/R2 read pairs to the reference sequence for which the annotation was improved using BLAST2GO version 4.0.7[34].

FeatureCounts version 1.4.6[35] was used to count the fragments mapped in the sense orientation to annotated *An. funestus* genes (automated predictions from gene set AfunF1.4, downloaded from VectorBase). EdgeR[36] was used to analyse the differential gene expression with pairwise comparisons performed.

The differences in total tag counts among samples were corrected by calculating normalisation factors using the "TMM" (Trimmed Mean *M*-values) method in edgeR[36] with default parameters. The *P* values associated with logFC were adjusted for multiple testing using the false-discovery rate (FDR) approach[37]. Differentially expressed genes were defined as those with an FDR-adjusted *P* value < 5% and> twofold absolute difference in expression level. Tag counts and the total CDS lengths were used to calculate the fragments per kilobase of gene sequence per million mapped reads.

Further analyses of the RNAseq data were performed using the Strand NGS software (Strand Life Sciences, version 3.0) following RNA alignment and RNA-seq analysis pipeline with standard parameters. Genes differentially expressed in each country and between different countries (generated from Venn diagrams) were detected using DESeq normalisation with a FC > 2. Multiple test correction used Benjamini–Hochberg method with a false discovery rate of 5% (adjusted *P* value < 0.05).

Quantitative reverse transcriptase PCR. The expression patterns of the most differentially expressed detoxification genes between countries (Cameroon, Uganda, Malawi, Ghana and Benin) were validated using qRT-PCR. The primers are listed in Supplementary Table 4. Total RNA was extracted from three biological replicates from mosquitoes that survived 1 h exposure to permethrin in each country. One microgram was used as the template for cDNA synthesis using Superscript III (Invitrogen) with oligo-dT20 and RNase H according to the manufacturer's instructions. The qRT-PCR amplification was performed following standard protocol[11,38] after establishing the standard curves for each gene to assess PCR efficiency and quantitative differences between samples using serial dilution. The relative expression level and FC of each target gene was established for comparisons to Cameroon; these were calculated according to the $2^{-\Delta\Delta CT}$ method incorporating the PCR efficiency[39] after normalisation with the housekeeping genes ribosomal protein S7 (*RSP7*; AFUN007153) and actin 5C (AFUN006819).

Analysis of *CYP6P9b* polymorphism patterns Africa-wide. Due to the high up-regulation of *CYP6P9b*, the polymorphism of its 5′UTR between *CYP6P9b* and *CYP6P5* was analysed by amplifying and sequencing a 1-kb fragment, following same protocol as for *CYP6P9a*[18] in 15 mosquitoes in different regions of the continent. This included southern (Mozambique, Malawi and Zambia), central (DR Congo and Cameroon), eastern (Kenya and Uganda), and western Africa (Benin, Nigeria and Ghana) and in the two laboratory strains FANG and FUMOZ. The primers are presented in Supplementary Table 4. ClustalW[40] was used to align the sequences, and the polymorphisms were identified in DnaSPv5.10[41]. The maximum likelihood tree was constructed using MEGA 7.0 with bootstrapping (500 replicates)[42].

The 1 kb 5′UTR fragment of *CYP6P9b* was also amplified in mosquitoes collected in Mozambique before the scale up of bed nets (pre-intervention) in 2000 and in mosquitoes collected in 2016 in the same region after the deployment of long-lasting insecticidal nets in these countries. The PCR products were cloned and sequenced, and the sequencing data was analysed as described above while also constructing a haplotype network using TCS[43].

Promoter activity of 5′-flanking region sequences of *CYP6P9b*. To investigate the regulatory regions controlling *CYP6P9b*, the intergenic region between this gene and the preceding gene *CYP6P5* was used to design primers (6P9dplF and

6P9a/b) to amplify this region in the laboratory colonies FANG and FUMOZ. The PCR products were then cloned in to PJET1.2 (Thermo Scientific, St. Leon-Rot, Germany) and sequenced using the Pjet1.2 forward and reverse sequencing primers.

The promoter activities of the 5′-flanking region sequences were evaluated using a luciferase reporter assay. Primers (KpnI_6P9bF1.0 and HindIII_6P9a/b) were used to amplify the 5′-flanking region sequences for the resistant and the susceptible strains cloned in Pjet1.2 and then sub-cloned into the PGL3-basic (Promega, Madison, WI, USA) firefly luciferase vector between the Kpn1 and Hind111 restriction sites to form the recombinant promoter PGL3 for testing.

The recombinant constructs were transfected in *An. gambiae* cell line 4a-2 cell line (MRA-917 MR4, ATCC® Manassas Virginia) maintained at 25 °C in Schneider's *Drosophila* medium supplemented with 10% heat inactivated foetal calf serum and 1% penicillin/streptomycin. One day prior to transfection, about 5×10^5 cells were plated in each well of 24-well plates and allowed to reach 60–70% confluence. At about 60% confluence, the constructs were introduced in the cells using Qiagen Effectene Transfection Reagent by co-transfecting 600 ng recombinant reporter constructs (*CYP6P9b* 5'flanking region in pGL3-Basic), LRIM promoter in pGL3 and pGL3 without promoter with 1 ng actin-Renilla internal control in 60 ml DNA condensation buffer, 4.8 ml enhancer and 6 ml Effectene in triplicates. Two independent replicates were carried out. Two days after incubation at 25 °C, the cells were washed with PBS and lysed in 100 ml passive lysis buffer (Promega); a luminometer (EG & G Berthold) was used to measure the luciferase activity, which was normalised to Renilla luciferase activity.

To localise the enhancer elements responsible for the up-regulation of *CYP6P9b* in the resistant as opposed to the susceptible populations, progressive 5′ deletion fragments of the 5′ flanking region from −978 to +97 (from the predicted transcription start site) was performed. Serially deleted fragments −630 to +97, −440 to +97, −181 to +97 and −128 to +97 were constructed using HindIII_6P9a/b common reverse primer, and each of the forward primer KpnI_6P9bF(1.0), KpnI_6P9bF(0.6), KpnI_6P9bF(0.4), KpnI_6P9bF(0.3) and KpnI_6P9bF(0.1) was tested to check if the deleted fragments −978 to −631, −630 to −441, −440 to −182 and −181 to −129 enhanced the promoter activity.

Design of PCR-RFLP diagnostic for *CYP6P9b* genotyping. The SNP-RFLPing 2[44] tool was used to design a PCR-RFLP that could discriminate between the *CYP6P9b* promoter of the resistant (FUMOZ) and the susceptible (FANG) strains. Primers 6P9brflp_0.5F and 6p9brflp_0.5R were designed to amplify a region in the promoter common both to FUMOZ and FANG. Each PCR reaction was performed in a 15-µL volume consisting of 1× buffer A, 25 mM $MgCl_2$, 25 mM dNTPs, 10 mM of each primer 1U KAPA Taq polymerase (Kapa Biosystems, Boston, MA, USA). The following PCR amplification conditions were used initial denaturation step of 3 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 60 s at 72 °C with 10 min at 72 °C for final extension according to the KAPA kit instructions. Five microlitre of the PCR product was digested by adding 1 µL of CutSmart buffer 0.2 µL of *Tsp*45I restriction enzyme (New England Biolabs) and 3.8 µL of water. The mix was incubated at 65 °C for 2 h. The digested product was migrated on 2% agarose and stained with Midori Green Advance DNA Stain (Nippon genetics Europe GmbH) and visualised using a gel imaging system to confirm the product sizes; there was one band at 550 bp for the resistant (undigested) sample and two bands at 400 bp and 150 bp for the susceptible samples.

To validate the robustness of the PCR-RFLP to detect the pyrethroid resistance in the field population, the F_8 progeny from a cross between highly resistant (FUMOZ) and highly susceptible (FANG) strains previously used for QTL mapping[14] were genotyped and correlated with the resistance phenotype established using the odds ratio and Fisher's exact test.

The pattern of geographical distribution of the resistant allele of *CYP6P9b* allele across the continent was assessed by genotyping the CYP6P9b_R in 30–50 field-collected females of *An. funestus* from several countries in Africa using the DNA-based diagnostic assay.

Impact of CYP6P9b-on LLINs' efficacy using hut trials. The study was performed in Mibellon (6°4′60″ N and 11°30′0″ E)—a village in the Adamawa region of Cameroon where 12 experimental huts have recently been built with concrete bricks following the specific design for experimental hut from the West Africa region[45].

The study used a hybrid *An. funestus* strain generated from reciprocal crossing between the highly pyrethroid resistant strain FUMOZ-R (CYP6P9b_R) and the fully susceptible FANG strain (CYP6P9b_S)[9]. The F5 and F6 generations were used for the release experiments in the huts after the initial reciprocal crossing between the laboratory susceptible (FANG and resistant (FUMOZ) strains using 50 males and 50 females of both strains. The susceptibility of these strains to insecticides is reported by Weedall et al[13] using pyrethroids (0.75% permethrin and 0.05% deltamethrin), DDT (4%) and the carbamate and bendiocarb (0.1%) according to WHO protocol[45].

The following three treatments were compared in the experimental huts: (i) untreated polyethylene net; (ii) PermaNet 2.0® (deltamethrin incorporated into polyethylene net); and (iii) PermaNet 3.0® (PBO + Deltamethrin incorporated into polyethylene net). To simulate a worn net, six 4 cm × 4 cm holes were made on each net according to WHO guidelines. The hybrid FANG/FUMOZ strain was released in each hut for 6 nights (80 mosquitoes per hut).

Three adult volunteers were recruited from the Mibellon village to sleep under the nets and collect mosquitoes in the morning. Each volunteer provided a written consent to participate in this study and were also given chemoprophylaxis during the trial. Ethical approval was obtained from the National Ethic Committee of the Ministry of the Health in Cameroon.

Mosquito collection was performed in the morning by each volunteer in their respective room using glass tubes. Mosquitoes were collected in three compartments including the main room (the walls, floor and the hut ceiling), inside of each net and in the exit traps (veranda). A single bag was used for each compartment to avoid the risk of mixing samples from different areas. A sugar solution was provided to mosquitoes found alive and they were kept in paper cups for 24 h to assess the delayed mortality. The status of collected mosquitoes was recorded in observation sheets either as alive/blood fed, dead/blood fed, alive/unfed and dead/unfed.

An untreated net was used as control to estimate the effect of each treatment by assessing several parameters including the induced exophily (the proportion of mosquitoes exiting the room early through the exit traps), the mortality rates (which provides an indication of the potential mass killing effect of the bed nets) and the blood feeding rate (which estimates the level of personal protection).

A direct comparison to the untreated control net was used to establish the effect of both treated nets. A logistic regression model using the Wald statistic that follows a chi-squared distribution (with $df = 1$) was used to assess the statistical significance of the difference.

The CYP6P9b_R resistance allele was genotyped in a subset of each treatment to determine the impact of the *CYP6P9b*-mediated metabolic resistance to pyrethroids on the effectiveness of the bed nets. The following were included: the dead, alive, blood-fed, and unfed mosquitoes in the veranda, in the net and in the room. The association between the mutation and the performance of each net was assessed using the Vassar stats (http://vassarstats.net/) with a 2 × 2 contingency table for the odds ratio calculation based on Fisher's exact probability test.

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

Data availability

RNAseq: PRJEB24351 and PRJEB10294; *CYP6P9b sequences*: GenBank MK457459-MK457689. Data analysed in this study are available on public repositories or available within the article and its Supplementary Information files. Further details are available from the authors upon request.

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Author contributions

C.S.W. conceived and designed the study; J.M.R. and C.S.W. performed the field collection and resistance bioassays; H.I. and C.S.W. performed the sample preparation for all RNAseq sequencing; G.D.W. and C.S.W. analysed the next-generation sequencing data with assistance from J.H. M.J.W., J.M.R. and Ma.T. performed qRT-PCR; L.M.J.M. and C.S.W. characterised *CYP6P9a* promoter and analysed the genetic diversity of *CYP6P9a*; L.M.J.M. and C.S.W. designed the PCR-RFLP diagnostic assay; L.M.J.M. and Mi.T. generated the lab crosses and performed the validation of the PCR-RFLP; B.D.M. performed the experimental hut experiments with CSW and genotyped *CYP6P9a* with M.J.W. and Ma.T.; C.S.W. wrote the paper with assistance from L.M.J.M. and J.H.; all authors read and approved the final draft of the paper.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to C.S.W.

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MALARIA VECTOR CONTROL

A cytochrome P450 allele confers pyrethroid resistance on a major African malaria vector, reducing insecticide-treated bednet efficacy

Gareth D. Weedall[1,2], Leon M. J. Mugenzi[3,4], Benjamin D. Menze[1,3,4], Magellan Tchouakui[3,4], Sulaiman S. Ibrahim[1,5], Nathalie Amvongo-Adjia[4,6], Helen Irving[1], Murielle J. Wondji[1,3,4], Micareme Tchoupo[3,4], Rousseau Djouaka[7], Jacob M. Riveron[1,3,4], Charles S. Wondji[1,3,4]*

Metabolic resistance to insecticides such as pyrethroids in mosquito vectors threatens control of malaria in Africa. Unless it is managed, recent gains in reducing malaria transmission could be lost. To improve monitoring and assess the impact of insecticide resistance on malaria control interventions, we elucidated the molecular basis of pyrethroid resistance in the major African malaria vector, *Anopheles funestus*. We showed that a single cytochrome P450 allele (*CYP6P9a*_R) in *A. funestus* reduced the efficacy of insecticide-treated bednets for preventing transmission of malaria in southern Africa. Expression of key insecticide resistance genes was detected in populations of this mosquito vector throughout Africa but varied according to the region. Signatures of selection and adaptive evolutionary traits including structural polymorphisms and cis-regulatory transcription factor binding sites were detected with evidence of selection due to the scale-up of insecticide-treated bednet use. A cis-regulatory polymorphism driving the overexpression of the major resistance gene *CYP6P9a* allowed us to design a DNA-based assay for cytochrome P450–mediated resistance to pyrethroid insecticides. Using this assay, we tracked the spread of pyrethroid resistance and found that it was almost fixed in mosquitoes from southern Africa but was absent from mosquitoes collected elsewhere in Africa. Furthermore, a field study in experimental huts in Cameroon demonstrated that mosquitoes carrying the resistance CYP6P9a_R allele survived and succeeded in blood feeding more often than did mosquitoes that lacked this allele. Our findings highlight the need to introduce a new generation of insecticide-treated bednets for malaria control that do not rely on pyrethroid insecticides.

INTRODUCTION

Prevention of malaria relies heavily on the use of insecticide-based vector control interventions, most notably pyrethroid-based long-lasting insecticidal bednets. These tools have been credited with more than 70% of the decrease in malaria mortality in the past 15 years, having helped avert more than 663 million clinical cases of malaria (*1*). However, resistance of mosquito vectors to insecticides, particularly pyrethroids, is threatening their continued effectiveness. Unless it is managed, the recent gains in reducing malaria transmission could be lost (*2*). Elucidating the genetic basis and evolution of insecticide resistance among mosquito vectors of malaria is crucial for designing resistance management strategies and preventing the resurgence of malaria (*2*).

Without genetic information on insecticide resistance genes and associated molecular markers in the mosquito vector, it is difficult to track and anticipate the course of insecticide resistance or assess its impact on malaria transmission and the effectiveness of control tools such as long-lasting insecticide-treated bednets. The current inability to track metabolic resistance in this way in all major malaria vectors in Africa including *Anopheles gambiae* and *Anopheles funestus* is a major obstacle to the design of rational, evidence-based resistance management strategies. Of the four classes of insecticides deployed in public health, pyrethroids are the most widely used and are the main class recommended for use in insecticide-treated bednets. Therefore, understanding the mechanisms conferring pyrethroid resistance on mosquitoes is of critical importance.

Two major causes of insecticide resistance are metabolic resistance and target-site insensitivity (*3*). Target-site insensitivity to pyrethroids is due to knockdown resistance (*kdr*) caused by a modification of the insecticide target (a sodium channel) in mosquitoes (*4*). In contrast, metabolic resistance remains less well characterized despite posing a greater risk to malaria control interventions (*5*). Although candidate resistance genes have been detected (*6*–*9*), it has proved difficult to dissect the molecular bases of metabolic resistance and to detect associated molecular markers because of the size of gene families involved in detoxification, redundancy among their members, and the multiple mechanisms through which metabolic resistance can arise (*10*). Cytochrome P450 monooxygenases have consistently been associated with pyrethroid resistance but, unlike *kdr* (*4*), there is no DNA-based marker to track P450-mediated resistance and to assess its impact on malaria control strategies.

Here, we elucidated the complex molecular basis and genomic evolution of metabolic resistance to pyrethroids in the major African malaria vector *A. funestus*. We detected key DNA-based markers of pyrethroid resistance and designed a field-applicable diagnostic assay. We used this assay to track pyrethroid resistance across Africa and to demonstrate that this metabolic resistance in mosquitoes reduced the efficacy of insecticide-treated bednets for preventing malaria transmission.

[1]Department of Vector Biology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK. [2]School of Natural Sciences and Psychology, Liverpool John Moores University, Liverpool L3 3AF, U.K. [3]LSTM Research Unit at the Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale (OCEAC), P.O. Box 288, Yaoundé, Cameroon. [4]Centre for Research in Infectious Diseases (CRID), P.O. Box 13591, Yaoundé, Cameroon. [5]Department of Biochemistry, Bayero University, PMB 3011, Kano, Nigeria. [6]Centre for Medical Research, Institute of Medical Research and Medicinal Plants Studies (IMPM), P.O. Box 13033, Yaoundé, Cameroon. [7]International Institute of Tropical Agriculture (IITA), Cotonou 08 BP 0932, Benin.
*Corresponding author. Email: charles.wondji@lstmed.ac.uk

RESULTS

RNA sequencing transcriptional profiling of mosquitoes identifies candidate pyrethroid resistance genes

To identify genes associated with pyrethroid resistance in the mosquito vector *A. funestus* Africa-wide, we performed RNA sequencing (RNA-seq)–based transcriptional profiling of mosquitoes from four different African regions: southern (Malawi), East (Uganda), West (Ghana), and Central (Cameroon). We compared these RNA-seq profiles to those from a laboratory colony of mosquitoes (FANG) that were susceptible to all insecticides. The quality metrics and the alignment parameters are presented in table S1. The number of differentially expressed genes between each of the four populations and the FANG susceptible strain is shown in Venn diagrams (Fig. 1A and fig. S1A); the expression profile is shown in the volcano plots for each country (fig. S1, B to E). After quality control and analyses (table S1), Gene Ontology (GO) enrichment was performed to assess the generic metabolic terms associated with resistance. Permethrin-resistant mosquitoes in Malawi showed enrichment of gene ontologies associated with cytochrome P450 genes in genes overexpressed relative to the fully susceptible FANG strain. These GO terms included heme binding, tetrapyrrole binding, oxidoreductase activity, and iron binding (fig. S2A). These GO terms among others were also enriched in the overexpressed gene set in mosquitoes from Ghana (fig. S2B). In Ugandan mosquitoes, more GO terms were enriched among the overexpressed genes but were not directly associated with detoxification activities (fig. S2C).

Pronounced differences in the expression of key candidate genes were observed among *A. funestus* from the four different African regions (Fig. 1, A to C; fig. S1, A to E; and table S1). Genes encoding cytochrome P450 were frequently overexpressed (adjusted $P < 0.05$) with the genes *CYP6P9a* (AFUN015792; 60.5-fold) and *CYP6P9b* (AFUN015889; 23.9-fold) showing increased overexpression in Malawi mosquitoes compared to those from other regions (<7-fold; Fig. 1, B and C). Other cytochrome P450s were overexpressed more in mosquitoes from one region than in mosquitoes from other African regions (Fig. 1, B and C) including *CYP9K1* (AFUN007549), which was highly overexpressed in mosquitoes from Uganda (5.2-fold), only moderately overexpressed in those from Ghana (2.9-fold), and not overexpressed in mosquitoes from Malawi or Cameroon compared to FANG mosquitoes. *CYP6P5* (AFUN015888) was overexpressed in mosquitoes from Ghana (6.3-fold), Cameroon (5.8-fold), and Uganda (4.1-fold), but not in those from Malawi compared to FANG mosquitoes. The duplicated *CYP6P4a* and *CYP6P4b* were highly overexpressed in mosquitoes from Ghana (44.8- and 23.9-fold, respectively), moderately so in those from Malawi and Uganda (<6-fold), and not overexpressed in those from Cameroon compared to FANG mosquitoes. *CYP325A* was highly overexpressed in mosquitoes from Cameroon (26.9-fold) but less so in those from other regions (<6-fold) compared to FANG mosquitoes. Other

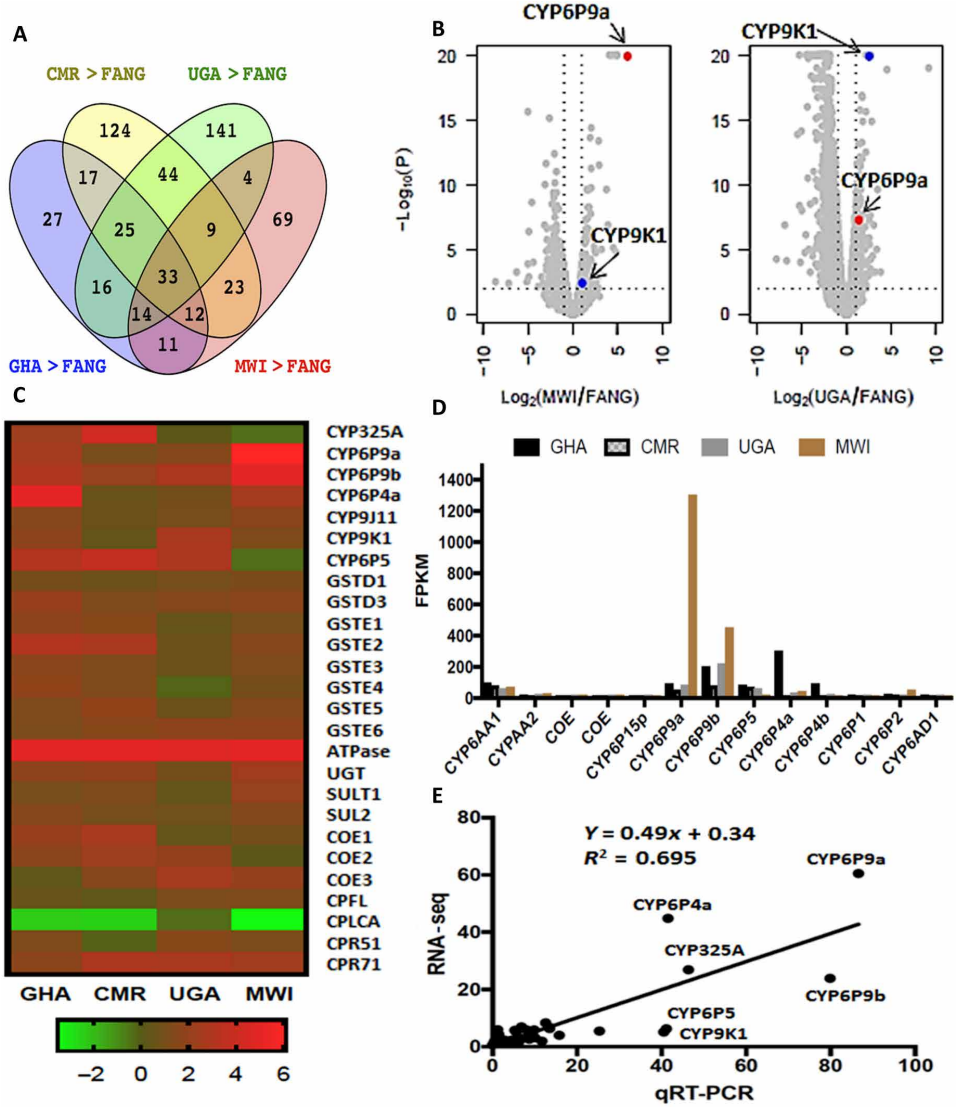

Fig. 1. RNA-seq transcriptional profiling of pyrethroid-resistant populations of *A. funestus* Africa-wide. (**A**) The Venn diagrams show the number of differentially up-regulated genes in *A. funestus* mosquitoes from different countries in Africa relative to the pyrethroid-susceptible FANG laboratory strain at a false discovery rate of <0.01 and a fold change of >2. (**B**) Volcano plots of differential gene expression between pyrethroid-exposed mosquitoes from four different countries in Africa (Cameroon, CMR; Malawi, MWI; Ghana, GHA; and Uganda, UGA) and the susceptible FANG strain. The volcano plots highlight differences in the expression of key resistance genes (e.g., *CYP6P9a* and *CYP9K1* in mosquitoes from different African countries). (**C**) Heatmap showing the differential expression of candidate detoxification genes among mosquitoes from different African countries. (**D**) Fragments per kilobase of transcript per million mapped reads (FPKM) for genes from the *rp1* pyrethroid resistance QTL. The histogram highlights differences among mosquitoes from four different African countries. (**E**) Correlation between RNA-seq and qRT-PCR data. The data shown are mean ± SEM ($n = 3$).

cytochrome P450s were moderately overexpressed, including two paralogous genes, *CYP9J11* and *CYP6N1*, which were up-regulated in mosquitoes from southern and West Africa, whereas *CYP315A1* was overexpressed in mosquitoes from all sites but Malawi.

Other detoxification-associated gene families were also overexpressed, including a cluster of glutathione *S*-transferase epsilon genes (*GSTe1*, *GSTe3*, *GSTe4*, *GSTe5*, and *GSTe2*), known dichlorodiphenyltrichloroethane (DDT) resistance genes (*11*) that were up-regulated in mosquitoes from all regions except East Africa (table S2). The *GSTD1* gene was overexpressed in mosquitoes from Malawi, Ghana, and Uganda, suggesting a role for this gene in these regions but not in Cameroon where it was not significantly overexpressed (table S2). A carboxylesterase (AFUN002514) was also overexpressed in mosquitoes from Central and West Africa with 5.5- and 3.6-fold expression, respectively, in mosquitoes from Cameroon and Ghana (table S2). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) with 15 genes confirmed these regional differences, with a close correlation observed between qRT-PCR and RNA-seq results for the four countries when compared to FANG mosquitoes ($R^2 = 0.85$; $P < 0.001$) (Fig. 1E and fig. S3, A to C). Control mosquitoes not exposed to insecticides also showed a strong correlation ($R^2 = 0.695$, $P = 0.002$ in mosquitoes from Malawi) with permethrin-resistant samples used for RNA-seq (Fig. 1E), supporting a constitutive expression of these candidate resistance genes to confer resistance. However, differences were observed for the expression of some genes such as the *CYP6Z1* P450, which, with qRT-PCR (but not RNA-seq), exhibited an up-regulation in Malawi (FC 66) and Ghana (FC13.4) mosquitoes (fig. S3). This gene has previously been shown to be overexpressed in southern Africa using microarray and qRT-PCR (*7*), suggesting that RNA-seq could have missed or that the primers were not efficient with the susceptible FANG strain.

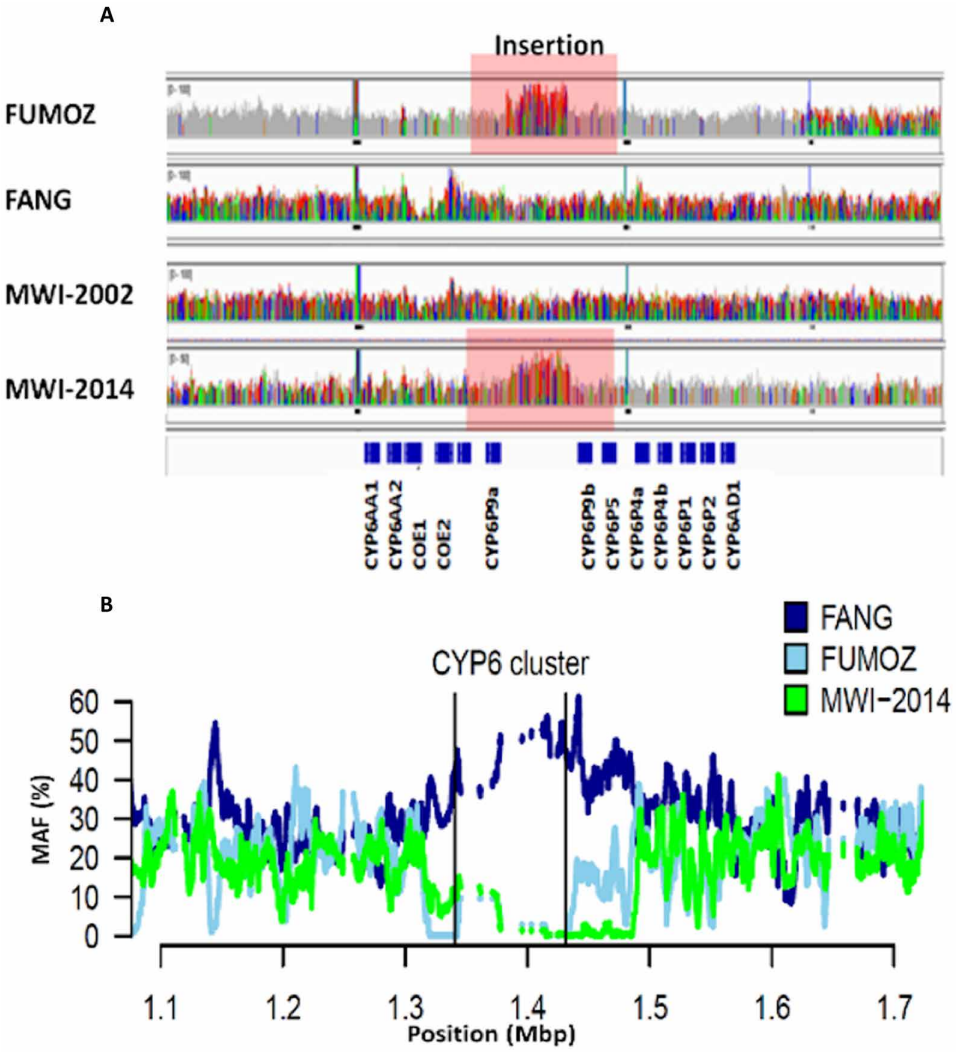

Fig. 2. Genetic signatures in the mosquito genome associated with pyrethroid resistance. (**A**) Contrasting polymorphism patterns among resistant FUMOZ and susceptible FANG mosquito strains. Also shown are contrasting polymorphism signatures between pre-bednet intervention (MWI-2002) and post-bednet intervention (MWI-2014) mosquito samples from Malawi. Data were aligned to 120 kb *rp1* bacterial artificial chromosome sequence (Integrative Genomics Viewer screenshot). Each row shows the alignment depth (on a log scale for display purposes); coverage depth is capped at >100×. Gray columns represent bases identical to the reference sequence, whereas colored columns indicate variant sites with a minor allele frequency > 10%. The genes of the cytochrome P450 cluster are indicated below the panel. An increased read coverage was observed between *CYP6P9a* and *CYP6P9b* in pyrethroid-resistant mosquito samples indicating a 6.5-kb insertion. (**B**) Shown is the major gene signature after a selective sweep through the mosquito genome detected around the *rp1* QTL pyrethroid resistance region on the 2R chromosome. This signature contained cytochrome P450 genes from the *CYP6* cluster and was found in mosquitoes from southern Africa but not in the susceptible FANG strain, after plotting minor allele frequency (MAF).

Whole-genome polymorphism analysis of mosquitoes collected in the field identifies insecticide resistance loci

Metabolic resistance could be conferred by point mutations in coding and cis-/trans-regulatory regions of the mosquito genome. Therefore, we scanned the whole genome for pyrethroid resistance–related signatures of selective sweeps (that is, a marked reduction in genetic diversity in a genomic region because of intense selection of a predominant allele) in the highly pyrethroid-resistant population of mosquitoes from Malawi to detect pyrethroid resistance loci. We performed pooled-template whole-genome sequencing on field-collected population samples from southern Africa where high expression of *CYP6P9a* and *CYP6P9b* genes has been observed. We also comparatively assessed the genomes of mosquitoes from Malawi and compared these genomes to those of the laboratory pyrethroid-resistant FUMOZ-R strain and the pyrethroid-susceptible FANG strain. We detected contrasting patterns of polymorphisms between the genomes of these strains and also between the genomes of the

MWI-2002 and MWI-2014 mosquito samples collected in Malawi before and after bednet intervention, respectively (Fig. 2A and table S3). The major selective sweep for genomes of Malawi and FUMOZ-R mosquitoes was on scaffold KB119169, spanning the *rp1* pyrethroid resistance region on chromosome arm 2R (Fig. 2B). Plotting minor allele frequencies across the region revealed a valley of reduced genetic diversity around the cluster of cytochrome P450 genes on the *rp1* pyrethroid resistance QTL, correlating a selected *rp1* haplotype with *CYP6P9a* overexpression (Fig. 1D). This selective sweep appeared to be at or near fixation in a contemporary Malawian mosquito population as well as in the FUMOZ-R strain, with little diversity observed around the cytochrome P450 gene cluster in this highly pyrethroid-resistant mosquito population (Fig. 2B). No reduced diversity was observed in the pyrethroid-susceptible FANG strain, suggesting an association between this selective sweep and pyrethroid resistance in line with the very low *CYP6P9a* expression in FANG mosquitoes (Fig. 2, A and B). These results are consistent with previous reports of selection on highly overexpressed resistance genes (*9*).

Complex evolution of the *rp1* cluster of *CYP6* genes associated with pyrethroid resistance

As the *rp1* gene cluster was consistently associated with pyrethroid resistance, a fine-scale analysis of this locus (120 kb) was performed, revealing evidence of complex molecular evolution that was most likely under insecticide-driven selection. Inspection of pooled-template whole-genome alignments showed two anomalous features in the 8.2-kb sequence between *CYP6P9a* and *CYP6P9b* (Fig. 2A and fig. S4A). In some samples, the coverage depth was greater than for the surrounding sequence, and some samples showed read pairs in the correct relative orientation but with greater than expected insert sizes. This was indicative of a large indel, that is, either a "deletion" in the sequenced genome or an "insertion" in the reference genome (fig. S4A). This insertion corresponded to 6545 base pairs (bp) and appeared to be fixed in the FUMOZ mosquito colony sample with evidence that the inserted sequence was homologous to another region nearby (on the same assembly scaffold) in the genome (fig. S4B). In contrast, the FANG pyrethroid-susceptible mosquito strain showed evidence of the deletion form of the indel (fig. S4A). This insertion, nearly fixed across the genomes of mosquitoes from southern Africa (26 of 27), was absent from the genomes of mosquitoes collected from elsewhere on the continent in which only a 1.7-kb intergenic region was observed after PCR (table S4). RNA-seq data showed that the inserted region was a transcribed region, showing evidence of splicing and containing three microRNAs but no cytochrome P450 genes.

We assessed the composition of this 6.5-kb insert to elucidate its role by sequencing the full 8.2-kb *CYP6P9a/b* intergenic region and analyzing it using GPMiner, a program for identifying promoter regions and annotating regulatory features (*12*). This insert contained

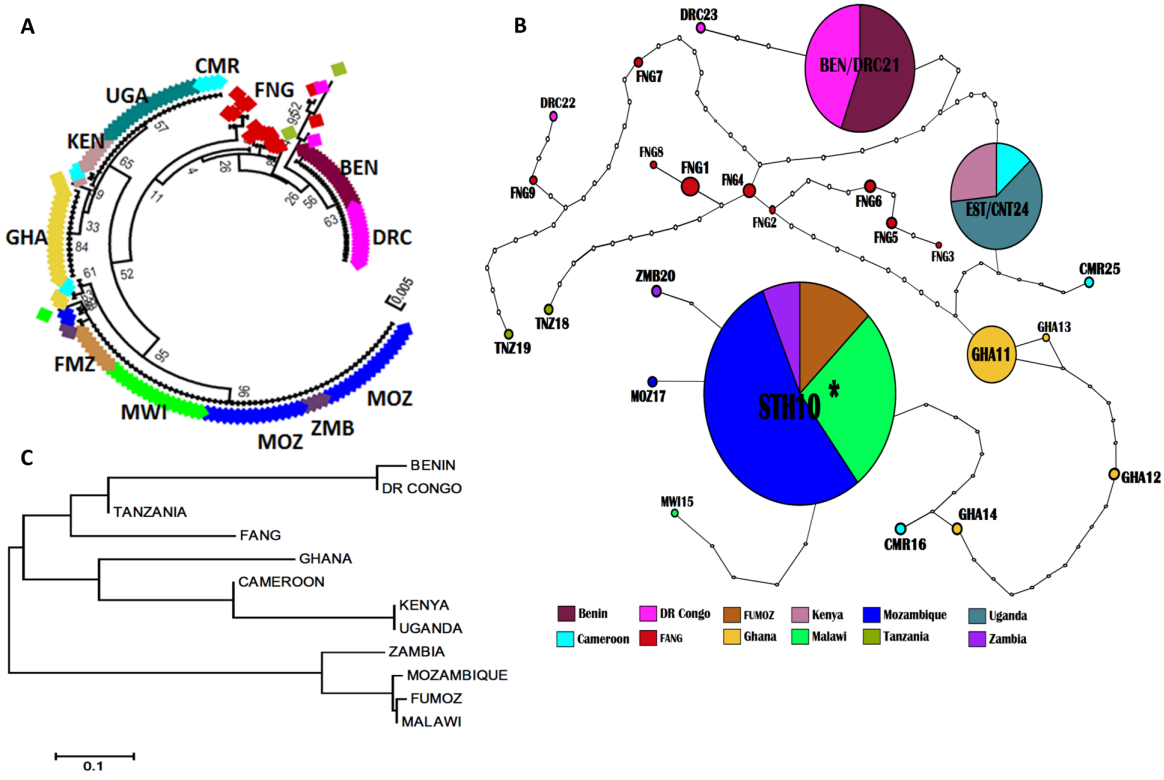

Fig. 3. Genetic diversity of the *CYP6P9a* 5UTR region in mosquitoes from different regions of Africa. (**A**) Molecular phylogenetic analysis of the *CYP6P9a* 5′UTR region in genomes of *A. funestus* mosquitoes collected across Africa, generated by the ML method. The evolutionary history of *CYP6P9a* promoter haplotypes of mosquitoes from across Africa was inferred using the ML method based on the Tamura three-parameter model. (**B**) Africa-wide TCS network for the *CYP6P9a* haplotypes showing four predominant regional haplotypes in southern Africa (STH10), West Africa (Ghana, GHA11), Central Africa (Benin/Democratic Republic of Congo, BEN/DRC21), and East/Central Africa (EST/CNT24). "*" indicates ancestral haplotype. Lines connecting haplotypes and each node represent a single mutation event (respective polymorphic positions are given on each branch). (**C**) Neighbor-joining phylogenetic tree of *CYP6P9a*-based genetic distance between 10 different populations of *A. funestus* mosquitoes from across Africa (N_{ST} estimates). MOZ, Mozambique; ZMB, Zambia; TNZ, Tanzania; DRC, Democratic Republic of Congo; FNG, FANG mosquito strain; FMZ, FUMOZ mosquito strain.

abundant binding sites for transcription factors including a CpG island (1.3 kb); several GATA, TATA (35), CCAAT (12), and GC (11) boxes; and overrepresented oligonucleotides. It also contained several binding sites for key transcription factors associated with xenobiotic detoxification, including Cap-n-Collar-C (CnCC) (51 sites) and muscle aponeurosis fibromatosis (MafK), suggesting that this insertion may be able to drive *CYP6P9a/b* overexpression. The insertion also contained a microsatellite (FUNR) between 6082 and 6482 bp, which was only 80 bp from the 5′ untranslated region (5′UTR) of *CYP6P9a*. Previous genotyping of this marker in mosquitoes Africa-wide revealed marked differences associated with a pyrethroid resistance profile (*13*). The FUNR microsatellite marker was not present within the 1.7-kb intergenic region between *CYP6P9a* and *CYP6P9b* in the genome of the pyrethroid-susceptible FANG mosquito strain. It has been shown in other insects such as aphids that microsatellite loci are involved in up-regulation of cytochrome P450, conferring insecticide resistance (*14*).

CYP6P9a regulatory region polymorphisms associated with pyrethroid resistance

To detect cis-regulatory mutations controlling *CYP6P9a/b*-based pyrethroid resistance, we compared an 800-bp sequence immediately upstream of *CYP6P9a* in mosquito samples from across Africa. Genomes of mosquitoes from several locations exhibited low or no polymorphisms in this region (fig. S5A). The sample with the highest diversity was the fully susceptible FANG mosquito strain, with diversity indices supporting selection acting on the *CYP6P9a* gene in field populations of mosquitoes resistant to pyrethroids. Despite the low diversity in the 800-bp sequence upstream of *CYP6P9a* observed in genomes of mosquitoes from different regions, mosquito populations from southern Africa consistently exhibited a different polymorphism pattern to those from other regions, including the presence of an AA insertion 8 bp upstream of a putative CCAAT box present only in southern Africa mosquito samples through an A/C substitution (fig. S5A). The AA insertion located 359 bp from the start codon of *CYP6P9a* was tightly associated with other polymorphisms in a haplotype (STH10) that was nearly fixed in the genomes of southern Africa mosquitoes (63 of 68), reflecting the marked selective sweep observed around this gene in this African region. Analysis of the phylogenetic tree revealed four clusters of haplotypes in mosquitoes from different regions: southern Africa (Malawi, Mozambique, and Zambia plus the FUMOZ-R strain), East-Central Africa (Kenya, Uganda, and Cameroon), West Africa (Ghana), and West-Central Africa (Benin and Democratic Republic of Congo) (Fig. 3A). The FANG pyrethroid-susceptible strain formed its own cluster that was divergent from the other clusters.

Closer analysis of the haplotypes using a haplotype network confirmed the presence of four major haplotypes corresponding to these four geographical clusters: STH10 in southern Africa, EST/CNT24 in East-Central Africa, BEN/DRC21 in West-Central Africa, and GHA11 in Ghana only (West Africa) (Fig. 3B). Unexpectedly, the other three geographical regions compared to southern Africa also exhibited predominant haplotypes in the mosquito populations to near fixation, contrary to previous data where they were found to be more polymorphic (*13*). This result suggested that resistance to pyrethroid beyond southern Africa could have been selected through *CYP6P9a* or other genes in the vicinity of the *rp1* QTL region. The neighbor-joining phylogenetic tree showing the genetic distances based on the N_{ST} genetic differentiation index between different countries (Fig. 3C) correlated with the polymorphism patterns showing countries clustering according to the haplotype diversity patterns from the maximum likelihood (ML) tree (Fig. 3A) or the Templeton, Crandall, and Sing (TCS) network (Fig. 3B).

CYP6P9a regulatory region changes selected for by insecticide-treated bednet scale-up

We next assessed whether the differences observed in the 5′UTR and upstream region of the *CYP6P9a* gene in mosquitoes from southern Africa compared to those from other regions were a result of selective pressure from insecticides. We compared mosquito samples from southern Africa before the scale-up of insecticide-based interventions, such as use of long-lasting insecticide-treated bednets, and mosquito samples after scale-up of bednet use. Thirty-nine clones of the 800-bp fragment upstream of *CYP6P9a* were obtained and sequenced from mosquitoes from Mozambique and Malawi before the bednet intervention and were compared to 52 clones obtained after the bednet intervention. Preintervention mosquito samples showed highly polymorphic regions in the 800-bp fragment (table S5 and fig. S5B). Before bednet intervention, there were many segregating sites (61 in Malawi mosquitoes and 25 in Mozambique mosquitoes), many haplotypes (19 of 30 and 12 of 30, respectively), and high nucleotide diversity ($\pi = 0.027$ and 0.012) (table S5 and fig. S5B). By contrast, in the mosquito samples from Malawi and Mozambique obtained after the bednet intervention, there was low diversity as revealed by a number

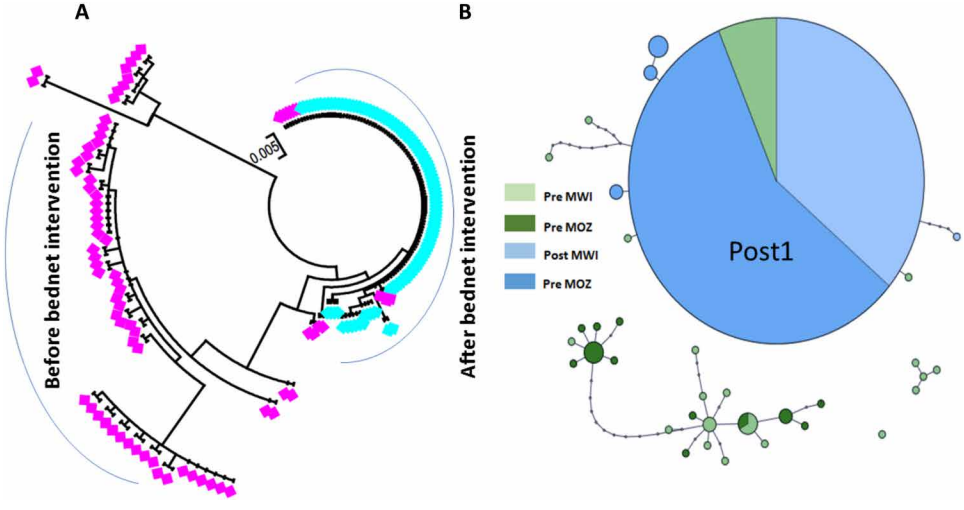

Fig. 4. Impact of bednet usage on the genetic diversity of the *CYP6P9a* promoter in mosquitoes. (**A**) Shown is the ML phylogenetic tree of *CYP6P9a* in *A. funestus* mosquitoes from Malawi collected before and after introduction of pyrethroid-treated bednets. This tree shows a cluster of highly diverse haplotypes (pink) before bednet intervention but a nearly fixed haplotype (blue) after bednet intervention. (**B**) TCS haplotype network in mosquitoes collected in Malawi (MWI) and Mozambique (MOZ) before bednet intervention (Pre) and after bednet intervention (Post). The network reveals a major resistant haplotype after intervention (Post1), but a very diverse set of haplotypes before bednet intervention.

of polymorphic sites (S) of 4 and 3 and a haplotype number of 2 and 4, respectively, with extremely low nucleotide diversity (π = 0.00066 and 0.0008). These differences were reflected on the ML phylogenetic tree, showing that mosquito samples taken before bednet intervention not only clustered together but also were more diverse and showed several haplotypes (Fig. 4A). In contrast, mosquito samples obtained after bednet intervention showed a markedly reduced haplotype number (Fig. 4A). A haplotype network confirmed that the major haplotype associated with pyrethroid resistance and now nearly fixed in all southern Africa mosquito populations was present in the pre-bednet intervention samples but at a much lower frequency of only 4 of 39 (10.2%) (Fig. 4B). This was compared to a much higher frequency of 44 of 52 (84.6%) in post-bednet intervention samples, with other haplotypes being only one or two mutational steps away from the predominant one (Fig. 4B). The pre-bednet intervention haplotypes were polymorphic and were separated by a number of mutational steps, whereas the post-bednet intervention mosquito samples showed a markedly reduced diversity (Fig. 4B). A detailed analysis of the polymorphisms between pre- and post-bednet intervention mosquito samples revealed that the AA insertion as well as the CCAAT box were now fixed (52 of 52) in all southern Africa mosquito populations after bednet intervention. The CCAAT box was also present in pre-bednet intervention samples but only at a very low frequency (7 of 24 and 0 of 15 for Malawi and Mozambique mosquito populations, respectively). Furthermore, a second binding site for the Nrf2:MafK xenobiotic transcription factor was found only in post-bednet intervention samples and was linked to both the AA insertion and the CCAAT box. These major modifications suggest that scale-up of use of insecticide-treated bednets is likely to have been a major factor driving evolution in *A. funestus* mosquito populations in southern Africa.

A molecular marker associated with overexpression of a pyrethroid resistance gene

Having confirmed that genomic changes upstream of *CYP6P9a* were associated with pyrethroid resistance in mosquitoes, we next searched for the mutations responsible for the overexpression of *CYP6P9a* in pyrethroid-resistant mosquitoes. We used a luciferase assay to assess the role of polymorphisms found in the 800-bp sequence upstream of the translation start site of *CYP6P9a* (including the 5′UTR). The 800-bp sequence upstream of the *CYP6P9a* translation start site in both FUMOZ and FANG mosquito strains was successfully cloned and sequenced. To narrow down the region containing the regulatory motifs, four different-sized fragments (800, 500, 300, and 150 bp) immediately upstream of the translation start codon were cloned upstream of a reporter gene in a pGL3 vector. These constructs were used in luciferase reporter gene assays. The assays demonstrated that although 800-bp insertions from both FUMOZ and FANG mosquito strains drove reporter gene expression, expression driven by the FUMOZ strain insertion was three times higher than that driven by the FANG strain insertion (fig. S6A). This finding supported a role for this region in the differential expression of *CYP6P9a* between pyrethroid-resistant and pyrethroid-susceptible mosquitoes. Progressive deletion of the 800-bp pGL3-FZ-*CYP6P9a* was performed to identify the major regulatory elements driving this differential gene expression. The first deletion from 800 to 500 bp did not affect the activity of the fragment. However, cutting the fragment from 500 to 300 bp (removing the AA insert and the CCAAT box) resulted in a 33% reduction of *CYP6P9a* activity in the FUMOZ strain ($P < 0.001$) (Fig. 5A). Subsequent deletion from 300 to 150 bp, which removed the AA insert, the CCAAT box, and the pyrethroid resistance–specific CnCC/MafK binding site, led to an 89% reduction in *CYP6P9a* activity ($P < 0.001$). This showed that

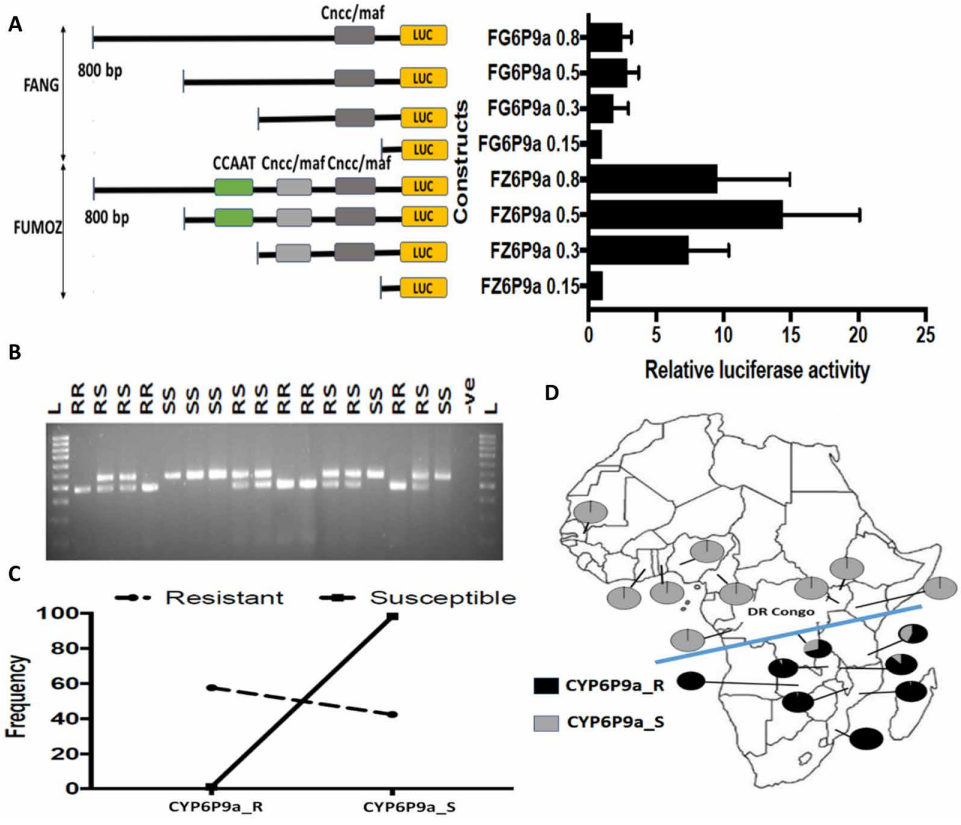

Fig. 5. A DNA-based assay to detect cytochrome P450–mediated metabolic-based pyrethroid resistance in mosquitoes. (**A**) Luciferase promoter assay for detecting the *CYP6P9a* 5′ flanking region. Shown are progressive serial deletions in an 800-bp fragment of the promoter region of *CYP6P9a* enabling detection of the variants causing pyrethroid resistance (mean ± SD; $n = 6$). (**B**) Agarose gel showing *CYP6P9a* fragments from the Taq I PCR-RFLP assay distinguishing the three genotypes of pyrethroid resistance: RR (homozygous resistance), RS (heterozygous resistance), and SS (susceptible). (**C**) Significant correlation ($P < 0.0001$) between the *CYP6P9a* resistance allele (*CYP6P9a*_R) and pyrethroid (permethrin) resistance. (**D**) Africa-wide distribution of the *CYP6P9a* resistance allele (*CYP6P9a*_R) in *A. funestus* mosquitoes showing near fixation in mosquitoes from southern Africa. The blue line represents the geographical limit of the spread of the *CYP6P9a*_R allele in Africa. Circles represent the frequency distribution of alleles in each location.

both the CCAAT box and the CnCC/MafK binding sites were key regulatory enhancer elements driving the overexpression of *CYP6P9a*.

A DNA-based diagnostic assay to detect *CYP6P9a*-mediated pyrethroid resistance

To design a DNA-based diagnostic assay to detect *CYP6P9a*-mediated pyrethroid resistance, we screened the most active portion (500 bp) for the presence of restriction site polymorphisms that could be used to design a simple restriction fragment length polymorphism PCR (PCR-RFLP) assay. We found a restriction site for the Taq I enzyme (cut site, 5′-TCGA-3′) spanning an A/G mutation located 18 bp upstream of the AA insertion (fig. S6B) and completely linked with the CCAAT box and other regulatory elements on the resistance haplotype. The Taq I enzyme cut the 450-bp fragment from the putative pyrethroid resistance haplotype into two fragments of 350 and 100 bp; the putative pyrethroid-susceptible haplotype remained uncut (Fig. 5B), allowing us to genotype the resistance allele (CYP6P9a_R) in single mosquitoes. To validate the robustness of this PCR-RFLP assay to detect pyrethroid resistance, we used F_8 progeny from a cross between highly resistant (FUMOZ) and highly susceptible (FANG) mosquito strains. The genotyping of 46 mosquitoes that were highly resistant to permethrin (alive after 180 min of exposure) (*15*) revealed 9 RR, 35 RS, and only 2 SS genotypes. By contrast, 42 highly pyrethroid-susceptible mosquitoes (dead after 30 min of permethrin exposure) had 0 RR, 1 RS, and 41 SS genotypes. Therefore, the odds ratio (OR) of the likelihood of surviving exposure to permethrin when homozygous for the resistance allele (RR) of the *CYP6P9a* gene (with the CCAAT box and CnCC/MafK binding sites) increased to 922 ($P < 0.0001$) compared to mosquitoes homozygous for the susceptible allele (SS) (Fig. 5C), thus demonstrating the reliability of this DNA-based diagnostic assay.

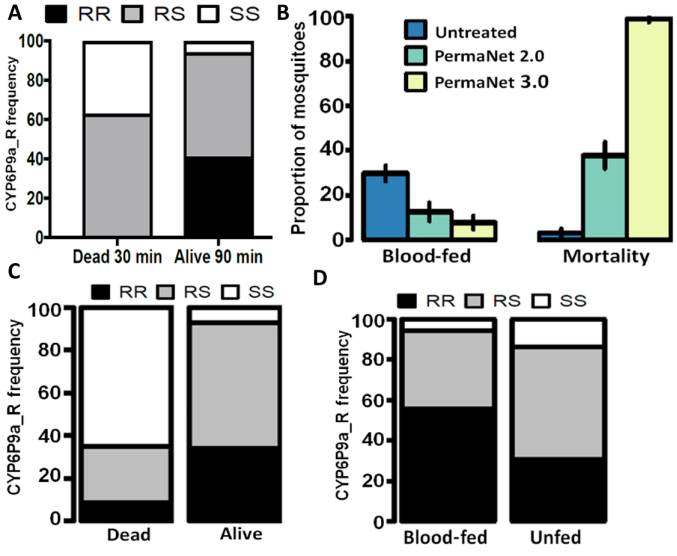

Fig. 6. Impact of *CYP6P9a*-mediated metabolic-based pyrethroid resistance on the efficacy of insecticide-treated bednets. (**A**) *CYP6P9a* genotypes correlate with pyrethroid resistance in the hybrid mosquito strain FUMOZ (pyrethroid resistant)/FANG (pyrethroid susceptible). This suggests that the FANG/FUMOZ hybrid mosquito strain can be used to assess the impact of *CYP6P9a*-mediated pyrethroid resistance on bednet efficacy. Mosquitoes were exposed to insecticide-treated bednets for 30 or 90 min to define pyrethroid-resistant and susceptible individuals. (**B**) Shown are blood feeding and mortality rates (mean ± SD; $n = 4$) for the FANG/FUMOZ hybrid mosquito strain after release-recapture studies in experimental field huts in Cameroon. Huts had untreated control bednets (blue), PermaNet 2.0 bednets (green), or PermaNet 3.0 bednets (yellow). (**C**) Shown is the *CYP6P9a* genotype (RR, RS, and SS) frequency in dead and alive mosquitoes after exposure to PermaNet 2.0 bednets. The *CYP6P9a*_R allele was associated with the ability of mosquitoes to survive exposure to pyrethroid-treated PermaNet 2.0 bednets ($P < 0.0001$). (**D**) Shown is the *CYP6P9a* genotype (RR, RS, and SS) frequency in mosquitoes that did or did not take a blood meal after exposure to PBO-treated PermaNet 3.0 bednets. The *CYP6P9a*_R allele was associated with the ability of pyrethroid-resistant mosquitoes to take a blood meal from individuals lying under PBO-treated PermaNet 3.0 bednets.

Geographical distribution of the *CYP6P9a* resistance allele across Africa

Genotyping of the CYP6P9a_R allele across Africa revealed that it was nearly fixed in mosquitoes from southern Africa and was present at an intermediate frequency (55.7%) in mosquitoes from Tanzania (East Africa) (Fig. 5D). However, CYP6P9a_R was absent from mosquitoes from Central/West Africa (Fig. 5D and fig. S6C). In the Democratic Republic of Congo, a geographical contrast was observed with the CYP6P9a_R allele present in mosquitoes from the eastern part of the country but absent from mosquitoes from the western part of the country including the capital Kinshasa (Fig. 5D). This pattern suggested a new allele/haplotype that arose in mosquitoes in southern Africa and that spread northward. Regional differences in CYP6P9a_R distribution were similar to those reported for previous markers (*7*, *11*).

Impact of *CYP6P9a*-mediated pyrethroid resistance on bednet efficacy in an experimental field hut trial

To assess the impact of the *CYP6P9a*-R haplotype on the effectiveness of long-lasting insecticide-treated bednets, we opted to use lab strains as this mutation is nearly fixed in the field in southern Africa. We crossed the highly resistant laboratory strain FUMOZ-R (where *CYP6P9a*_R is fixed) with the fully susceptible laboratory strain FANG (where *CYP6P9a*_R is completely absent). Using reciprocal crosses between the two strains, we generated a hybrid strain at the F_4 generation that we used for semi-field studies in experimental huts.

The bioassays performed with the reciprocal FANG/FUMOZ strains revealed that both hybrid strains were resistant to pyrethroids and carbamates and moderately resistant to DDT (93% mortality) (fig. S7A). As expected, resistance was lower than in the fully resistant strain FUMOZ_R, with a mortality rate of 76.1 to 80.7% when exposed to permethrin. However, a difference was observed for deltamethrin with a higher mortality rate recorded for the strain generated from crossing female FUMOZ_R to male FANG (48.5%) than in the strain from female FANG and male FUMOZ_R (77.3%). This difference could indicate the role of some candidate genes in the X chromosome for deltamethrin resistance (*CYP9K1*, for instance). The resistance pattern was similar for the carbamate bendiocarb in both reciprocal strains.

Before any field studies were conducted with the hybrid FANG/FUMOZ strain, the role of the *CYP6P9a*_R allele in observed pyrethroid resistance was confirmed. World Health Organization (WHO) bioassays showed a mortality of 39.0 and 42.3% after 30 min of exposure and mortality rates of 81.3 and 86.3% after 90 min of exposure, respectively, to permethrin and deltamethrin (Fig. 6A and fig. S7B). The OR of surviving exposure to permethrin when homozygous for

Table 1. Experimental field hut study with the FANG/FUMOZ mosquito strain. For each comparison, estimates not sharing the same symbols (§ or ‡) are statistically different at $P < 0.05$. ns, not significant.

	Untreated nets	PermaNet 2.0	PermaNet 3.0
Total mosquitoes	356	270	322
% Exophily (CI)	11.8 (8.45–15.15)	16.7 (12.2–21.1), ns	15.8 (11.85–19.8), ns
% Blood feeding (CI)	29.5 (24.7–34.2)	14.8 (10.6–19.05)§	6.8 (4.1–9.6)§‡
% Blood feeding inhibition	–	49.8§	76.84§‡
% Personal protection (total blood-fed)	– (105)	61.9 (40)§	79.04 (22)§‡
% Mortality after blood feeding (no. dead)	1 (1)	40.0 (16)§	95.5 (21)§‡
% Mortality corrected (CI)		33.3 (27.7–38.9)§	98.7 (97.5–99.9)§‡

the resistant *CYP6P9a*_R allele (RR) was high at 693 [confidence interval (CI), 88 to 5421; $P < 0.0001$] compared to the homozygous susceptible allele (SS) (fig. S7, C and D). The OR was 131 (CI, 27 to 978; $P < 0.0001$) when comparing RR to RS, indicating that the resistance conferred by *CYP6P9a* was additive.

Females from the F_4 generation of the FANG/FUMOZ hybrid strain were used in a release-recapture experiment in huts in Cameroon with PermaNet 2.0 bednets treated with deltamethrin or PermaNet 3.0 bednets treated with deltamethrin and piperonyl butoxide (PBO), a cytochrome P450 inhibitor, or control nets that were untreated. After four consecutive nights of mosquito release and then recapture, analysis of the mosquitoes collected showed no induced exophily (mosquitoes exiting the room) with either PermaNet 2.0 or PermaNet 3.0 bednets compared to the control ($P > 0.05$) (Table 1). The percentage of females that had taken a blood meal was lower for both pyrethroid-treated bednets compared to untreated control bednets. PermaNet 3.0 bednets showed a lower number of female mosquitoes taking a blood meal than did PermaNet 2.0 bednets ($P < 0.001$) (Fig. 6B). This was reflected in the percentage inhibition of blood feeding, which was higher for PermaNet 3.0 (76.8%) than for PermaNet 2.0 (49.8%) bednets ($P < 0.0001$). Treated bednets provided greater personal protection from blood feeding than did the untreated bednets, with greater protection from PermaNet 3.0 (79.0%) than from PermaNet 2.0 (61.9%) bednets (Table 1). Analysis of mosquito mortality rates revealed higher mortality of the hybrid FANG/FUMOZ strain exposed to PermaNet 3.0 (98.7%) compared to PermaNet 2.0 (33.3%) bednets ($P < 0.001$) (Fig. 6B); low mortality was observed for mosquitoes exposed to control untreated bednets (6.2%). Mosquitoes that had taken a blood meal through the bednets showed a higher mortality rate after exposure to PermaNet 3.0 (95.5%) compared to PermaNet 2.0 (40%) bednets (Table 1).

The role of *CYP6P9a* in the loss of PermaNet 2.0 bednet efficacy

Genotyping of the *CYP6P9a* marker allowed us to assess the impact of cytochrome P450–based resistance in mosquitoes on the loss of efficacy of the pyrethroid-only treated PermaNet 2.0 bednet. Most of the mosquitoes released in the huts containing PermaNet 3.0 bednets died because of PBO inhibition of cytochrome P450s including *CYP6P9a*. Therefore, the impact of the *CYP6P9a* genotypes on the ability of mosquitoes to survive exposure to long-lasting insecticide-treated bednets was studied using the pyrethroid-only PermaNet 2.0 bednet. To avoid confounding effects from blood feeding, bednet entry, or exophily status, the distribution of the *CYP6P9a* genotypes was assessed first only among unfed mosquitoes collected in the huts. We observed significant differences in the frequency of the three genotypes between dead and alive mosquitoes ($\chi^2 = 375$; $P < 0.0001$) (Fig. 6B). Analysis of the correlation between each genotype and mortality revealed that *CYP6P9a* homozygous resistant mosquitoes (RR) were better able to survive exposure to PermaNet 2.0 bednets than were homozygous susceptible mosquitoes (SS) (OR, 34.9; CI, 15.8 to 77.1; $P < 0.0001$) (table S6). Similarly, possessing the heterozygous (RS) *CYP6P9a* genotype also conferred a significant survival advantage compared to the homozygous SS genotype (OR, 19.9; CI, 9.7 to 40.9; $P < 0.0001$). Although a higher frequency of alive mosquitoes with the RR genotype was observed compared to the RS genotype, this difference was not significant (OR, 1.75; CI, 0.82 to 3.7; $P = 0.26$). Overall, a single *CYP6P9a* resistance allele (R) conferred a greater ability to survive than did a susceptible allele (OR, 6.25; CI, 3.3 to 11.7; $P < 0.0001$) (Fig. 6C). The same association between *CYP6P9a*_R and the ability to survive exposure to PermaNet 2.0 bednets was observed when analyzing all collected samples although with lower ORs (e.g., for RR versus SS; OR, 10.8; CI, 5.6 to 20.8; $P < 0.0001$) (table S6). The impact of the *CYP6P9a* resistance gene on the ability of mosquitoes to blood feed was also assessed. We found that the distribution of the three genotypes was significantly different among blood-feeding and unfed mosquitoes for both PermaNet 2.0 ($\chi^2 = 16.9$; $P < 0.0001$) and PermaNet 3.0 bednets ($\chi^2 = 30.5$; $P < 0.0001$) (Fig. 6D). Homozygous RR mosquitoes were significantly more likely to blood feed when exposed to PermaNet 3.0 bednets than either susceptible SS mosquitoes (OR, 4.54; $P < 0.0001$) or heterozygous RS mosquitoes (OR, 2.6; $P = 0.0012$) (Fig. 6D). A similar trend was observed for PermaNet 2.0 bednets, although this was not significant (fig. S7, E and F).

DISCUSSION

This study investigated the genetic basis of metabolic resistance to pyrethroids in the mosquito *A. funestus*, a major malaria vector in Africa. We detected a DNA-based resistance marker for cytochrome P450–mediated metabolic resistance to pyrethroids and designed a field-applicable diagnostic assay to detect and track the spread of insecticide resistance across Africa. We established a direct impact of metabolic-mediated pyrethroid resistance on the efficacy of insecticide-treated bednets in an experimental field hut trial. Gene expression analyses underlined the importance of cytochrome P450 monooxygenases for pyrethroid resistance in the *A. funestus* vector of malaria in Africa, as has been previously reported for other *A. funestus* populations (*9*, *15*) and other mosquito species (*16*–*18*). Important heterogeneities in gene expression were observed among mosquito populations from different geographical regions of Africa.

A cluster of *CYP6* genes in the *rp1* pyrethroid resistance QTL showed the greatest differences in expression in mosquitoes from different locations. The southern Africa mosquitoes showed marked overexpression of *CYP6P9a* and *CYP6P9b*, whereas West African mosquitoes from Ghana showed overexpression of *CYP6P5* and *CYP6P4a/b*. Such differences showed that the molecular basis of pyrethroid resistance in mosquitoes varied across the African continent, as previously suggested for this species (*19*) and for other vectors of malaria such as *A. gambiae* (*8*, *20*), possibly due to a combination of variation in selective pressures and restricted gene flow among regions (*13*, *21*).

The association of the *rp1* QTL locus with pyrethroid resistance was further supported by the detection of several adaptive evolutionary features across this locus including signatures of selective sweep, large structural variations in the 6.5-kb insertion, and cis-regulatory polymorphisms. The selective sweep detected in the *rp1* QTL locus in mosquitoes from southern Africa coincided with increased expression of the duplicated *CYP6P9a* and *CYP6P9b* genes encoding cytochrome P450s in mosquitoes from Malawi. This supported a key role for these genes in pyrethroid resistance (*9*). However, the presence of a predominant haplotype for the *CYP6P9a* 5′UTR in the other three African regions suggests that resistance to pyrethroid beyond southern Africa could have been selected through *CYP6P9a* or other genes in the vicinity of the *rp1* QTL region. The hypothesis of a possible hitchhiking effect here rather than the direct involvement of *CYP6P9a* was supported by the important differences observed between the four major haplotypes with more than 20 mutational steps of difference between them. Therefore, it is likely that resistance conferred by the *rp1* locus occurred through independent selective events with different genes. This is supported by RNA-seq data showing that although *rp1* genes are overexpressed in mosquitoes Africa-wide, the main genes are different. *CYP6P4a* is predominant in mosquitoes from Ghana but not in those from other geographical regions; *CYP6P5* is predominant in mosquitoes from Cameroon and Uganda, whereas *CYP6P9a* is the major gene overexpressed in mosquitoes from southern Africa. Selective sweeps associated with insecticide resistance have been reported recently in *A. gambiae* mosquitoes from across Africa (*22*).

The cis-regulatory changes that we detected in *CYP6P9a* included binding sites for transcription factors such as CnCC and MafK, which have recently been shown to be involved in insecticide resistance in *A. gambiae* (*23*) and other insects (*24*). This suggested that cis-regulatory modifications may be important drivers of resistance to insecticides. However, because cis-regulatory elements and enhancers are able to drive expression of genes from distant locations and can be upstream or downstream of these genes, future studies will need to establish the role of the 6.5-kb insertion in the overexpression of *CYP6P9a* and *CYP6P9b*. The strong association of these regulatory variations with pyrethroid resistance enabled us to design a DNA-based diagnostic tool for detecting metabolic-mediated pyrethroid resistance in mosquitoes, which should facilitate the detection and management of this major resistance mechanism in the field. This molecular assay for cytochrome P450–based metabolic resistance to pyrethroids in a malaria vector comes two decades after a DNA-based diagnostic test was designed for target-site resistance (*kdr*) (*4*) and should facilitate the study of the impact of metabolic resistance on pyrethroid resistance and malaria control interventions. Although SNPs associated with pyrethroid resistance have been detected in the dengue vector *Aedes aegypti*, no markers have been reported (*25*). Another DNA-based diagnostic test has been designed for the glutathione *S*-transferase gene (*GSTe2*) in *A. funestus* to demonstrate resistance to both pyrethroids and DDT in West/Central Africa, but it used a single amino acid change (L119F) in the coding region of the gene and not the putative causative variant regulating gene expression as done here for *CYP6P9a* (*11*).

The CYP6P9a_R allele is present principally in mosquitoes from southern Africa where it is nearly fixed in the mosquito population, but it is completely absent from mosquitoes from other regions. This difference among African populations of *A. funestus* has previously been observed, most notably for the distribution of other resistance markers such as the *L119F-GSTe2* (*11*) and the *A296S-RDL* alleles conferring resistance to the insecticide dieldrin (*26*), which are present in mosquitoes from West/Central and East Africa but are completely absent from those in southern Africa. In contrast, the *N485I-ace-1* carbamate resistance allele is present only in mosquitoes from southern Africa (*7*). Furthermore, patterns of F_{ST}-based genetic differentiation among populations of this species indicate a restriction of gene flow and increased genetic divergence in mosquitoes from southern Africa compared to those from other regions (*13*, *21*). However, there seems to be a gradual increase of CYP6P9a_R frequency from south to north in the southern Africa region as seen in Malawi, with 98% in the south (Chikwawa), 90% in the center (Salima), and 78% in the north of the country (Fulirwa) (fig. S6C). This correlates with previous observations that *CYP6P9a* overexpression was lower in the north of southern Africa and that the spread of pyrethroid resistance likely originated from far south and is spreading northward (*27*). It will be important to monitor the spread of such alleles across the African continent as there is the risk that super-resistant mosquitoes could be generated if, for instance, *CYP6P9a*-mediated pyrethroid resistance becomes combined with the *GSTe2*-based DDT resistance seen in West/Central Africa. The Democratic Republic of Congo will be particularly important to monitor as both insecticide resistance mechanisms are present in this country (*28*).

Using a new DNA-based diagnostic CYP6P9a_R assay, we assessed the direct impact of metabolic-mediated pyrethroid resistance in mosquito vectors of malaria on the efficacy of insecticide-treated bednets. We showed that cytochrome P450–mediated pyrethroid resistance reduced the efficacy of insecticide-treated bednets. Our findings help to clarify the debate about whether pyrethroid resistance directly affects the efficacy of insecticide-treated bednets (*29*). Bednets containing the insecticide synergist PBO (such as PermaNet 3.0 bednets in this study), which inhibits the activity of cytochrome P450 enzymes, provided better efficacy than did bednets treated with pyrethroid alone (PermaNet 2.0 bednets). Despite the high mosquito mortality observed here with the PBO-treated PermaNet 3.0 bednets, the CYP6P9_R allele resulted in pyrethroid-resistant mosquitoes being more likely than susceptible mosquitoes to bite individuals under the bednet and potentially transmit malaria. This suggests that efforts to eliminate malaria in Africa will be impeded unless the overreliance on pyrethroid-treated bednets to control transmission is addressed.

There are several limitations to our study. It is important to highlight that the DNA-based assay designed in this study detects pyrethroid resistance principally in mosquitoes collected in southern Africa and only applies to *A. funestus*. Therefore, further studies are needed to detect similar markers in mosquitoes from other regions of Africa and in other mosquito species such as *A. gambiae* s.l. to track cytochrome P450–mediated resistance to pyrethroids across

Africa. Furthermore, even though *CYP6P9a* exhibited a strong correlation with the pyrethroid resistance phenotype, it might not be the only gene involved and further studies should be performed to establish the contribution of other genes, notably *CYP6P9b*. Last, the impact of metabolic-mediated resistance has been assessed in this study in a hybrid mosquito lab strain, which might not be identical to mosquitoes in the field; thus, future studies will need to use field populations of mosquitoes.

MATERIALS AND METHODS

Study design

The objectives of this study were to detect key genetic variants conferring metabolic-mediated pyrethroid resistance on the mosquito *A. funestus*, a major vector of malaria in Africa, and to design a simple DNA-based assay to monitor such resistance in field populations of mosquitoes from different African regions. Transcriptome profiling of *A. funestus* populations from four African regions—southern (Malawi), East (Uganda), West (Ghana), and Central (Cameroon)—was analyzed to detect key candidate resistance genes. Because metabolic-based pyrethroid resistance could also be conferred by point mutations in coding and cis-/trans-regulatory regions, we performed comparative whole-genome sequencing of pyrethroid (permethrin)–resistant and –susceptible mosquitoes collected in the field to screen for genomic resistance regions and polymorphisms. To comprehensively detect resistance loci, we also scanned the whole genome for pyrethroid resistance–related signatures of selective sweeps in mosquitoes from southern Africa. Because the *rp1* QTL was consistently associated with pyrethroid resistance, a fine-scale analysis of this locus was performed to detect potential structural variants associated with resistance such as indels and copy number variations. To detect cis-regulatory mutations potentially controlling *CYP6P9a/b*-mediated pyrethroid resistance, we compared an 800-bp sequence immediately upstream of *CYP6P9a* in resistant and susceptible mosquitoes. The role of insecticide-based interventions in the selection of *CYP6P9a* cis-regulatory changes was assessed by sequencing the genomes of mosquitoes collected from before and after the introduction of widespread insecticide-treated bednet usage. To establish the specific mutations controlling *CYP6P9a* overexpression, a comparative luciferase assay based on pyrethroid-resistant and pyrethroid-susceptible *CYP6P9a* promoter sequences was performed. To design a DNA-based assay to detect *CYP6P9a*-mediated pyrethroid resistance, we screened the *CYP6P9a* promoter for restriction sites and used this information in a PCR-RFLP assay to assess pyrethroid resistance in mosquitoes collected from across Africa.

Last, we assessed the impact of *CYP6P9a*-mediated metabolic-based pyrethroid resistance on the effectiveness of long-lasting insecticide-treated bednets using experimental field huts in Cameroon, Central Africa. The following three bednet treatments were compared in the experimental huts: untreated control polyethylene bednets, PermaNet 2.0-treated polyethylene bednets (deltamethrin), and PermaNet 3.0-treated polyethylene bednets (PBO + deltamethrin). To simulate a worn bednet, six holes (4 cm by 4 cm) were made in each bednet, according to WHO guidelines (*30*). The hybrid FANG/FUMOZ mosquito strain was released in each hut for six nights (80 mosquitoes per hut). Three adult volunteers were recruited from the Mibellon village in Cameroon to sleep under the bednets and to collect mosquitoes in the morning. They were provided with a written consent form and given chemoprophylaxis during the trial to prevent malaria. Ethical approval was obtained from the National Ethics Committee of the Ministry of Health in Cameroon.

Collection and rearing of mosquitoes

Two *A. funestus* laboratory colonies were used in the study. The FANG colony is a fully insecticide-susceptible colony derived from Angola (*31*). The FUMOZ colony is a multi-insecticide–resistant colony derived from southern Mozambique. Mosquitoes were collected from four primary locations across the continental range of *A. funestus*. Mosquitoes were collected in March 2014 from Obuasi (5°56′N, 1°37′W) in Ghana (*32*), in February 2015 from Mibellon (6°46′N, 11°70′E) in Cameroon, in March 2014 from Tororo (0°45′N, 34°5′E) in Uganda (*33*), and in January 2014 from Chikwawa (16°1′S, 34°47′E) in southern Malawi (*34*).

Collected mosquitoes were kept until fully gravid and forced to lay eggs using the forced-egg laying method (*35*). All F_0 females that laid eggs were morphologically identified as belonging to the *A. funestus* group according to a morphological key (*36*). Parents (F_0) and egg batches were transported to the Liverpool School of Tropical Medicine under a Department for Environment, Food and Rural Affairs (DEFRA) license (PATH/125/2012). Eggs were allowed to hatch in cups and mosquitoes were reared to adulthood in the insectaries under conditions described previously (*35*). Insecticide resistance bioassays on these samples have been previously described (*32*–*34*).

Transcription profiling of pyrethroid resistance using RNA-seq

Total RNA was extracted from pools of 10 female mosquitoes (alive after 1 hour of permethrin exposure) using the Arcturus PicoPure RNA Isolation Kit (Life Technologies), according to the manufacturer's instructions (Supplementary Materials and Methods). Pools of libraries were sequenced, eight per lane of the HiSeq 2500 (Illumina, San Diego, CA, USA) at 2 × 125-bp paired-end sequencing. Sequence library preparation and sequencing were done at the Centre for Genomic Research (CGR), University of Liverpool. RNA-seq data were analyzed as described previously (*37*). This involved an initial processing, quality assessment of sequences, and alignment to the reference sequence using the AfunF1.4 annotation. Differential gene expression analysis was performed using edgeR and the Strand NGS program (Strand Life Sciences, version 3.0).

Whole-genome sequencing of mosquito samples from southern Africa

Genomic DNA was extracted from whole mosquitoes from F_0 Malawi samples (2002 and 2014), the pyrethroid-resistant FUMOZ-R laboratory strain, and the fully susceptible FANG strain using the DNeasy kit (Qiagen, Hilden, Germany). For each sample, genomic DNA was extracted from individuals and pooled in equal amounts to form pools of DNA. These were sequenced on an Illumina HiSeq 2500 (2 × 150 bp, paired-end). Initial processing and quality assessment of the sequence data were performed as for RNA-seq data. Alignment of POOLseq R1/R2 read pairs and R0 reads to the reference sequence (the same as that used for RNA-seq alignment) as well as variant calling were performed as described previously (*13*).

Analysis of polymorphisms in the *CYP6P9a* promoter region

To detect potential causative mutations conferring pyrethroid resistance on *A. funestus*, the polymorphism of the cis-regulatory region of the pyrethroid resistance gene *CYP6P9a* was analyzed.

Detection of the causative mutations driving up-regulation of CYP6P9a

An 800-bp region upstream of the start codon of *CYP6P9a* was amplified and directly sequenced in 15 field-collected mosquitoes each from 10 countries across different regions of Africa including southern (Mozambique, Malawi, and Zambia), East (Uganda, Kenya, and Tanzania), Central (Democratic Republic of Congo and Cameroon), and West (Benin and Ghana). Primers are listed in table S7. Amplification and purification of PCR products were performed as previously described (*13*). Sequences were aligned using ClustalW (*38*), whereas haplotype reconstruction and polymorphism analysis were done using DnaSP version 5.10 (*39*), MEGA (Molecular Evolutionary Genetics Analysis) (*40*), and TCS (*41*).

Investigation of the content of the CYP6P9a and CYP6P9b intergenic region

The entire 8.2-kb intergenic region between both genes was amplified for the FUMOZ and FANG strains in two to three fragments using primers listed in table S7. PCR was performed using the Phusion polymerase following the manufacturer's instructions. PCR products were purified and cloned into pJET1.2 plasmid.

Assessing the impact of insecticide-treated bednet intervention scale-up on changes in polymorphisms of the CYP6P9a promoter region

The same 800-bp region upstream of the *CYP6P9a* was amplified in mosquitoes collected before (pre-intervention) the scale-up of insecticide-treated nets and also after the scale-up (post-intervention) in Malawi (2002 and 2014) and Mozambique (2000 and 2016). The PCR products were cloned and sequenced, and sequencing data were analyzed as described above.

Genotyping of the *CYP6P9a* resistance allele using a PCR-RFLP assay

A restriction site (5′-TCGA-3′) for the Taq I enzyme at the A/G mutation located 18 bp of the AA insertion and completely tight with the CCAAT box on the resistance haplotype was used to design a PCR-RFLP assay to genotype the CYP6P9a_R allele. The RFLP6P9aF forward primer, 5′-TCCCGAAATACAGCCTTTCAG-3′, and the RFLP6P9aR reverse primer, 5′-ATTGGTGCCATCGCTAGAAG-3′, were used to amplify a partial *CYP6P9a* upstream region containing the restriction site. Ten microliters of the digestion mix made of 1 μl of CutSmart buffer, 0.2 μl of 2 U of Taq I restriction enzyme (New England Biolabs, Ipswich, MA, USA), 5 μl of PCR product, and 3.8 μl of dH_2O was incubated at 65°C for 2 hours. Restriction digest was separated on 2.0% agarose gel (Fig. 3D).

Validation of the DNA-based diagnostic test

To validate the robustness of the PCR-RFLP to detect the pyrethroid resistance in field population, F_8 progeny from a cross between highly resistant (FUMOZ) and highly susceptible (FANG) strains previously used for QTL mapping (*15*) were genotyped, and correlation with resistance phenotype was established using OR.

Geographical distribution of the resistance *CYP6P9a* allele across Africa

The geographical distribution of the resistant *CYP6P9a* allele across Africa was established by genotyping the CYP6P9a_R in 30 to 50 field-collected females of *A. funestus* from several countries in Africa using the PCR-RFLP.

Luciferase reporter assay for the *CYP6P9a* core promoter region

The region immediately 5′ of *CYP6P9a* from both FUMOZ and FANG strains was amplified using primers 6P9a1F and 6P9R a/b. These primers gave an 817-bp product for both FUMOZ and FANG, which were cloned into pJET1.2 (Thermo Fisher Scientific) and sequenced. Primers were designed (table S7) to obtain constructs of progressive serial 5′ deletions of the *CYP6P9a* promoter of 800, 500, 300, and 150 bp for the different primers. The primers incorporated either the Sac I or Mlu I (for the FUMOZ) and Kpn I or Hind III (for the FANG) to facilitate cloning in the pGL3 basic vector. Products were amplified with Phusion polymerase (Thermo Fisher Scientific) and cloned into pJET 1.2. The product was then excised from pJET1.2, ligated to pGL 3 basic (Promega), and sequenced. Plasmids were then extracted using Midiprep kit (Qiagen) to obtain high concentrations for the transfection. Dual luciferase assay was undertaken using *A. gambiae* 4a-2 cell line (MRA-917 MR4; American Type Culture Collection, Manassas, VA). Approximately 5×10^5 cells (600 μl) were subcultured from a T75 culture and seeded in each well of a 24-well plate 1 day before transfection and allowed to reach 60 to 70% confluency.

Transfection of the construct was carried out using the Qiagen Effectene transfection reagent, and the promoter activity was measured using the Dual Luciferase Reporter Assay (Promega, Madison, WI, USA). Reporter constructs (600 ng; *CYP6P9a* upstream sequences in pGL3-Basic), pGL3 without insert, and LRIM promoter in pGL3 basic were cotransfected with 1 ng of actin-*Renilla* internal control in 60 ml of DNA condensation buffer, 4.8 ml of enhancer, and 6 ml of Effectene in triplicate. After 48 hours of incubation at 25°C, the cells were washed with phosphate-buffered saline and harvested in 100 ml of passive lysis buffer (Promega), and luciferase activity was measured on a luminometer (EG & G Berthold, Wildbad, Germany). *Renilla* luciferase activity was used to normalize the construct luciferase activity. The values obtained after measuring the firefly (LAR II) luciferase activity, which represent the activity of the promoter, were divided by the corresponding *Renilla* luciferase activity values and the ratio used to compare different promoters.

Evaluating the impact of *CYP6P9a*-based pyrethroid resistance on the efficacy of insecticide-treated bednets using experimental field huts

Study area and field hut description

The study was performed in Mibellon (6°4′60″N and 11°30′0″E), a village in the Adamawa region of Cameroon where we recently built 12 experimental huts of concrete bricks, following the specific design for experimental huts from the West Africa region (*42*).

Mosquito strains

The study was carried out with a hybrid strain generated from reciprocal crossing between the highly pyrethroid-resistant strain FUMOZ-R (CYP6P9a_R) and the fully susceptible FANG strain (CYP6P9a_S) (*43*). After the initial F_1 generation obtained from the reciprocal crosses of 50 males and 50 females of both strains, the hybrid strain was reared to F_5 and F_6 generations, which were used for the release in the huts.

Susceptibility of the hybrid FANG/FUMOZ mosquito strain

WHO bioassays were carried out to assess the susceptibility profile of the two reciprocal hybrid strains for the pyrethroids (0.75% permethrin and 0.05% deltamethrin), DDT (4%), and the carbamate bendiocarb (0.1%). The bioassays were performed according to WHO protocol (*42*).

Mosquito collection in field huts

Early in the morning, mosquitoes were collected using glass tubes from the room (the floor, walls, and roof of the hut), inside the bednet, and from the exit traps on the veranda. Each compartment of the hut had its own bag to avoid mixture between samples. Surviving mosquitoes were provided with sugar solution and held for 24 hours in paper cups after which delayed mortality was assessed. Samples were recorded in observation sheets as dead/blood-fed, alive/blood-fed, dead/unfed, and alive/unfed.

The effect of each treatment was expressed relative to the control (untreated bednets) by assessing induced exophily (the proportion of mosquitoes that exited early through the exit traps because of the treatment); the mortality rate, an indicator of the potential killing effect of the insecticide-treated bednets; and the rate of blood feeding, an indicator of insecticide resistance and personal protection.

Genotyping of the CYP6P9a metabolic resistance marker

To establish the impact of the *CYP6P9a*-mediated metabolic resistance to pyrethroids on the effectiveness of the insecticide-treated nets, the PCR-RFLP diagnostic assay was used to genotype a subset of each treatment including the dead, alive, blood-fed, and unfed mosquitoes on the veranda, in the net, and in the room.

Statistical analysis

Genes that were differentially expressed in each country and between different countries (generated from Venn diagrams) were detected using DESeq normalization with a fold change of >2. A multiple test correction was then performed using the method of Benjamini and Hochberg, at a false discovery rate of 5% (adjusted $P < 0.05$). Statistical significance of the luciferase assays was assessed after an unpaired Student's *t* test with respective replicates between the FUMOZ and FANG mosquito strains. Correlation between the *CYP6P9a*_R allele and the pyrethroid resistance phenotype was established using OR and Fisher's exact test. The effect of pyrethroid-treated bednets was established by direct comparison to untreated control bednets. The statistical significance of the difference was estimated by a logistic regression model using Wald statistic that follows a χ^2 distribution (with df = 1). OR and Fisher's exact test were used to assess the impact of *CYP6P9a*_R on the ability of mosquitoes to survive and blood feed after exposure to insecticide-treated bednets.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. Differential gene expression among four permethrin-exposed mosquito populations in Africa and the FANG strain.
Fig. S2. GO enrichment of up-regulated genes using BLAST2GO.
Fig. S3. qRT-PCR validation of the expression profile of the main detoxification genes differentially expressed between resistant and susceptible mosquito samples using RNA-seq.
Fig. S4. Insertion of a 6.5-kb intergenic fragment between *CYP6P9a* and *CYP6P9b* in mosquitoes from southern Africa.
Fig. S5. Genetic diversity patterns of an 800-bp cis-regulatory genomic fragment of *CYP6P9a* before and after scale-up of insecticide-treated bednet use.
Fig. S6. Design of a DNA-based diagnostic assay to detect and track pyrethroid resistance in mosquitoes across Africa.
Fig. S7. Impact of *CYP6P9a*-mediated metabolic-based pyrethroid resistance on the efficacy of insecticide-treated bednets using experimental field huts.
Table S1. Descriptive statistics of RNA-seq sequence read data and alignments for different samples.
Table S2. Detoxification-associated genes differentially expressed among four pyrethroid-resistant mosquito populations and the FANG susceptible mosquito strain.
Table S3. Descriptive statistics of whole-genome POOLseq sequence read data.
Table S4. Counts of reads aligned at the left and right breakpoints of the 6.5-kb insertion supporting different haplotypes.
Table S5. Population genetic parameters of the 800-bp fragment upstream of *CYP6P9a*.
Table S6. Correlation between *CYP6P9a* genotypes and mosquito mortality (PermaNet 2.0 bednets) and blood feeding after the experimental field hut trial with the FANG/FUMOZ mosquito strain.
Table S7. Primers used for characterization of the *CYP6P9a* promoter.
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A cytochrome P450 allele confers pyrethroid resistance on a major African malaria vector, reducing insecticide-treated bednet efficacy

Gareth D. Weedall, Leon M. J. Mugenzi, Benjamin D. Menze, Magellan Tchouakui, Sulaiman S. Ibrahim, Nathalie Amvongo-Adjia, Helen Irving, Murielle J. Wondji, Micareme Tchoupo, Rousseau Djouaka, Jacob M. Riveron and Charles S. Wondji

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Tracking insecticide resistance

Malaria prevention relies extensively on mosquito control using insecticide-treated bednets. However, insecticide resistance in mosquito vectors of malaria threatens control of this disease. In a new study, Weedall *et al.* detected the major genes conferring insecticide resistance on the *Anopheles* mosquito vector of malaria in Africa. They found a DNA marker in a gene (cytochrome P450) encoding an enzyme that breaks down the insecticides used for treating bednets. The authors then designed a simple test allowing this resistance to be tracked and showed that mosquitoes carrying this resistance marker were better able to survive and to take a blood meal after exposure to insecticide-treated bednets in a field hut study in Cameroon.