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Assessment of vectorial competency and resistance to insecticides of *Anopheles gambiae sensu stricto* in the Moyen Ogooué Province of Gabon

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DEDICATION

This work is dedicated to my late Father Albert Sambe

and to all my family members gone too soon

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1 Introduction

Malaria remains the most important parasitic disease in the world. According to the World Health Organization (WHO) there were an estimated 249 million cases of malaria in 2022 in the 85 countries where malaria is still endemic [1]. The WHO African Region bears the brunt of the cases with an estimated 233 million cases occurring in this region accounting for 94% of all cases. Sub-Saharan Africa (sSA), with 580 000 out of 608 000 deaths in 2022, recorded 95.3% of malaria mortality [1]. Malaria is the leading cause of death among children in sSA with those under 5 accounting for 76% of all deaths [1]. Children under 5 and pregnant women constitute the most vulnerable groups toward which most control methods are directed [1].

The deployment of insecticide-treated nets (ITNs) alongside indoor residual spraying (IRS), and prompt treatment of clinical malaria cases with artemisinin based combination therapy (ACT) in the early 2000s led to a halving of *Plasmodium falciparum* infection prevalence and a 40% decrease in clinical disease between 2000 and 2015 [2]. It is estimated that those interventions had averted 663 million clinical cases since 2000 with ITNs responsible for 68% of the cases averted [2]. However, since 2015, there has been an increase in the number of cases and deaths due to malaria which was exacerbated by the COVID19 pandemics and the ensuing lockdown measures implemented. Lockdown measures led to an additional 11 million and 55 000 malaria cases and deaths, respectively, between 2019 and 2020 [1]. The rebound in cases observed since 2015 may be linked to a lower coverage and loss of efficacy of current control tools but also to their limits at further reducing malaria cases and deaths [2] especially in the context of increasing reports of resistance of both vectors and parasites [3]. Resistance of malaria vectors to pyrethroids, the main class of insecticides used in impregnating bed nets, has been reported in most sub Saharan African (sSA) countries with models predicting high levels of pyrethroid resistance across Africa [4]. The impact that this resistance may have on malaria transmission and its potential to lead to operational failures of vector control interventions are still not clearly understood. However, there is evidence of a decrease in the efficacy of Long Lasting Insecticidal Nets (LLINs) in areas with pyrethroid resistant malaria vectors [5–7]. In addition, local emergence or the potential spread of artemisinin resistant malaria from countries in Southeast Asia such as Myanmar and Thailand [1], to Africa is a looming threat that may have severe consequences on malaria control. An increasing number of reports on the emergence of partial resistance characterized by delayed clearance of parasites, and not yet treatment failure, to artemisinin based combination therapies in countries

such as Rwanda, Ethiopia, Uganda and Eritrea [8–13] are warning signs of the potential dangers ahead.

The challenges posed by the adaptations of both the vectors and the parasites must be counteracted by the introduction and the development of control strategies that are adapted to local settings and the divergence from the one-size-fits-all approach. Malaria transmission usually involves more than one *Anopheles* species, thus the need to perform entomological surveys in order to identify the primary and secondary vectors. Malaria vectors usually vary in their human biting rates, their trophic preferences, their vectorial capacity and their resting habits [14] as well as their susceptibility to insecticides. Entomological surveys are therefore important for the selection of the appropriate vector control measures. In addition, they allow for the monitoring of the efficacy and effectiveness of vector control measures at reducing transmission during the implementation phase. Moreover, despite the fact that *P. falciparum* is the most common and deadly species, the contribution of other species such as *P. vivax*, *P. malariae and P. ovale* is also relevant. However, over the years these species, especially *P. malariae* and *P. ovale,* have drawn less attention and are neglected malarial species despite the higher than expected prevalence and distribution for the former [15, 16] and the potential for relapses due to liver hypnozoites for the latter [17]. Although conclusive evidence reports on hypnozoite-induced relapse of *P. ovale* are scarce, recent reports support this hypothesis [18, 19] using molecular evidence [18]. Thus, there is the need for more studies on their contribution to malaria transmission as well as the development of protocols to fill the gaps in knowledge about these species that have no continuous *in vitro* culture.

The mass distribution of LLINs has been shown to have a great impact on the vector control of malaria. However, the success of such measures entails that baseline information on the vector species, their distribution, density, bionomics and their susceptibility to insecticides are available in order to introduce and monitor vector control strategies. Gabon has never implemented a program for mass distribution of LLINs and information on the transmission of malaria are scarce especially in the Moyen Ogooué Province. Systematic data on the susceptibility of malaria vectors to insecticides is inexistent in this region. Moreover, most studies on vector competency of *Anopheles* species to *P. malariae* are outdated and were done with the Uganda I/CDC strain of *P. malariae* using non-human primate. Thus, there is a need to address gaps in entomological data on malaria transmission in the Moyen Ogooué Province of Gabon by investigating malaria vector bionomics and developing specific methods to investigate the mosquito-stages of *P. malariae* using local field isolates.

1.1 Malaria in Gabon

Gabon is a country located on the west coast of Central Africa characterized by a low population density and a high level of transmission of malaria, with 100% of the population considered to be at high risk of malaria [1]. Malaria in Gabon is mainly caused by *P. falciparum* followed by *P. malariae* and the two *P. ovale* species [20]. The number of cases of malaria in 2022 was estimated at 550 748 leading to 424 deaths in Gabon [1]. There was a steady decrease in reported cases from 2000 to 2008, with this period coinciding with the adoption by Gabon of the multi-pronged strategy suggested by the WHO to control malaria. This strategy includes vector control interventions such as ITNs, intermittent preventive treatment, diagnostic testing and treatment with quality-assured ACTs [1, 21]. The artemether-lumefantrine and artesunate-amodiaquine combinations were adopted as first and second line treatments for uncomplicated malaria while artesunate was recommended for the treatment of severe malaria [1]. The introduction of these new measures was supported by fundings from the Global Fund [21]. Since then, there has been no new significant campaigns of LLINs which may explain the rebound of cases observed with a 2.2-fold increase in the number of cases compared to the figures in 2008 [1]. Therefore, Gabon is not yet on track to achieve the global technical strategy target of reducing by 75% the malaria incidence and mortality by 2025 compared to those of 2015 [22]. Thus, this upward trend raises important questions and emphasizes the need for a thorough examination of the factors contributing to this significant increase in cases over the years.

1.2 Malaria control in Gabon

Since 2003, Gabon has adopted the multi-pronged strategy suggested by the WHO[1]. It is estimated that almost 100% of the malaria cases in Gabon in 2022 were treated with ACT combinations. In addition, intermittent preventive treatment of pregnant women (IPTp) is implemented. Studies in both rural and urban areas have reported IPTp with sulfadoxine-pyrimethamine (IPTp-SP) coverage of more than 50% [23, 24], with IPTp-SP offered to 84.1% of the women during antenatal care [23].

However, the implementation of vector control interventions which is the most effective control interventions, is still poor in Gabon. Indeed, the country has the lowest ITNs coverage in Central Africa with less than 20% of the population having access to a ITN [1]. This could be a result of the targeted distribution policy of LLINs to pregnant women and children under 5 instead of a mass distribution approach in the country. This low LLINs coverage could also

be due to the lack of funding as Gabon, up to 2022, is not receiving funds for malaria control from major donors such as the Global Fund, PMI/USAID and World Bank. Government funding contribute the largest share for the control of this disease in the country [1]. The resulting low LLIN coverage may partly explain the increase in cases recorded in Gabon contrary to other countries where multiple rounds of distribution have been carried out.

1.3 Malaria parasites and their life cycle

Malaria is caused by five species of the genus *Plasmodium*, namely *P. falciparum, P. vivax, P. ovale,* and *P. malariae,* which are the human species and *P. knowlesi,* which causes malaria in macaques but can also be transmitted to humans in some areas of Southeast Asia [25].

Malaria parasites are transmitted through bites of infected female *Anopheles* mosquitoes [26]. When an infected female *Anopheles* mosquito bites a human, she takes in blood. At the same time, she injects saliva that contains the infectious form of the parasite, the sporozoite, into the skin and ultimately the bloodstream. The sporozoites infect liver cells where the first stage of development in humans takes place, this is called the exo-erythrocytic phase (A) of the life cycle (figure 1). Sporozoites mature into schizonts, which then rupture and release merozoites. After this replication in the liver, the parasites undergo asexual multiplication in the erythrocytes. The erythrocytic cycle (B) starts when merozoites infect red blood cells and develop into trophozoites. The trophozoites then mature into schizonts, followed by egress of merozoites that infect new erythrocytes. Some parasites, less than 5 % of the total parasite biomass, differentiate into sexual erythrocytic stages (gametocytes) in natural infections [27]. Blood stage parasites are responsible for the clinical manifestations and complications of the disease.

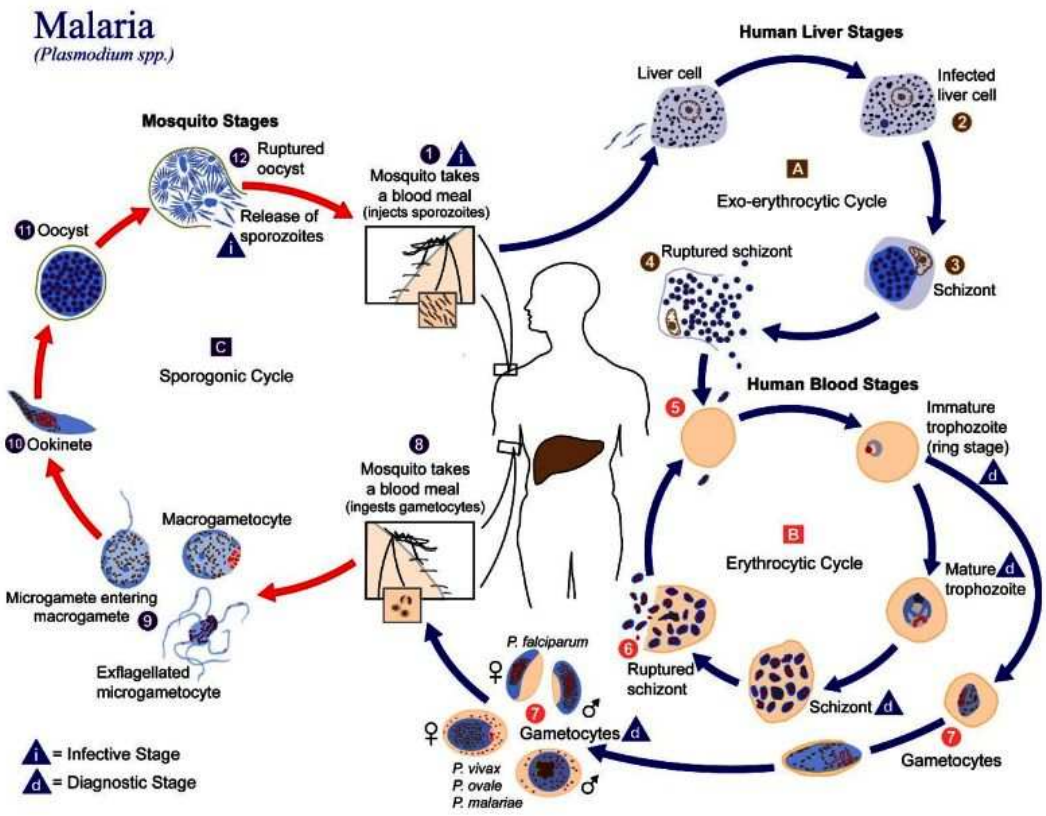

Figure 1: Life cycle of the malaria parasite. Source: https://www.cdc.gov/dpdx/malaria /index.html (Public Domain)

During a blood meal on a gametocyte-infected individual, male (microgametocytes) and female (macrogametocytes) gametocytes are ingested by an *Anopheles* mosquito, the male gametocyte immediately produces up to eight flagellate microgametes by a process termed exflagellation, marking the beginning of the sporogonic cycle (C). The microgametes fertilize the female gametocyte to form zygotes and the resulting motile ookinete penetrates the midgut wall of the mosquito [14] where they develop into round oocysts. Inside the oocyst, the nucleus divides repeatedly, with the formation of thousands of sporozoites and enlargement of the oocyst. When the sporozoites are fully formed, the oocyst bursts, releasing the sporozoites into the mosquito's body cavity [14]. The sporozoites migrate to the salivary glands from where they are injected into a human host when the mosquito feeds.

1.4 Clinical features

The clinical symptoms of malaria are unspecific and a typical of a systemic inflammatory reaction. Amongst others, headache, fatigue, muscle aches, loss of appetite, nausea and gastrointestinal symptoms are found. Potential complications include severe anaemia, cerebral

malaria, respiratory distress, hyperparasitaemia, kidney failure, haemolysis, dysentery, pulmonary oedema, and spleen rupture.

The onset of symptoms of malaria usually occurs usually 9–14 days after the infecting bite, it can also be longer in previously exposed individuals. Species such as *P. vivax* and *P. ovale* may form hypnozoites that result in relapses for months to years [28]. The manifestation of symptoms especially fever is synchronized with the bursting of infected erythrocytes which is followed by the release of parasite molecules at regular time intervals, which depends on the malaria parasite species [29]. The recurrence of fever is peculiar to each species and has been used as diagnostic feature with fevers occurring every two days for *P. ovale* and *P. vivax* (tertian malaria) while for *P. malariae,* fevers occur every three days (quartan malaria) [29]. In *P. falciparum* malaria, symptoms are typically less synchronous as there are multiple overlapping cycles of schizogony. This results in a less regular fever patterns compared to other plasmodial species [30].

1.5 Malaria transmission

1.5.1 Distribution of *Anopheles* mosquitoes

There are over 500 known species of *Anopheles* among which about 70 are competent vectors of human malaria [31]. Sub-Saharan Africa (sSA) is most affected by malaria and this is partly because Africa has the most effective and efficient Dominant Vector Species (DVS) of human malaria [32–34]. DVS are defined as species responsible for malaria transmission in an area because of their abundance, their propensity for feeding on humans, their mean adult longevity or any combination of these and other factors that increase overall vectorial capacity [31]. Most major malaria vectors in Africa are members of sibling complexes which are defined as a taxonomic groups of morphologically identical, closely related species [35]. The main sibling complexes are the *Anopheles gambiae* complex, the *funestus* group and the *nili* complex. In addition, *An. moucheti*, a species which is mainly found in forested areas, is also considered as a DVS because of its highly anthropophilic and endophilic behaviour. It usually acts as a secondary vector [36–38].

1.5.2 Malaria transmission in Gabon

Information on malaria vectors in Gabon is scarce with data reported from only few regions of the country. Approximately 40 potential vectors of malaria have been recorded in Gabon [39] with only few involved in malaria transmission. In most studies, members of the *An. gambiae*

s.l. complex, namely *An. gambiae s.s.* and *An. melas,* were identified as the main malaria vectors [40–44]. This was not the case in Akou (Franceville) and Benguia where *An. funestus* was identified as the primary vector [42, 43]. Moreover, other *Anopheles* species found infected with human *Plasmodium* species were *An. nili*, *An. marshallii, An. moucheti* and *An. hancocki* in areas where they were acting as secondary vectors [40, 42, 43]. In addition to their involvement in the transmission of human *Plasmodium* species, *An. marshallii*, *An. moucheti* and *An. funestus* were also found infected with primate *Plasmodium* raising the concern of transmission of simian malaria to humans [39, 45].

Meanwhile, *An. gambiae* and *An. moucheti* were identified as the main vector species in the Moyen Ogooué Province. Other potential vector species such as *An. paludis, An. funestus, An. hancocki* and *An. coustani* are also present but were not found infected with human plasmodial species [40, 46]. Most studies were carried out in the provincial capital Lambaréné and surrounding rural communities.

1.5.3 The *Anopheles gambiae* complex

The *Anopheles gambiae* complex comprises nine cryptic species [47–50] which are morphologically undistinguishable but different in their role as malaria vectors in Africa [51, 52]. This complex includes three of the main major malaria vectors in Africa namely *An. gambiae*, *An. coluzzii*, *An. arabiensis* [49, 53]. Among the six other species only the coastal species and salt water tolerant *An. melas* (in West Africa) and *An. merus* (in East Africa) are considered as malaria vectors of importance as the other species namely *An. bwambae* are restricted geographically while *An. quadriannulatus*, *An. amharicus* and the recently described *Anopheles fontenillei* sp.n. [50] are zoophilic [49].

Polymerase Chain Reaction assays have been developed to distinguish between members of this complex; the most widely used methods are the PCR-RFLP by Fanello et al. (2002) and the SINE200 PCR protocol developed by Santolamazza et al. (2008).

1.5.4 Life Cycle of the Vector

Like all mosquitoes, anophelines develop through four stages (figure 2) in their life cycle: egg, larva, pupa, and adult.

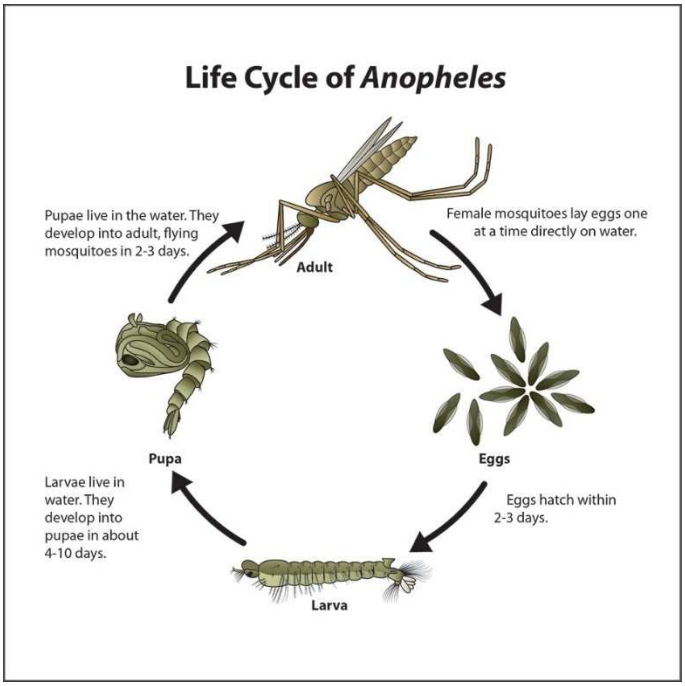

Figure 2: Life cycle of *Anopheles* species. Source: https://www.cdc.gov/mosquitoes/about/life-cycle-of-anopheles-mosquitoes.html (Public Domain)

1.5.4.1 Egg

Adult females lay up to 200 eggs per oviposition which are laid singly directly on water [32] The eggs have floats on either side and are not resistant to drying. They hatch within 2-3 days. *Anopheles sp.* have different preferential sites for egg laying, which can be temporary pools, streams, swamps and rivers [14].

1.5.4.2 Larva

A larva hatches from the egg after 1–2 days and generally floats below and parallel to the water surface, where it breathes air. It feeds by filtering food particles from the water [14]. There are four instars stages before the larva reaches the pupal stage. The total time spent in the larval stage is about eight to ten days at tropical temperatures [14].

1.5.4.3 Pupa

Pupae are comma-shaped, do not feed and are the last aquatic stage. This stage lasts for 2 – 3 days. At the end of the pupal stage, the skin splits, followed by the emergence of the adult mosquito which rest temporally on the water's surface until it flies [14].

1.5.4.4 Adult

Once *Anopheles* mosquito emerges from pupa, the females mate once. Sperm is stored in the spermatheca and released during fertilization. The spermatheca plays an important role in

sperm maturation and activation as it also protects sperm from mechanical damage and contact with the haemolymph [55]. After mating the female takes a blood meal to begin a gonotrophic cycle, which will lead to the laying of its first batch of eggs [14]. Interestingly, it has been demonstrated in Sao Tomé that a proportion of females feed before mating [56].

The feeding and resting habits of mosquitoes are of great importance in vector control programmes and must be well understood. Although most anopheline mosquitoes bite at night [57], some bite shortly after sunset while others bite later, around midnight or the early morning. This biting pattern is usually observed with most major *Anopheles* species that act as malaria vectors. They bite mostly bite late in the night to avoid host defensive behaviour. However, studies are reporting shifts in biting time and/or feeding location as a response to the long-term use of vector control measures such as LLINs [58].

1.6 Malaria vector control

Vector control of malaria is mainly based on the distribution of long-lasting insecticidal nets (LLINs) and on indoor residual spraying of insecticides. Until recently, only four classes of insecticides were used in malaria vector control namely organochlorides, pyrethroids, organophosphates, and carbamates. Insecticides from the pyrethroid class are used for impregnation of all bed nets currently approved by the WHO.

1.6.1 Insecticides used in malaria vector control

1.6.1.1 Organochlorides

Dichlorodiphenyltrichloroethane (DDT), hexachlorocyclohexane (HCH), aldrin and dieldrin were the most commonly used organochlorines. They affect the peripheral nervous system by disrupting nerve impulse transmission which leads to the knockdown and eventually the death of the insects [59]. DDT especially was widely used in both agriculture and disease control until 2001 when its use was banned from all applications except disease control [60] because of its negative effects on the environment and human health [61].

1.6.1.2 Pyrethroids

Pyrethroids are used in the form of alphacypermethrin, bifenthrin, cyfluthrin, deltamethrin, lambda-cyhalothrin, permethrin and etofenprox [62]. They are widely used because of their low toxicity to humans and to non-target species, their knock-down effect, relative longevity and low cost [60]. They affect both the peripheral and central nervous system of insects by acting on the sodium channels (figure 3), keeping them open and therefore allowing continuous sodium influx that causes the insect to become hyperexcitable leading to the knockdown of the

insect [59]. In addition, due to their irritant effect, pyrethroids cause a repellency response which leads to undirected flight, shorter landing time and feeding inhibition [60, 63]. The hyperexcitability and the repellency (excito-repellency) caused by exposure to pyrethroids guarantee the maintenance of the efficacy of nets even when they are torn [63].

There are two types of pyrethroids: type I and type II. Type I pyrethroids, such as permethrin, are effective knockdown agents as they induce repetitive firing in axons leading to paralysis of the insects. On the other hand, Type II pyrethroids which contain a cyano group at the α-benzylic position as exemplified by deltamethrin, trigger a pronounced convulsive phase, leading to more effective killing as the depolarization of nerve axons and terminals becomes irreversible [64].

1.6.1.3 Organophosphates

Organophosphates comprise a wide range of chemicals. Fenitrothion, malathion, and pirimiphos-methyl are used for indoor residual spraying (IRS) in vector control [62]. They work by inhibiting acetylcholinesterase, which prevents the breakdown of the neurotransmitter acetylcholine, leading to neuromuscular overstimulation and, ultimately, the death of the vector [65]. Although they are highly effective, they do not trigger an excito-repellency effect and have a shorter residual activity than pyrethroids and DDT [60]. Additionally, organophosphates used in malaria control are considerably more expensive than other insecticides and some of them need toxicological monitoring to prevent accidental overexposure during spraying [60].

1.6.1.4 Carbamates

Carbamates are used for IRS vector control in the form of bendiocarb [62]. Their mode of action is similar to that of organophosphates. Carbamates are highly effective but have a lack of excito-repellency effect and have a short residual activity like organophosphates [65]. Carbamates are also more expensive than pyrethroids and DDT.

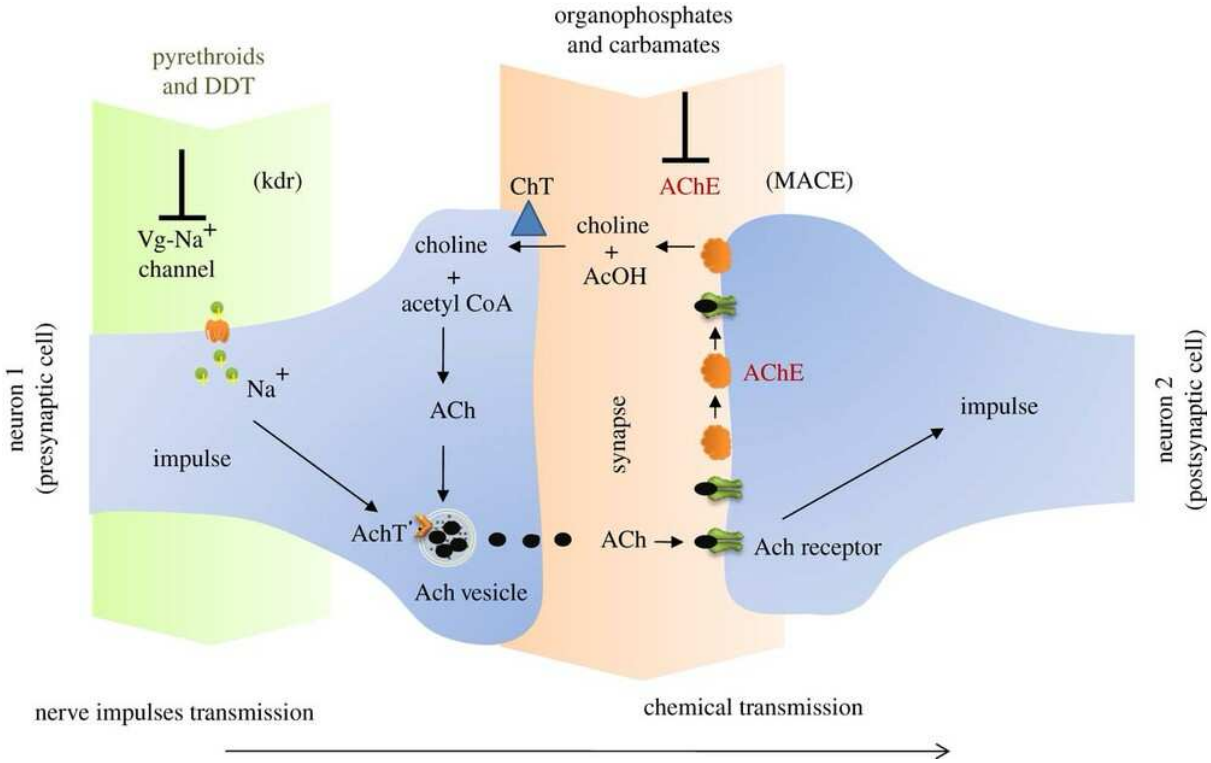

Figure 3 : **Biochemical target sites of synthetic insecticides.** Source: David et al., 2013. Philosophical Transactions of The Royal Society B. (CC BY 3.0)

1.6.2 Indoor residual spraying

Indoor residual spraying (IRS) involves applying insecticides to the inner surfaces of dwellings with the aim of targeting indoor resting mosquitoes. The primary effect of IRS is to kill mosquitoes that enter houses and rest on the treated surfaces. Consequently, IRS is less effective for controlling vectors that rest outdoors, though it may still impact outdoor-biting mosquitoes that enter homes to rest after feeding. When properly implemented, IRS is a highly effective intervention, offering community-wide protection by rapidly reducing vector populations, decreasing their density and longevity, and lowering their vectorial capacity [14].

All the four classes of insecticides are recommended for IRS (table 1) and the choice of one is based on its formulation, its biological effectiveness and the susceptibility of the target organism. Other considerations for the choice of an insecticide for IRS are the methods of application, its safety for humans, its toxicity to non-target organisms, the registration status of the pesticide for the required use and its cost [66].

1.6.3 Long Lasting Insecticidal Nets

A Long Lasting Insecticidal Net (LLIN) is a factory-treated mosquito net which has insecticide incorporated into or coated onto the fibres [62]. The WHO requires that these nets retain their effective insecticidal activity without re-impregnation for at least 20 WHO standard washes under laboratory conditions and three years of recommended use under field condition [67].

LLINs act as physical barriers thus preventing vector human contact and providing personal protection [57]. With the addition of insecticides, their effectiveness is improved and they remain effective even when they are torn [68] as non-treated nets with holes tend to increase malaria transmission [69] . The conventionally treated nets were the first to be used for vector control but the need for re-treatment and the loss of insecticidal activity after washing led to the development and adoption of LLINs which result in a more durable protection. LLINs remain the most widely used intervention method and have contributed the most to decrease in the number of cases [2]. It is estimated that more than 2.3 billion LLINs have been produced and distributed worldwide at an annual rate of 300 – 400 million [70].

Pyrethroids remain the main class of insecticides used in impregnating LLINs and are present in all the 23 WHO qualified/prequalified LLINs either solely or in combination with other additives [71]. The intensive use of pyrethroids in agriculture and LLINs has led to the development of resistance of target vectors and to a decline of efficacy of LLINs. Therefore, WHO currently recommends the use of pyrethroid-piperonyl butoxide (PBO) nets, pyrethroid-chlorfenapyr and pyrethroid-pyriproxyfen nets in areas with pyrethroid resistant mosquitoes [1]. PBO is a synergist compound that inhibits cytochrome P450 enzymes from detoxifying the pyrethroid thus ensuring that more insecticide reach their site of action [72]. On the other hand, chlorfenapyr, a halogenated pyrrole-based insecticide with a different mode of action [73] from pyrethroids is used with the idea that mosquitoes will not be resistant to both insecticides when used in combination. Pyriproxyfen is a juvenile hormone mimic and growth inhibitor which is used to sterilize mosquitoes that survived contact with nets [74].

1.7 Factors impacting malaria transmission

Malaria transmission can be affected by many factors, most importantly factors related to environment, vectors and humans.

1.7.1 Environmental factors

Environmental factors such as rainfall and temperature can greatly impact malaria transmission. Rainfall has been identified as one of the major driving factors in malaria transmission as rains lead to more available breeding sites for mosquitoes especially for those that are known to prefer small and temporary breeding sites such as *An. gambiae s.s.* [75]. It also affects those mosquitoes that tend to breed in larger and more stable water bodies such as *An. coluzzii* and *An. funestus.* This increase in mosquito number is usually accompanied by an increase in malaria transmission although this relationship has been shown to be species-specific [76]. However, excessive rainfall may reduce transmission as breeding sites can be washed away.

Temperature also affects many factors of malaria transmission dynamics such as development rates and survivorship of the larvae and adults as higher temperature tends to decrease the time for mosquito maturation and thereby increase the feeding frequency [77]. Temperature also acts on the replication of the parasite within the mosquito vector. High temperature can reduce the time needed for pathogen development [76, 78]. Indeed, it has been suggested by predictive models that maintaining *P. falciparum* infected mosquitoes at fluctuating temperature near the lower limit of 19–20 °C could enhance malaria transmission while higher temperatures between 35–37 °C, the upper survival limit for both vectors and pathogens, could reduce transmission [76].

1.7.2 Vector – Pathogen interaction

The passage of the *Plasmodium* parasites through the mosquito is an obligatory step for it to complete its life cycle. The ingested gametocytes go through various steps starting in the midgut up to the salivary glands. The passage in *Anopheles* has been identified as one of the most suitable target for transmission blocking strategies as throughout the life cycle, as the oocyst stage constitutes a parasite bottleneck, where parasites are lowest during this stage before expanding at sporozoite stage [79]. This has led to many studies to identify the mechanisms that are involved in the parasite survival such as thioester-containing protein 1 (TEP1) which is involved in the killing of ookinetes by melanization and lysis [80]. Most of the work on this topic has been done on *P. falciparum* and *P. berghei,* a mouse *Plasmodium* species. Other species can be considered as scientifically and clinically neglected plasmodial species, despite increasing evidence of their non-negligible contribution to the overall burden of malaria with a prevalence of up to 32% recorded in areas in sSA [15].

The fact that there is no continuous in vivo culture of plasmodia other than *P. falciparum*, has greatly limited the capacity to investigate them and there is therefore a need to establish protocols that will allow to study the mechanisms involved in the transmission of these parasites.

1.7.3 Resistance to pyrethroids and impact on control programmes

The Insecticides Resistance Action Committee defines resistance as "the selection of a heritable characteristic in an insect population that results in the repeated failure of an insecticide product to provide the intended level of control when used as recommended". Four types of resistance have been identified: metabolic, target site, behavioural and cuticular, with the two most common and well-studied being metabolic and target site resistance [81].

1.7.3.1 Metabolic resistance

Metabolic resistance is the most common mechanism of resistance found in insects. It occurs due to alterations in a mosquito's enzyme system, leading to faster detoxification or breakdown of insecticides, which prevents the insecticide from reaching its target [82]. In malaria vectors, three enzyme systems are considered significant: esterases, mono-oxygenases, and glutathione S-transferases. Resistant strains may have higher levels or more efficient forms of these enzymes [83]. Among them, esterase detoxification enzymes, also known as P450 oxidases, are the most prevalent, capable of metabolizing a wide range of insecticides. To date, this is the only resistance mechanism that has caused failure in a vector control program using pyrethroids in the Republic of South Africa [84].

Metabolic resistance has been reported in many countries all over Africa in most of the major vectors [85–93]. Thus, the recommendation by the WHO for the use of pyrethroid-PBO LLINs which have demonstrated their efficacy in randomised trials [94–96]. Data on metabolic resistance of vectors in Gabon are currently absent with most studies only screening vectors for genes of target site resistance.

1.7.3.2 Target site resistance

Target site resistance occurs when the site of action of an insecticide (typically within the nervous system) is modified in resistant strains, such that the insecticide no longer binds effectively and the insect is therefore unaffected, or less affected, by the insecticide [81].

Two mutations (*Kdr-w* and *Kdr-e*) at the domain II of the voltage-gated sodium channel gene have been identified as providing cross-resistance to pyrethroids and DDT in *An. gambiae*. s.s.

[97, 98]. These mutations lead to a substitution of a leucine (TTA) for phenylalanine (TTT) in the *Kdr-w* found mainly in West Africa [97] and a substitution of a leucine (TTA) for serine (TCA) in the *Kdr-e* which is widespread in East Africa [98]. These two mutations have been reported in almost all the sSA countries usually accompanied with a loss of knock down effect of pyrethroids and DDT. In Gabon, these mutations have been reported in Libreville [44, 99], Benguia [100], Port-Gentil [44] and in Mouila and its surrounding areas [101].

Several mutated forms of acetylcholinesterase also called modified acetylcholinesterase (MACE) are responsible for target site resistance to organophosphate and carbamate insecticides in *An. gambiae s.l.* [81]. Resistance results from a single nucleotide substitution in the *ace-1* gene encoding the enzyme, conferring a Glycine to Serine (G119S) amino acid substitution (termed *ace-1*^{R}) in the oxyanion hole of the enzyme [102]. This mutation has been detected in vectors from Benin, Burkina Faso [103], Cameroon [104–106], Côte d'Ivoire [107]. This mutation was also detected in Gabon albeit in low proportion [44] while it was totally absent in Mouila and its surrounding areas [101].

1.7.4 Implications of pyrethroid resistance on malaria control programmes

Pyrethroid resistance could lead to control failure defined by the WHO, as an epidemiological event where resistance is identified as the cause of rising malaria transmission [60]. However, the presence of resistant vectors does not automatically indicate control failure. In fact, to date, resistance has been only once linked to control failure in a vector control program using pyrethroids. This occurred in South Africa, where bioassays revealed that *An. funestus* mosquitoes were resistant to pyrethroids used in indoor residual spraying with the emergence of this resistance coinciding with a roughly fourfold increase in malaria cases in the country [84].

However, despite the widespread distribution of insecticide resistance, its impact on malaria transmission is still not fully elucidated. Indeed, it is challenging to isolate the effect of the failure of any single control tool on malaria transmission given the wide range of control measures currently in use. However, insecticide resistance likely contributed largely to the increase of cases observed since 2016 as this coincided with the spread of insecticide resistance to most endemic countries [108, 109]. Other factors that could have played a role are drug resistance, climate change, funding gaps and limited deployment of control measures.

A meta-analysis of data from trials of treated and untreated nets, for example, indicated that roughly half of the protection came from the physical barrier of the net, while the other half

was due to the chemical barrier [110]. Thus, a failure of insecticides used in impregnating to act on *Anopheles* vectors may have significant consequences. Indeed, some studies showed a loss of efficacy of bed nets in areas with resistant mosquitoes [6, 7, 63, 111] with LLINs failing to provide protection against mosquito bites.

1.7.5 Resistance management

Resistance management measures are important tools in reducing the negative impact of insecticide resistance on control measures. WHO recommends four strategies to deal with resistance of target vectors:

- Rotations: this strategy is based on the assumption that insecticide resistance to one insecticide is a rare phenomenon and thus that resistance to two insecticide is likely improbable. The strategy is based on the rotational use of two or more insecticides from different classes with different mode of action.
- Mixture: it is the simultaneous use of two or more insecticides which can be applied by using a single formulation of more than one insecticide, two or more insecticide formulations mixed in the same spray tank or a LLIN impregnated with more than one insecticide. This strategy has allowed for the production of LLINs with combination such as PBO + pyrethroids, Deltamethrin or Alphacypermethrin + Chlorfenapyr, Alphacypermethrin + Pyriproxyfen.
- Mosaics: it is done by spatially separated applications of different compounds against the same insect “mosaic” approach to resistance management.
- Combinations: Two or more insecticide-based vector control interventions are used in a house (e.g. pyrethroids on nets and an insecticide of a different class on the walls), so that the same insect is likely, but not guaranteed, to come into contact with the second insecticide if it survives exposure to the first.

Objectives

1. To assess species composition of malaria vectors, sporozoite rate, *Plasmodium* species composition, entomological inoculation rate (EIR) and presence of insecticide resistance genes in localities where intense clinical malaria research activities have been carried out for > 30 years

2. To establish experimental transmission of *P. malariae* from human blood to mosquitoes in a colony of *An. gambiae sensu stricto* mosquitoes, using fresh parasite isolates obtained from asymptomatic individuals with microscopic and molecular evidence of *P. malariae*

3. To assess the susceptibility to pyrethroids and organochlorides of malaria vectors from two different settings of the Province of Moyen-Ogooué and to determine the frequency of insecticide resistance genes

4. To evaluate the susceptibility profile of mosquitoes to pyrethroids, organophosphates and carbamates, to assess the level of resistance and the effect of piperonyl butoxide (PBO), a synergistic compound used to revert pyrethroid resistance

2 RESULTS

2.1 Chapter 1: Assessment of malaria transmission intensity and insecticide resistance mechanisms in three rural areas of the Moyen Ogooué Province of Gabon

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Assessment of malaria transmission intensity and insecticide resistance mechanisms in three rural areas of the Moyen Ogooué Province of Gabon

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Abstract

Background: Vector control is considered to be the most successful component of malaria prevention programs and a major contributor to the reduction of malaria incidence over the last two decades. However, the success of this strategy is threatened by the development of resistance to insecticides and behavioural adaptations of vectors. The aim of this study was to monitor malaria transmission and the distribution of insecticide resistance genes in *Anopheles* populations from three rural areas of the Moyen Ogooué Province of Gabon.

Methods: *Anopheles* spp. were collected using human landing catches in Bindo, Nombakélé and Zilé, three villages located in the surroundings of Lambaréné, during both the rainy and dry seasons. Mosquitoes were identified morphologically, and DNA was extracted from heads and thoraces. Members of the *Anopheles gambiae* complex were identified by molecular methods using the PCR SINE200 protocol and by sequencing of the internal transcribed spacer 2 region. Taqman assays were used to determine *Plasmodium* infection and the presence of resistance alleles.

Results: *Anopheles gambiae* sensu lato (97.7%), *An. moucheti* (1.7%) and *An. coustani* (0.6%) were the three groups of species collected. *Anopheles gambiae* sensu stricto (98.5%) and *An. coluzzii* (1.5%) were the only species of the *An. gambiae* complex present in the collection. Of the 1235 *Anopheles* collected, 1193 were collected during the rainy season; these exhibited an exophagic behaviour, and consistently more mosquitoes were collected outdoor than indoor in the three study areas. Of the 1166 *Anopheles* screened, 26 (2.2%) were infected with *Plasmodium* species, specifically *Plasmodium falciparum* (66.7%), *P. malariae* (15.4%), *P. ovale curtisi* (11.5%) and *P. ovale wallikeri* (3.8%). Malaria transmission intensity was high in Zilé, with an average annual entomological inoculation rate (aEIR) of 243 infective bites per year, while aEIRs in Bindo and Nombakélé were 80.2 and 17 infective bites per year, respectively. Both the *L1014F* and *L1014S* mutations were present at frequencies > 95% but no *Ace1G119S* mutation was found.

Conclusion: Our results demonstrate that malaria transmission intensity is heterogeneous in these three rural areas of Moyen Ogooué Province, with areas of high transmission, such as Zilé. The exophagic behaviour of the mosquitoes

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as well as the high frequency of resistance mutations are serious challenges that need to be addressed by the deployment of control measures adapted to the local setting.

Keywords: *Anopheles gambiae* complex, *Plasmodium* species, Entomological inoculation rate, Moyen Ogooué Province, Gabon

Background

Malaria remains a major public health issue in many malaria-endemic countries. Despite all the efforts put in place to control malaria, the disease still took an estimated 409,000 lives in 2019, mostly children under 5 years of age in sub-Saharan Africa [1]. Between 2000 and 2015, the incidence of malaria due to *Plasmodium falciparum* decreased by 40%, which a significant proportion of this decrease attributable to malaria vector control interventions, such as insecticide-treated nets (ITNs) and indoor residual spraying (IRS) [2]. However, there is evidence that members of the *Anopheles gambiae* complex and the *Anopheles funestus* group, the main malaria vectors in Africa [3], have developed physiological resistance to the insecticides used in malaria control programmes. Pyrethroids were until recently the sole class of insecticides used to impregnate bednets, and this is now a main factor driving the emergence and spread of insecticide resistance [4]. Furthermore, while the widespread use of ITNs has had a large effect in reducing endophilic/anthropophilic vectors, some evidence indicates that it may have led to changes in vector behaviour and mosquito population composition. Consequently, some malaria vectors have changed their biting activity from indoors to outdoors as well as their biting time, which reduces the effect of the two main interventions [5].

There is a need for data on local vector species prior to or following the introduction of vector control measures. In Gabon, ITNs are the main tools used in vector control, however, net ownership in 2019 was estimated to be below 20% [1]. Moreover, data on malaria transmission are lacking as only few entomological assessments have been carried out over the years [6–9] and distribution of insecticide resistance has received little attention [8–11]. Most of the studies carried out to date focussed mainly on a few parts of the country, with members of the *An. gambiae* complex identified as the main malaria vectors in Lambaréné, Libreville and Port-Gentil [7–9], and *An. funestus* found to be the primary and secondary vector in Akou and Benguia, respectively [6, 12]. Other mosquito species, such as *Anopheles nili*, *An. moucheti* and *An. hancocki*, have been reported to be secondary vectors [6, 7, 12].

Sylla et al. [7] reported that *An. gambiae* and *An. moucheti* are the main vector species in Moyen Ogooué Province, situated in the midwestern part of Gabon. However, no data are currently available on the distribution of insecticide resistance genes in malaria vectors. The aim of the present study was to assess species composition of malaria vectors, sporozoite rate, *Plasmodium* species composition, entomological inoculation rate (EIR) and presence of insecticide resistance genes in localities where intense clinical malaria research activities have been carried out for > 25 years [13].

Methods

Study sites

The study was carried out from May 2017 to August 2018 in three villages of Moyen Ogooué Province: Zilé (−0.703910, 10.340140), Nombakélé (−0.847490, 10.378100) and Bindo (−0.436095, 10.387816) (Fig. 1). Zilé is an area located approximately 12 km from Lambaréné and is surrounded by forest and rubber plantations. The houses, which are the homes of the plantations' workers and families, are constructed of concrete materials. Bindo is located approximately 61 km from Lambaréné, in an area of palm tree plantations and forest. The houses are constructed of concrete materials. Nombakélé is located along the National 1 road. The inhabitants of Nombakélé have diverse occupations, and the houses are mostly built with wooden planks.

Precipitation data were obtained from the World Weather Online website [14]. During the study period, Moyen Ogooué Province was subjected to a prolonged rainy season stretching from October to May, with a small decline in precipitation from January to February, with monthly precipitation > 200 mm. The dry season extended from June to September, with monthly rainfalls of < 200 mm.

Mosquito collection and identification

Mosquitoes were collected in both the rainy and dry seasons by overnight human landing catches (HLC), indoor and outdoor. A total of four rounds of collections were performed in the three study areas, two during the rainy seasons and two during the dry seasons, with the exception of Zilé where three collections were performed in the rainy seasons and one during the dry seasons. The mosquitoes were collected during 2 nights in each month of collection. Collectors were trained and informed by the investigators of the procedure and of the associated risks. All collectors signed an informed consent form.

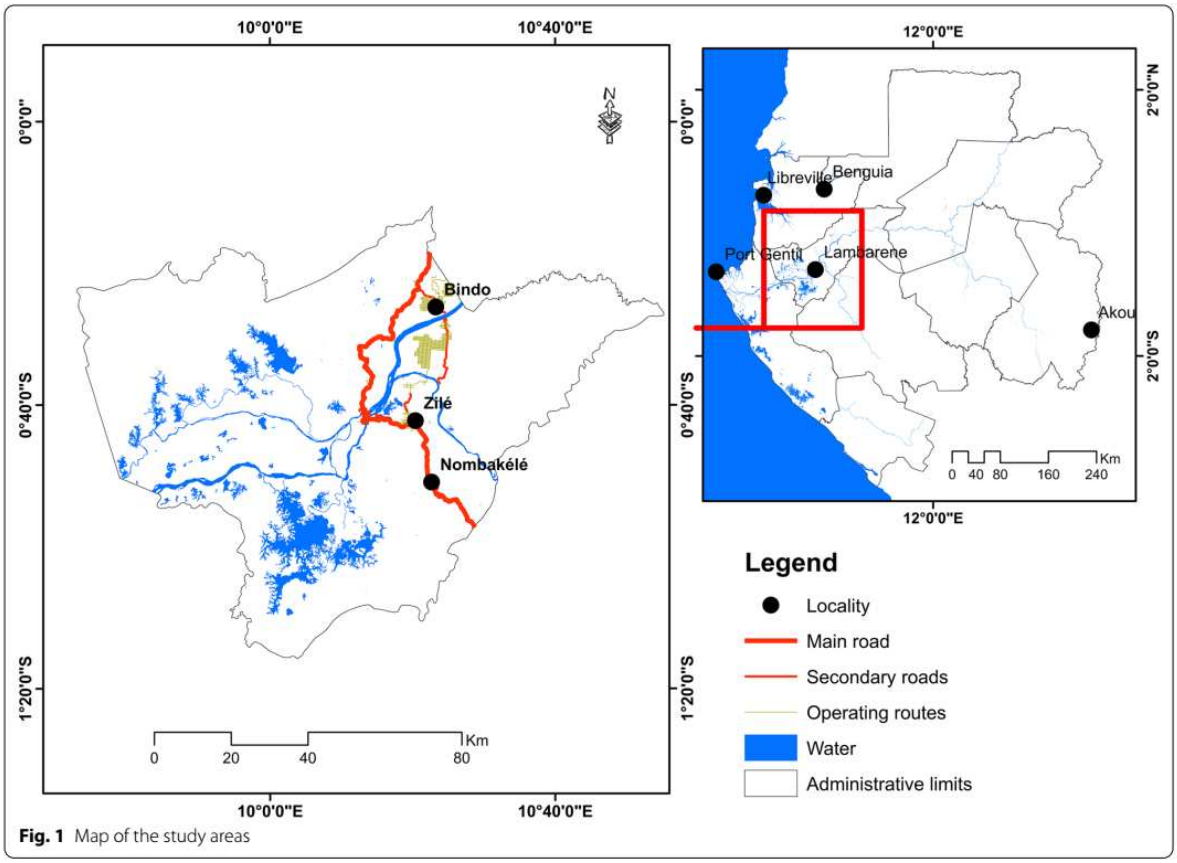

Fig. 1 Map of the study areas

They were instructed to contact the team in case they developed any symptoms of malaria during the 4 weeks following the HLC. The HLC method was used in this study after the results of a pilot test aimed at assessing the efficacy of CDC-light traps in catching mosquitoes were not satisfactory.

Four collectors were appointed each night at each collection point, two indoor and two outdoor at least 5 m away from the houses. Mosquito collections were performed during 2 nights in two selected houses at each of the study locations from 1800 hours to 0600 hours. Each collection period was divided into a 50-min collection time followed by a 10-min break, and this schedule was repeated for the duration of the night. Collectors switched houses and switched from indoor to outdoor every hour to minimize any bias due to the skills of the collectors or their attractiveness to mosquitoes.

Mosquitoes that landed on the exposed legs of the collectors were collected in glass tubes and pooled per collection hour.

Mosquitoes were transferred back to the Medical Entomology Laboratory of the Centre de Recherches Médicales de Lambaréné for morphological identification. *Anopheles* mosquitoes were identified using the morphological keys of Gillies and de Meillon [15] and Gillies and Coetzee [16]. Following morphological identification, the mosquitoes were preserved at − 20 °C in silica gel in Eppendorf tubes until transferred to the Institute of Tropical Medicine in Tübingen, Germany for further processing.

The mosquitoes were dissected, and the head/thorax separated from the abdomen. The head/thorax was ground in a FastPrep-24™ 5G sample disruption instrument and lysis system (MP Biomedicals LLC, Irvine, CA, USA), and DNA was extracted using the QIAamp DNA Mini and Blood Mini Kit (Qiagen®, Hilden, Germany) and the Quick-DNA Tissue/Insect Miniprep Kit (Zymo Research Corp., Irine, CA, USA). Mosquitoes that were morphologically identified as *An. gambiae* sensu lato (s.l.) were further analysed by PCR for species identification using the PCR-SINE200 protocol of Santolamazza et al. [17]. The PCR products were then electrophoresed in a 1.5% agarose gel.

Detection of *Plasmodium* spp. sporozoites

The extracted DNA was used to screen *Anopheles* mosquitoes for the presence of sporozoites using the protocol of Bass et al. [18] with slight modifications. This protocol allows for the simultaneous identification of *Plasmodium* spp. using one set of primers (PlasF: 5′-GCT TAG TTA CGA TTA ATA GGA GTA GCT TG-3′; PlasR: 5′-GAA AAT CTA AGA ATT TCA CCT CTG ACA-3′) and two probes, one labelled with the FAM fluorophore (Falci+; 5′-TCT GAA TAC GAA TGT C-3′) for the detection of *P. falciparum* and one labelled with the HEX fluorophore (OVM+; 5′-CTG AAT ACA AAT GCC-3′) for the detection of *Plasmodium ovale*, *P. vivax* and *P. malariae*. TaqMan assays were performed in the LightCycler 480 Instrument II system (Roche Applied Science, Penzburg, Germany). The cycling conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

The samples with positive assay results according to Bass et al. [18] were further differentiated using the nested qPCR protocol from Groger et al. [19] that allows for the differential identification of *Plasmodium* spp. The positive samples were pre-amplified using primers from Snounou et al. [20], and the PCR products were used as templates in a single-plex qPCR assay for each of the five species of *Plasmodium* that can cause malaria in humans (*P. falciparum*, *P. malariae*, *P. ovale curtisi*, *P. ovale wallikeri* and *P. vivax*) using previously described primers and probes [19]. The cycling conditions consisted of polymerase activation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s and 60 °C for 30 s.

Identification of insecticide resistance genes

A subsample of 118 mosquitoes were selected randomly and screened for the knockdown resistance gene (*Kdr*) and *Ace-1* genes using the protocol of Bass et al. [22] with slight modifications. This protocol enables the detection of knockdown mutations and wild-type (WT) alleles in two separates assays using one set of primers (kdr-forward: 5′-CAT TTT TCT TGG CCA CTG TAG TGA T-3′; kdr-reverse: 5′-CGA TCT TGG TCC ATG TTA ATT TGC A-3′) and three probes. One of the probes was labelled with the HEX fluorophore and was used to detect the WT allele (5′-CTTACGACTAAATTTC-3′) and the remaining two probes were labelled with the FAM fluorophore for the detection of the resistant alleles Knockdown West (KdrW; 5′-ACG ACA AAA TTT C-3′) and Knockdown East (KdrE; 5′-ACG ACT GAA TTT C-3′). The cycling conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s and 65 °C for 45 s.

The detection of the insensitive acetylcholinesterase (iAChe) mutation was performed using an assay that enables the WT allele and the mutant allele (S119) to be distinguished. The protocol uses one set of primers (ACE1-F: 5′-GGC CGT CAT GCT GTG GAT-3′; ACE1-R: 5′ -GCG GTG CCG GAG TAG A-3′) and two probes, one labelled with the HEX fluorophore for the detection of the susceptible allele (Ace1G119; 5′-TTC GGC GGC GGCT-3′) and one labelled with the FAM fluorophore for the detection of the resistant allele (Ace1S119; 5′-TTC GGC GGC AGC T-3′). The cycling conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 35 s.

Internal transcribed spacer 2 sequencing

Thirty-seven samples that failed to amplify using the PCR-SINE200 approach were amplified using the internal transcribed spacer 2 (ITS2) gene [22]. A subset of samples ($n=16$) identified either by molecular or morphological methods were also sequenced. As primers, the 5.8S ATC ACT CGG CTC GTG GAT CG and 28S ATG CTT AAA TTT AGG GGG TAGTC were used. The cycling conditions consisted of 95 °C for 2 min, 30 cycles of 95 °C at 30 s, 50 °C at 30 s and 72 °C for 1 min, with a final extension of 72 °C for 5 min. The PCR products were electrophoresed in a 1.5% agarose gel to confirm that the samples were amplified. The PCR products were cleaned using ExoSAP (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced using Sanger sequencing.

Sequence analysis for species identification

The sequences were cleaned and analysed with Bioedit v. 7.2.5. The consensus sequences generated were blasted in the NCBI Genbank. Multiple sequence alignment was performed using MUSCLE in MEGA v.10.2.6 with default parameters.

The evolutionary history was inferred by using the maximum likelihood method and Kimura 2-parameter model [23]. The bootstrap consensus tree was inferred from 1000 replicates [24] and taken to represent the evolutionary history of the taxa analysed [24]. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 63919)). Codon positions included were first, second, third positions and non-coding sites. Evolutionary analyses were conducted in MEGA [25].

Statistical analysis

Human biting rate (HBR) was calculated by dividing the total number of *Anopheles* mosquitoes collected by the number of collectors multiplied by the number of collection nights. The EIR was calculated by multiplying the HBR by the sporozoite rate. The seasonal daily EIRs were calculated by multiplying the average HBRs and sporozoite rate of the collections carried out for

Table 1 *Anopheles* species collected in Bindo, Nombakélé and Zilé

Anopheles spp.	Number of *Anopheles* spp. captured			
	Bindo	Nombakélé	Zilé	Total
An. coustani	–	1	6	7
An. moucheti	4	–	17	21
An. gambiae s.l.	356	74	777	1207
An. gambiae s.s	189 (97.4%)	65 (98.5%)	692 (98.7%)	946
An. coluzzii	4 (2.1%)	1 (1.5%)	9 (1.3%)	14
An. gambiae/An. coluzzii	1 (0.5%)	–	–	1

s.l. sensu lato, *s.s.* sensu stricto

each season in each study site. The seasonal monthly EIRs were determined by multiplying the seasonal daily EIRs by the number of days in each season (243 days for the rainy season [October–May] and 122 days for the dry season [June–September]). For this study, we performed two types of analysis: a descriptive analysis and a univariate explanatory analysis. The statistical analyses were carried out using R version 4.0.2 with a two-sided $P < 0.05\%$ indicating significance. Graphical presentation of data was done using GraphPad Prism Version 8.4.0 (GraphPad Software Inc.) A descriptive analysis was carried out on all the study data and the results were expressed as proportions. To achieve the objectives, logistic regression analysis was used to compare the number of mosquitoes collected per site and per season and the indoor/outdoor collections using binary data. We used the Pearson Chi-square (χ^2) test to compare the HBRs between the three sites and Tukey's method for multiple comparison.

Results

Human landing catches

A total of 1235 *Anopheles* spp. mosquitoes were collected in Bindo, Nombakélé and Zilé (Table 1). Overall, the highest HBR was recorded in Zilé, with 25 bites per person per night (b/p/n) (95% confidence interval [CI] 11.3–38.7), resulting in the collection of 800 mosquitoes, 64.8% of the total number of mosquitoes collected throughout the study period ($\chi^2 = 22{,}558.4$, $df = 3$, $P < 0.0001$). In Bindo and Nombakélé, the HBR was on average 11.25 (95% CI 3.6–18.9) and 2.34 (95% CI 0–5.5) b/p/n, respectively, yielding a total of 360 (29.1%) mosquitoes collected in Bindo and 75 (6.1%) mosquitoes in Nombakélé (Table 2). *Anopheles gambiae* s.l. was the most abundant species collected in the three areas, with a total of 1207 samples collected (97.7%). Other species collected were *An. moucheti* ($n = 21$, 1.7%) and *An. coustani* ($n = 7$, 0.6%).

Molecular identification of *Anopheles* spp.

Of the 1003 *An. gambiae* s.l. identified using molecular methods, 42 samples could not be analysed molecularly. *Anopheles gambiae* sensu stricto (s.s.) was the predominant species collected, comprising up to 97.4% (189/194), 98.5% (65/66) and 98.7% (692/701) of the mosquitoes identified in Bindo, Nombakélé and Zilé, respectively. *Anopheles coluzzii* ($n = 14$) was the second most common species identified and was found in proportions of < 2% in the three areas. One sample collected in Bindo was identified as a hybrid between *An. gambiae*/*An. coluzzii*.

Species identification by sequencing

The ITS2 sequences from 36 of the 37 samples that were morphologically identified as *An. gambiae* s.l., but which could not be identified using the SINE200 protocol, had a 99–100% identity to *An. gambiae* (Additional file 1). Two samples identified as *An. gambiae* s.l. and *An. moucheti* were identified as *An. moucheti* and *An. gambiae* s.l., respectively, after ITS2 sequencing. A phylogenetic analysis was performed using the closest hits in Genbank. The sequences obtained from the samples that failed to amplify formed a clade with a high bootstrap support of 96% that included the molecularly identified samples (*An. gambiae* s.s. and *An. coluzzii*) from our collections. Two other distinct clades were formed by samples morphologically identified as *An. moucheti* and *An. coustani* (Fig. 2).

Seasonal variations

More mosquitoes (1193/1235) were collected during the rainy seasons than during the dry seasons in all three localities (generalized linear model [GLM], $P < 0.0001$) (Fig. 3). The effect of season was particularly strong at Bindo where the HBR decreased from 22.13 b/p/n (95% CI 11.09–33.16) during the rainy season to 0.38 b/p/n (95% CI 0–0.81) during the dry season (Table 2). In Nombakélé, the HBR decreased from 9.25 b/p/n (95% CI 0–24.41) in the rainy season to 0.06 b/p/n (95% CI 0–0.20) in the dry season. In Zilé, the HBR during the rainy seasons was 31.88 (95% CI 15.10–48.65) b/p/n and dropped to 4.38 (95% CI 3.01; 5.73) b/p/n during the dry season (Table 2). However, in Zilé the HBR dropped in February 2018 (5.75 b/n/p) compared to the collections carried out in November 2017 (62.5 b/n/p) and May 2017 (27.38 b/n/p).

Similar to the findings for *An. gambiae* s.l., more *An. moucheti* ($n = 12$ vs. $n = 1$) and *An. coustani* ($n = 6$ vs.

Table 2 Summary of entomological indicators of malaria transmission in Bindo, Nombakélé and Zilé

Collection site	Human biting rate (b/p/n) [95% CI]	Sporozoite rate (%)	Entomological Inoculation rate			
			Night (ib/p/night)	Monthly (ib/p/month)	Seasonal (ib/p/number of months)	Annual (ib/p/year)
Bindo						
July 2017	0.13 [0; 0.52]	0	0	0	–	–
August 2018	0.63 [0; 1.62]	0	0	0	–	–
Dry season	0.38 [0; 0.81]	0	0	–	0	–
December 2017	30.88 [10.91; 50.84]	2.3	0.71	22.01	–	–
May 2018	13.38 [2.71; 24.04]	0	0	0	–	–
Rainy season	22.13 [11.09; 33.16]	1.5	0.33	–	80.2 ib/p/8 months	–
Annual average	–	–	–	–	–	80.2
Nombakélé						
July 2017	0.13 [0; 0.52]	0	0	0	–	–
July 2018	0	0	0	0	–	–
Dry season	0.06 [0; 0.20]	0	0	–	0	–
November 2017	9.25 [0; 24.41]	1.5	0.14	4.2	–	–
May 2018	0	0	0	0	–	–
Rainy season	4.63 [0; 11.28]	1.5	0.07	–	17 ib/p/8 months	–
Annual average	–	–	–	–	–	17
Zilé						
August 2017	4.38 [3.01; 5.73]	13.8	0.60	18.6	–	–
Dry season	4.38 [3.01; 5.73]	13.8	0.60	–	73.2 ib/p/4 months	–
May 2017	27.38 [7.75; 47.00]	1.5	0.41	12.71	–	–
November 2017	62.50 [40.08; 84.92]	2.7	1.69	50.70	–	–
February 2018	5.75 [0; 12.26]	0	0	0	–	–
Rainy season	31.88 [15.10; 48.65]	2.2	0.70	–	170.1 ib/p/8 months	–
Annual average	–	–	–	–	–	243.3

b/p/n Bites per person per night, *CI* confidence interval, *ib/p/night* infective bites per person per night

$n = 1$) were collected during the rainy seasons than during the dry seasons. The dominant species during the two seasons were *An. gambiae* s.l.

Hourly collection and biting behaviour

In all three study areas, more mosquitoes (80.73%) were collected during the second half of the night (from midnight [00 h] to 0600 hours) than during the first half (0600 hours to midnight [00 hours]). There was a steady increase in the number of bites from *An. gambiae* s.l throughout the night up to early morning when the peak biting times were recorded. Specifically, the peak biting times in Nombakélé and Zilé were between 0400 hours and 0500 hours, and in Bindo, between 0500 hours and 0600 hours (Fig. 4). The number of *An. moucheti* and *An. coustani* was very small for a clear pattern to be observed, but similar to the biting pattern observed for *An. gambiae* s.l., most of these two species were collected during the second half of the night.

Anopheles spp. collected in the three study areas exhibited a highly exophagic behaviour (Fig. 5). Consistently higher proportions of mosquitoes were collected outdoor than indoor in Bindo (58.6 vs. 41.4%; GLM, $P < 0.05$), Nombakélé (76 vs. 24%; GLM, $P < 0.05$) and Zilé (55 vs. 45%; GLM, $P < 0.05$).

Sporozoite rate and EIR

Out of the 1166 *Anopheles* mosquitoes screened, 26 (2.2%) were infected with *Plasmodium* spp. Of the 26 sporozoite-positive mosquitoes, 18 (69.2%) were infected with *P. falciparum*, four with *P. malariae* (15.4%), three (11.5%) with *P. ovale curtisi* and one (3.8%) with *P. ovale wallikeri*. *Plasmodium* spp. transmission was detected in three (May 2017, August 2017 and November 2017) of the four collections carried out in Zilé (Table 2). In Bindo and Nombakélé, infected mosquitoes were found only in collections performed in December 2017 and November 2017, respectively (Table 2). The highest infection rate was recorded during a collection carried out in the dry season in Zilé, with four of 29 (13.8%) *Anopheles* mosquitoes infected (Table 2).

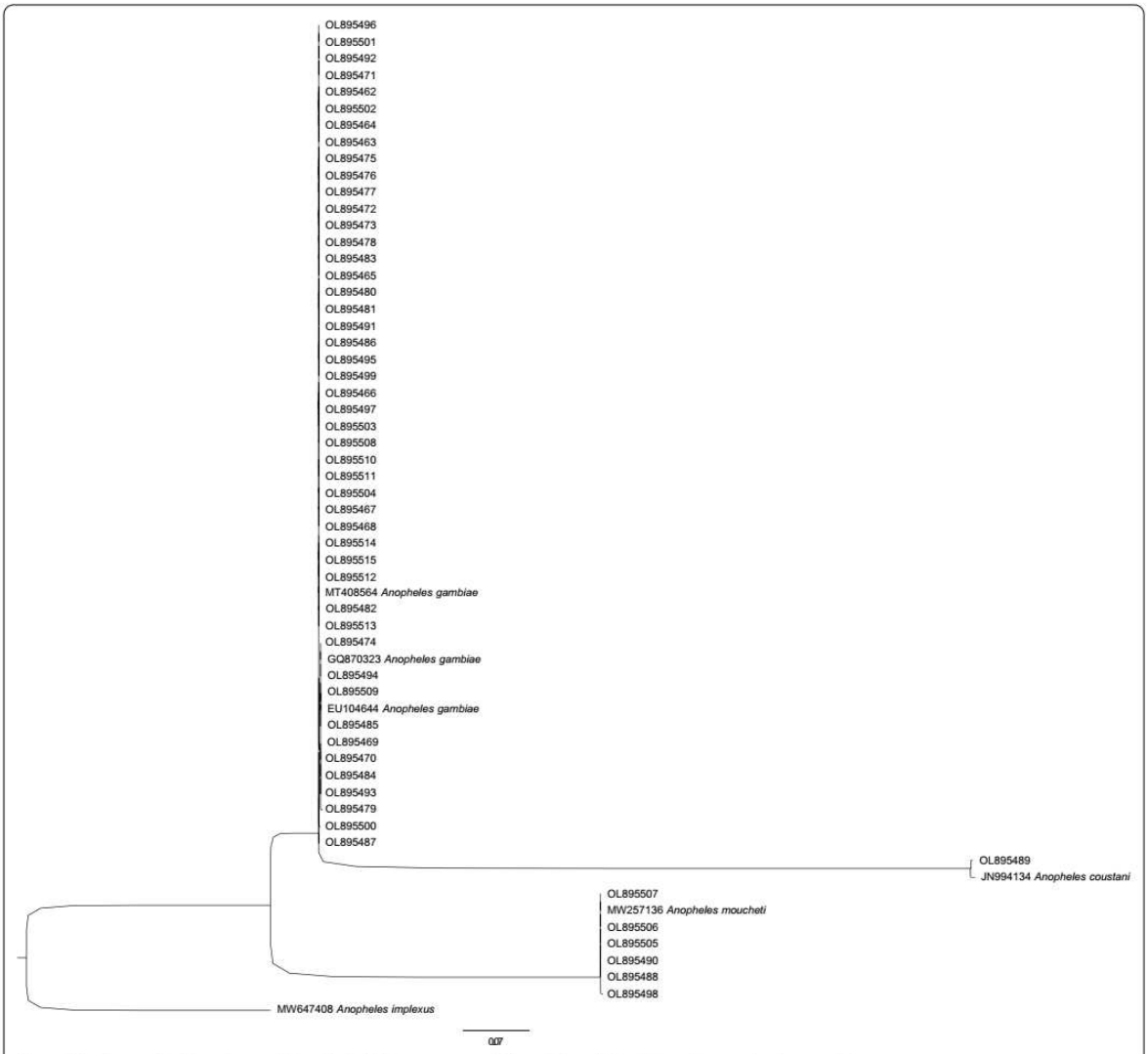

Fig. 2 Maximum-likelihood tree of *Anopheles* ITS2 sequences. Abbreviations: ITS2, Internal transcribed spacer 2

In Bindo and Nombakélé, the annual average EIR was 80.2 and 17 infective bites/person/year (ib/p/y), respectively (Table 2). These EIRs were recorded exclusively in collections carried out between November and December and were similar to the average number of infective bites received by a person in Bindo (80.2 ib/p/8 months) and Nombakélé (17 ib/p/8 months) during the rainy season. The daily EIR in Bindo and Nombakélé was 0.71 and 0.15 infective bites per person per night (ib/p/n), respectively (Table 2).

In Zilé, the annual average EIR was estimated to be 243.3 ib/p/y. The EIR in Zilé was 2.3-fold greater during the rainy season (168 ib/p/8 months) than during the dry season (72 ib/p/4 months) (Table 2). The highest daily EIR (1.69 ib/p/n) was recorded in November 2017, yielding a monthly average of 50.70 ib/p/month. There was a drop in the daily EIR to 0.41 ib/p/n in May 2017 (rainy season) which was lower than the one recorded in August 2017 (0.60 ib/p/n) (Table 2).

Anopheles resistance genes

A total of 118 *An. gambiae* s.l. were randomly selected for screening for the presence of the *Kdr* and *Ace-1*^{R} alleles. Of the 117 *An. gambiae* s.l. that were

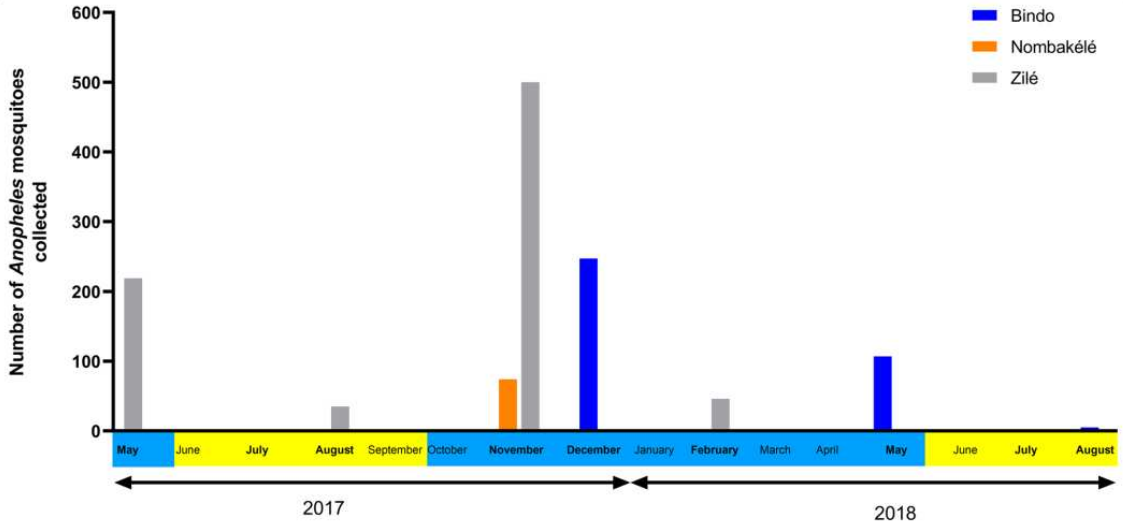

Fig. 3 Number of *Anopheles* spp. collected according to seasons in Bindo, Nombakélé and Zilé. Months shown in bold represent the months of collections. Blue and yellow shading indicates the rainy seasons and the dry seasons, respectively

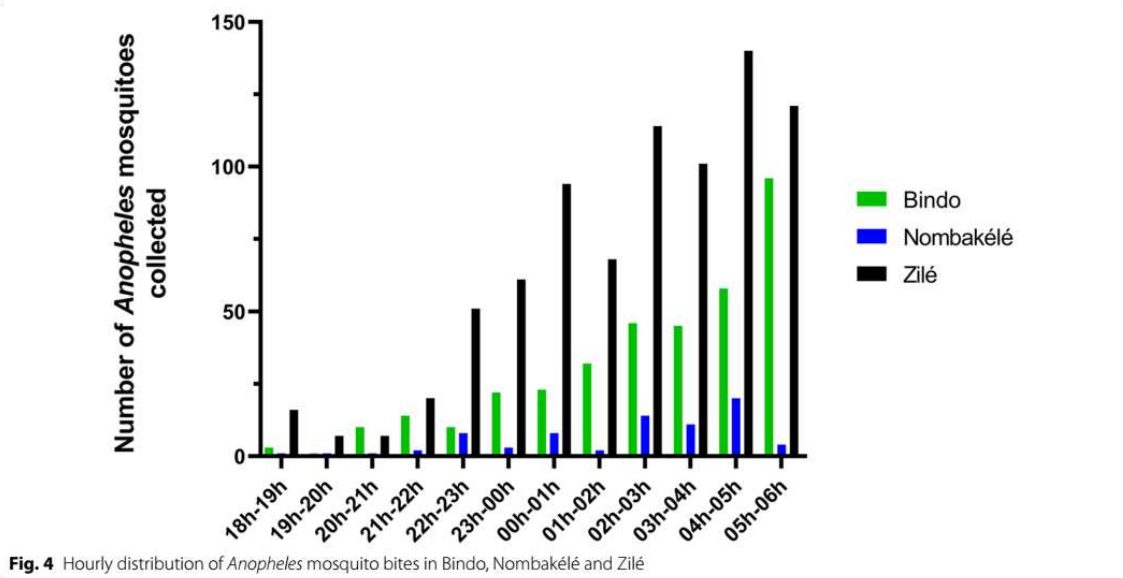

Fig. 4 Hourly distribution of *Anopheles* mosquito bites in Bindo, Nombakélé and Zilé

successfully amplified, 116 (99%) were either heterozygous or homozygous for the *Kdr L1014F* and *L1014S* mutations, which are associated with resistance to dichlorodiphenyltrichloroethane (DDT) and pyrethroids. Of the 116 *An. gambiae* s.l. carrying resistance mutations, 67 (57.3%) and 10 (8.5%) were homozygous for the *L1014F* and *L1014S* mutation, respectively, while 39 (33.3%) were heterozygous for both mutations. One *Anopheles* mosquito was homozygous for the WT allele *L1014L*. The *L1014F* mutation (73.9%) was the most common mutation found in mosquitoes, followed by the *L1014S* (25.2%) and *L1014L* (0.9%)

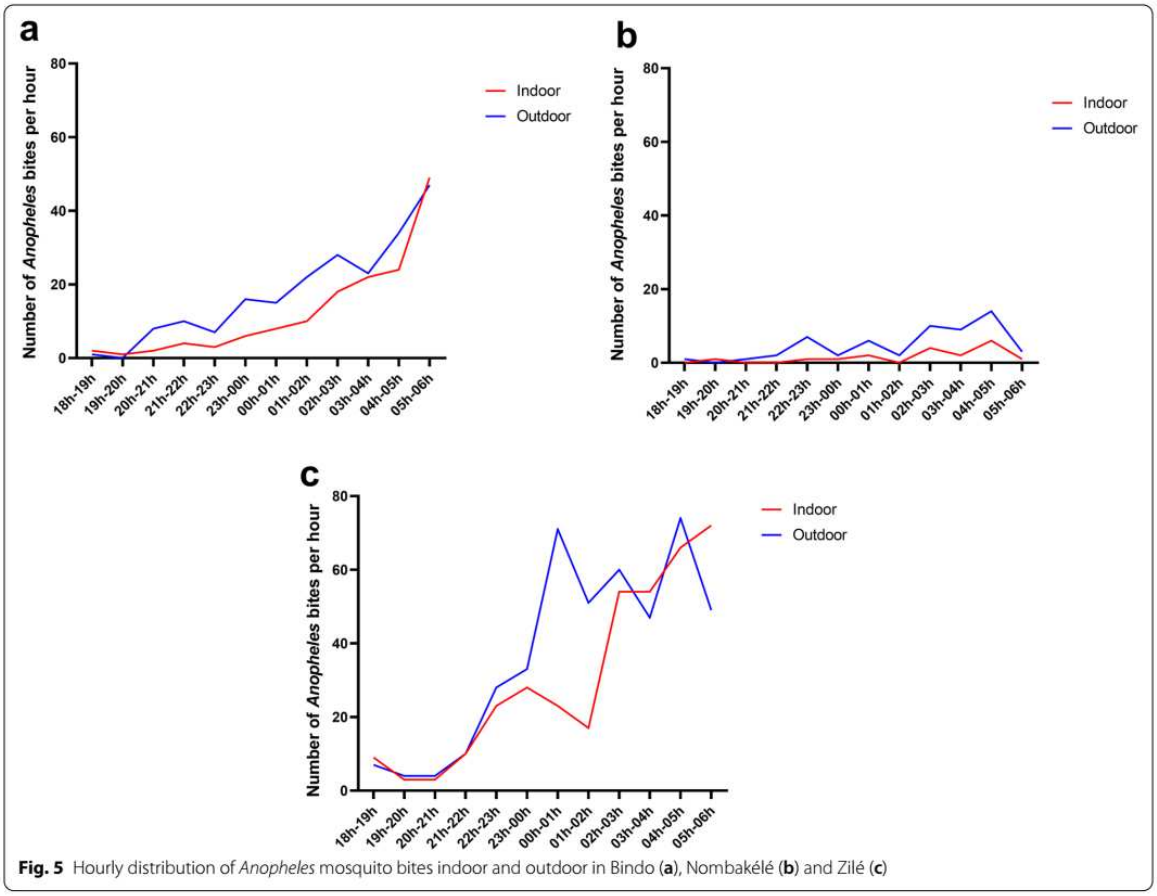

Fig. 5 Hourly distribution of *Anopheles* mosquito bites indoor and outdoor in Bindo (**a**), Nombakélé (**b**) and Zilé (**c**)

mutations. Allelic frequencies between the three villages were similar.

All of the mosquitoes were homozygous for *Ace1G119*, which is the WT (susceptible) allele for resistance to carbamates and organophosphates.

Discussion

Accurate and up-to-date data are key to the implementation of vector control measures that are efficient at reducing malaria transmission. To fill the gap in knowledge of malaria vectors in Gabon, we assessed the EIR as a measure of malaria transmission, as well as the distribution of insecticide resistance genes in *Anopheles* mosquitoes in three rural areas of Moyen Ogooué Province. Rural areas are usually hotspots of malaria transmission due to the abundance of breeding sites and the absence of adequate primary health care facilities.

ITS2 sequencing confirmed the presence of three groups of *Anopheles* species, namely *An. gambiae* s.l., *An. moucheti* and *An. coustani*, in accordance with the respective species identification based on morphological characters. *Anopheles gambiae* s.s. and *An. coluzzii* were the only species of the *An. gambiae* complex present, with the former being the dominant vector species, as previously reported in other areas of Gabon [8, 9, 11]. One sample was identified as a hybrid between *An. gambiae* s.s. and *An. coluzzii*. Hybrids of these two distinct species can occasionally be found, usually in frequencies of < 1% [26]. Other collected species, such as *An. moucheti* and *An. coustani*, may be acting as secondary vectors. The failure to amplify samples identified by sequencing of the ITS2 region as *An. gambiae* s.l. could be due to technical reasons.

Zilé had the highest HBR, followed by Bindo and Nombakélé. The high HBRs found in our study were expected based on previous collections performed in rural areas. The high HBRs is rural areas of Gabon compared to urban area are due to the availability of breeding sites

for malaria vectors in the former, as reported previously [8, 9]. That Zilé was home to permanent breeding sites established through human activities could explain the high density of vectors found in this area. In addition, the lack of implementation of comprehensive vector control measures could also explain the high HBRs recorded in our study areas. Gabon has not yet implemented a mass distribution campaign of long-lasting insecticidal nets (LLINs), unlike other countries in sub-Saharan Africa [27–31]. The low LLIN ownership is a result of the targeted policy that has focused solely on the provision of ITNs to pregnant women and children aged < 5 years [1].

Anopheles density was significantly higher during the rainy seasons than during the dry seasons, which was expected as there is an increase in the availability of breeding sites for mosquitoes during the rainy season. This is especially true for species like *An. gambiae* s.s., the dominant species in our study areas, which has been shown to prefer temporally variable and rain-dependent breeding sites [32]. Although, the proportion of *An. coluzzii* was < 2%, the species composition of the *Anopheles gambiae* complex should be monitored regularly as a shift in species composition may have epidemiological consequences, such as year-round malaria transmission [33]. *Anopheles coluzzii* prefers long-lasting breeding sites resulting from anthropogenic activities [32]. Most *Anopheles* mosquitoes were collected during the second half of the night (> 80%), with peak biting times early in the morning. This period of the night corresponds to the time when people are sleeping, thereby presenting a reduced risk from host defensive behaviour. This late night/early morning biting behaviour is a trait well known for *An. gambiae* that may increase their ability to transmit malaria [3, 34].

Anopheles gambiae s.l. exhibited a highly exophagic behaviour, and all a higher number of all three *Anopheles* species was consistently collected outdoor than indoor in the three study sites, as also previously reported in Libreville [9]. This exophagic behaviour may preclude the efficacy of indoor-focused vector control interventions, such as LLINs and IRS, to significantly reduce malaria transmission [35]. The observed exophagic behaviour in the absence of large-scale vector control interventions may be due to adaptations of local *Anopheles* spp. to human sleeping behaviour in combination with physiological resistance to insecticides, which are potential exacerbators of outdoor biting [36]. A growing number of studies have reported the switch in mosquito feeding behaviour from indoor to outdoor biting following vector control interventions. This switch may have implications on the current way interventions are designed, targeting mosquitoes at the source or while resting and feeding upon humans or livestock outside of houses [5].

Anopheles gambiae s.s. was the sole species infected with *Plasmodium* spp. Infected mosquitoes were found in three of the four collections performed in Zilé, while in Bindo and Nombakélé they were found only in collections performed during the rainy season (October–May). *Plasmodium falciparum* was found to be the most prevalent species infecting mosquitoes although there was a substantial proportion of infections by non-falciparum species. Specifically, *P. malariae* was the second most common species infecting mosquitoes, followed by *P. ovale* curtisi and *P. ovale wallikeri*, respectively. This distribution of *Plasmodium* species is similar to that reported in humans from rural settings of Gabon [37]. In comparison, no mosquito was found to be infected with more than one species although a high prevalence of coinfections in humans has been reported from neighbouring areas [37]. This finding suggests that people living in those areas develop infections concurrently following sequential bites from mosquitoes infected with different *Plasmodium* spp. and that mosquitoes from this area have the tendency to be infected by only one parasite species at a time after feeding on coinfected individuals. Our results should draw attention to these non-falciparum species as this is the first study to screen for all *Plasmodium* species in mosquitoes in Gabon; previous studies were based on *P. falciparum* circumsporozoite protein determined by enzyme-linked immunosorbent assay [6–9, 12]. Overall, the sporozoite rate was 2.3%, with the highest sporozoite rate (13.8%) surprisingly recorded during a collection carried out during the dry season in Zilé. Similar results were reported in Thailand by Rosenberg et al. [38] and were attributed to higher vector survival rates of mosquitoes during the dry season.

The EIR is used to measure the intensity of transmission of *Plasmodium* spp. by anopheline vectors [39]. In the present study, the transmission of malaria was different across study sites, with almost a perennial transmission in Zilé and intermittent transmission in Bindo and Nombakélé. The major contribution of the period between October to December to the overall burden of malaria transmission in our study areas was exemplified by the fact that the highest EIR in Zilé as well as the sole EIRs in Bindo and Nombakélé were recorded in collections carried out during this period. The annual average EIR (243.3 ib/p/y) recorded in Zilé was one of the highest ever recorded in Gabon and should be associated to a high infection rate in populations living in this area. Indeed, Beier et al. [41] reported that annual EIRs of ≥ 200 are regularly associated with a > 80% prevalence of *P. falciparum* in humans. Although the average annual EIRs recorded in Bindo and Nombakélé were lower than that in Zilé, the former suggest a *P. falciparum* prevalence in

humans of at least 50% [40]. Aside from the availability of breeding sites as mentioned above, this high transmission intensity in Zilé may be a consequence of the high prevalence of helminth infections, such as *Schistosoma haematobium* and *Trichuris trichiura* or hookworm, in populations living in these area compared to populations living in Bindo and Nombakélé [41]. These helminths, especially *S. haematobium*, have been shown to have an effect on *P. falciparum* infections in humans by increasing *P. falciparum* incidence, thus increasing its transmission intensity either alone [42–44] or in synergy with other helminths, such as *Trichuris trichiura* or hookworm [41].

Previous reports have shown the presence of the *Kdr* mutations in local vector populations [8, 9, 17, 45]. Libreville was the first coastal West African location where the presence of both *L1014F* (*Kdr-w*) and *L1014S* (*Kdr-e*) was observed [45]. These mutations were subsequently also found in mosquito populations from other areas, such as Benguia [46], Port-Gentil and Libreville [8] and Mouila [10]. The genotypic and allelic frequencies of the *Kdr* mutations observed in *An. gambiae* collected in the present study are similar to frequencies reported in previous studies in Gabon and suggest the presence of high level of resistance in these mosquito populations to pyrethroids and DDT [8, 9, 17, 45]. This potential resistance to pyrethroids, in the absence of a mass distribution of LLINs, may be driven by the use of these insecticides in agriculture, as previously described [47, 48]. However, our finding that all of the mosquitoes screened carried the susceptible allele for carbamate and organophosphate may hint to the susceptibility of mosquitoes to these classes of insecticides, suggesting that a combination strategies may be used as a tool to circumvent the effect that pyrethroid resistance may have on the efficacy of LLINs. Such studies have been conducted in Burkina-Faso [49] and Tanzania [50], with the results showing that simultaneous use of LLINs and net wall hangings treated with organophosphate improved malaria control.

Our study has a number of limitations, which mainly include the small sample size in terms of mosquitoes collected as well as the number of collections which did not allow us to fully assess the effect of seasonal variations on mosquito populations. In addition, the use of qPCR to screen for mosquito infections did not allow us to determine with confidence the infective status of mosquitoes. Although Foley et al. [51] reported that bisection of mosquitoes anterior to the junction of the thorax and abdomen eliminates the risk of false positives, it has been demonstrated that this risk is not totally eliminated, especially when using very sensitive PCR protocols [52].

Conclusion

The assessment of *Plasmodium* spp. distribution based on the results of the present study revealed a high prevalence of non-falciparum species in the mosquitoes collected, which should draw more attention to their contribution to the malaria burden in Gabon, particularly in Moyen Ogooué Province. From our results, it is obvious that the transmission of malaria was heterogenous in the three areas, where Zilé could be considered to be an area of high transmission. The combination of exophagic behaviour of mosquitoes and the high frequencies of *Kdr* mutations before the implementation of a mass distribution of LLINs may significantly impede the success of such a strategy to durably curb malaria transmission. Thus, there is a need to adopt vector control strategies that will include the use of other insecticide classes and new vector control tools.

Abbreviations

DDT: Dichlorodiphenyltrichloroethane; EIR: Entomological inoculation rate; HBR: Human biting rate; HLC: Human landing catches; *iAChE*: Insensitive acetylcholinesterase; IRS: Indoor residual spraying; ITN: Insecticide-treated net; ITS2: Internal transcribed spacer 2; *Kdr*: Knockdown resistance gene; LLIN: Long-lasting insecticidal net.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-022-05320-9.

Additional file 1. Sequence identification of *Anopheles* sp. using ITS2.

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Author contributions

AAA, BM, PGK, JCDA, UAN, TGW and STBS conceived and planned the study and its design. STBS, AGDN and BN performed the field activities with support from JRE, JFZ and YJH for the follow-up of the collectors. STBS and TGW carried out the laboratory analysis of the samples. RBM and STBS analysed the data. STBS drafted the manuscript. AAA, BM, PGK, SB, JCDA and TGW critically reviewed the manuscript. All authors contributed to the intellectual input to the study. All authors read and approved the final manuscript.

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Availability of data and materials

Raw data are archived and available on request from the corresponding author. The ITS2 sequences found in this study were deposited in the GenBank database with accession numbers OL895462–OL895515.

Declarations

Ethics approval and consent to participate
The study protocol was approved from the Institutional Review Board of the Centre de Recherches Médicales de Lambaréné (CEI-CERMEL: 009/2014).

Consent for publication
All authors concur with the submission presented by the corresponding author.

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

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Publisher's Note

2.2 Chapter 2: Experimental transmission of *Plasmodium malariae* to *Anopheles gambiae*

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BRIEF REPORT

Experimental Transmission of *Plasmodium malariae* to *Anopheles gambiae*

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Our current knowledge of the clinical burden, biology, and transmission of *Plasmodium malariae* is extremely scarce. To start addressing some of those questions, we experimentally infected *Anopheles gambiae* mosquitoes with fresh *P. malariae* isolates obtained from asymptomatic individuals in Lambaréné, Gabon. The proportion of mosquitoes infected via direct membrane feeding assay with either *P. malariae* monoinfections (16% [19 of 121]) or coinfections (28% [31 of 112]) was higher after serum replacement than in parallel groups without serum replacement (4% [4 of 102] and 4% [2 of 45], respectively; $P < .01$). Our results show that isolates from asymptomatic carriers can be used for experimental studies of *P. malariae* transmission.

Keywords. *Plasmodium malariae*; *Anopheles gambiae*; experimental transmission; Gabon.

Plasmodium malariae is 1 of the 5 species that cause human malaria. Infections with this species are characterized by a slow, 72-hour intraerythrocytic replication cycle, a unique ability to persist in the host for decades, and high fever peaks despite self-limiting asexual blood-stage density in nonimmune patients [1]. *P. malariae* prevalence can reach up to 30% in some endemic regions [2, 3]. High infection rates have also been observed in field-collected mosquitoes [3, 4]. *P. malariae* seems to be more prevalent than *Plasmodium ovale* spp. and usually occurs in coinfections with *Plasmodium falciparum* or *Plasmodium vivax*, with coinfection rates varying between 15% and 45% in Gabon [5]. An analysis of the landmark Garki project, however, revealed a striking temporal offset between *P. malariae* and *P. falciparum* prevalence and conversion rates, implying yet-undefined interactions of potential importance for malaria control strategies [6].

P. malariae infections in endemic areas are typically asymptomatic and submicroscopic [1] and are thus frequently undetected, especially in mixed infections. This will underestimate the number of true cases [7]. More sensitive polymerase chain reaction (PCR) protocols can dramatically improve the detectability of *P. malariae* infections [8]. Studies using molecular methods have found regional prevalence rates of *P. malariae* up to 40% [2]. It is important to note that *P. malariae* infections can also reach high prevalence in non-African endemic settings characterized by low *P. falciparum* prevalence rates [2]. The undetected persistence at low parasitemia of *P. malariae* infections and potentially, the existence of a monkey reservoir cause intermittent outbreaks of *P. malariae* malaria in South America [9]. Moreover, there is evidence for possibly natural refractoriness to standard antimalarial treatment regimens [7].

In stark contrast to *P. falciparum* and *P. vivax*, our knowledge of the experimental transmission of *P. malariae* is very limited, in part owing to a lack of a continuous in vitro culture protocol for *P. malariae*. Previous reports have shown that mosquito species such as *Anopheles stephensi*, *Anopheles freeborni, Anopheles gambiae,* and *Anopheles dirus* transmit *P. malariae*. However, most of these studies were carried out using monkeys infected with the Uganda I/CDC strain [10]. Only 1 study reported the infectivity of *Anopheles* species from human samples that were previously inoculated with a *P. malariae* clinical isolate [11].

Therefore, the current study aimed to establish experimental transmission of *P. malariae* in a colony of *A. gambiae* sensu stricto (ss; Kisumu strain) mosquitoes using fresh parasite isolates obtained from asymptomatic individuals with microscopic and molecular evidence of *P. malariae*, as either monoinfections or mixed infections. Because *P. malariae* oocysts are morphologically indistinguishable from other human malaria parasites, we used quantitative PCR (qPCR) to verify infections in both infecting isolates and infected mosquitoes.

METHODS

All procedures involving human subjects used in this study were approved by the national ethics committee of Gabon (no. 040/2018/SG/CNE), the institutional review board of CERMEL (CEI-014/2018), and the ethics committee of the medical faculty and the university clinics of the University of

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Tübingen (010/2018BO2). The study was carried out from April to October 2019 as a household-based survey in rural communities of the Moyen-Ogooué province of Gabon.

Finger-prick blood samples were collected from volunteers aged >3 years after provision of signed informed consent (Supplementary Methods). Individuals with microscopic evidence of *P. malariae* infections (with or without *P. falciparum* or *P. ovale*), a hemoglobin concentration of ≥6 g/dL and no antimalarial treatment for the last 3 months were invited to provide 5 mL of blood taken by venipuncture into a heparinized tube for direct membrane feeding assays (DMFAs). All malaria slide-positive participants were treated with artemether-lumefantrine (Coartem). A 500-µL aliquot was stored at –20°C in DNA/RNA Shield (Zymo Research) for total RNA extraction using the quick-DNA/RNA Microprep Plus Kit (Zymo Research), according to the manufacturer's instructions. The detection of *P. malariae* infections was performed using qPCR (Supplementary Methods and Supplementary Table 1).

Female adult *A. gambiae* ss (Kisumu strain) mosquitoes, 3–6 days old, were starved for 12 hours before blood feeding. DMFAs were performed in the negative air pressure biosafety level 3 area of the insectary to prevent accidental release of infected mosquitoes. The DMFA procedure was performed immediately after blood collection; 50 female mosquitoes per paper cup were fed via an artificial membrane (Parafilm). Two experimental groups were tested; the first group was allowed to feed on 1 mL of whole blood, and the second group was fed after serum replacement with 60% naive European AB serum to exclude transmission blocking activities in semiimmune serum. Mosquitoes were fed for 30 minutes in the dark. Afterward, unfed mosquitoes were removed and killed. Blood-engorged mosquitoes were provided with a 10% sugar solution and kept in standard insectary conditions (Supplementary Methods).

Mosquito infections were determined 10–12 days after blood feeding. At least 20 randomly selected mosquito midguts per cup were dissected and stained with mercurochrome (0.2% in phosphate-buffered saline). Oocysts were counted at x100 magnification under a light microscope (Leica DM750). In addition, 10 randomly selected fed mosquitoes per cup were stored in 500 µL of RNAlater solution at –20°C for molecular confirmation. After examination by microscopy, stained midguts were stored at –20°C. Sporozoites were isolated from salivary glands on day 21 from mosquitoes dissected in the serum replacement group. *Plasmodium* infections in *A. gambiae* mosquitoes fed with monoinfected or coinfected fresh isolates were confirmed using an established qPCR assay (Supplementary Table 1). The data analysis is described in the Supplementary Methods.

RESULTS

Among 647 participants screened in 110 household surveys, 15 *P. malariae* infections were identified by microscopy (2.3%). Subsequent analysis of this subset of 15 infections with qPCR revealed that 8 of the participants were monoinfected, 6 were coinfected with *P. falciparum,* and 1 was coinfected with *P. falciparum* and *P. ovale*.

At the time of membrane feeding, we detected median asexual parasite counts of 663/µL (range, 181–1203/µL) for *P. malariae*–monoinfected isolates and 447/µL (range, 251–820/µL) for coinfection isolates. The respective median *P. malariae* gametocyte counts were 71/µL (range, 17–236/µL) and 103/µL (0–601/µL) (Table 1).

We performed membrane feeding experiments with 13 isolates. A total of 847 female mosquitoes were successfully fed

Table 1. Susceptibility of *Anopheles gambiae* Mosquitoes to *Plasmodium malariae* Isolates From Asymptomatic Carriers

				Mosquitoes Infected/ Mosquitoes Dissected, No.		Infection Rate, %		Oocysts per Mosquito, Mean No.	
Isolate No.	*Plasmodium* Species qPCR	Parasite Count, No./µL	Gametocyte Count, No./µL	WB	SR	WB	SR	WB	SR
103	*P. malariae*	322	115	1/20	2/20	5	10	0.3	1.4
106	*P. malariae*	663	17	1/21	9/20	4.8	45	0.05	3.3
325	*P. malariae*	1203	71	…	4/16	…	25	…	1.9
379	*P. malariae*	805	30	0/10	0/14	0	0	0	0
416	*P. malariae*	181	25	0/19	0/20	0	0	0	0
568	*P. malariae*	1178	236	1/18	2/11	5.5	18.2	0.6	0.2
625	*P. malariae*	423	88	1/14	2/20	7.1	10	0.07	0.11
90	*P. malariae–P. falciparum*	447	230	0/20	10/40	0	25	0	1.3
91	*P. malariae–P. falciparum*	820	601	0/6	18/35	0	51.4	0	15.1
139	*P. malariae–P. falciparum*	468	103	0/13	2/13	0	15.4	0	0.3
205	*P. malariae–P. falciparum*	251	6	2/6	0/8	33.3	0	0.5	0
323	*P. malariae–P. falciparum*	309	0	…	1/16	…	6.2	…	0.06
109	*P. malariae–P. falciparum–P. ovale*	1523	30	4/10	5/21	40	23.8	2.6	1.1

Abbreviations: qPCR, quantitative polymerase chain reaction; SR, serum replacement; WB, whole blood; …, no experiment.

in 2 groups (whole blood [n = 319] and serum replacement [n = 528]). Of the mosquitoes that survived until day 12 after feeding (survivorship, 68% [578 of 847]), a minimum of 20 mosquitoes were examined per isolate and per experimental group. The majority of *P. malariae*–positive isolates were infective—that is, at least 1 mosquito was found to harbor oocysts (15% [65 of 441]), (Figure 1, Table 1, and Supplementary Material). Two isolates failed to yield mosquito infections (Table 1). The presence of *P. malariae* oocysts in midguts of a subset of mosquitoes 10–12 days after feeding was confirmed in 4 of 4 separate experiments, using qPCR. None of these mosquitoes was positive for *P. falciparum* after feeding with coinfected *P. malariae–P. falciparum* samples.

Infection rates were lower after feeding with whole-blood isolates compared with serum replacement isolates (4% [4 of 102] vs 16% [19 of 121]; $P = .004$). A similarly pronounced difference was also seen with coinfected isolates (4% [2 of 45] vs 28% [31 of 112], respectively; $P = .001$) (Figure 1A). Serum replacement also increased the intensity of mosquito infections with *P. malariae*–monoinfected isolates (geometric mean, 2.9; 7 independent experiments, representing a total of 121 mosquitoes) compared with whole-blood isolates (1.6; 6 independent experiments, representing a total of 102 mosquitoes; $P = .004$) (Figure 1B). Likewise, the number of oocysts per mosquito fed with coinfected isolates was higher in the serum replacement group (geometric mean, 5.1; 5 independent experiments, representing a total of 112 mosquitoes) than in the whole-blood group (1.3; 4 independent experiments, representing a total of 45 mosquitoes; $P < .001$) (Figure 1B).

Salivary gland dissections were performed on day 21 in a total of 30 mosquitoes from 4 batches fed with serum replacement isolates. We estimated sporozoite numbers per mosquito at 53 700 and 1350 for mosquitoes fed on 2 *P. malariae*–monoinfected isolates and 2500 and 9580 for mosquitoes infected with 2 coinfected isolates.

P. malariae gametocytes were detected in 92% of isolates (12 of 13). Gametocytemia was correlated both with the prevalence of mosquito infection (Pearson $r = 0.66$; $P = .02$) (Figure 1C)

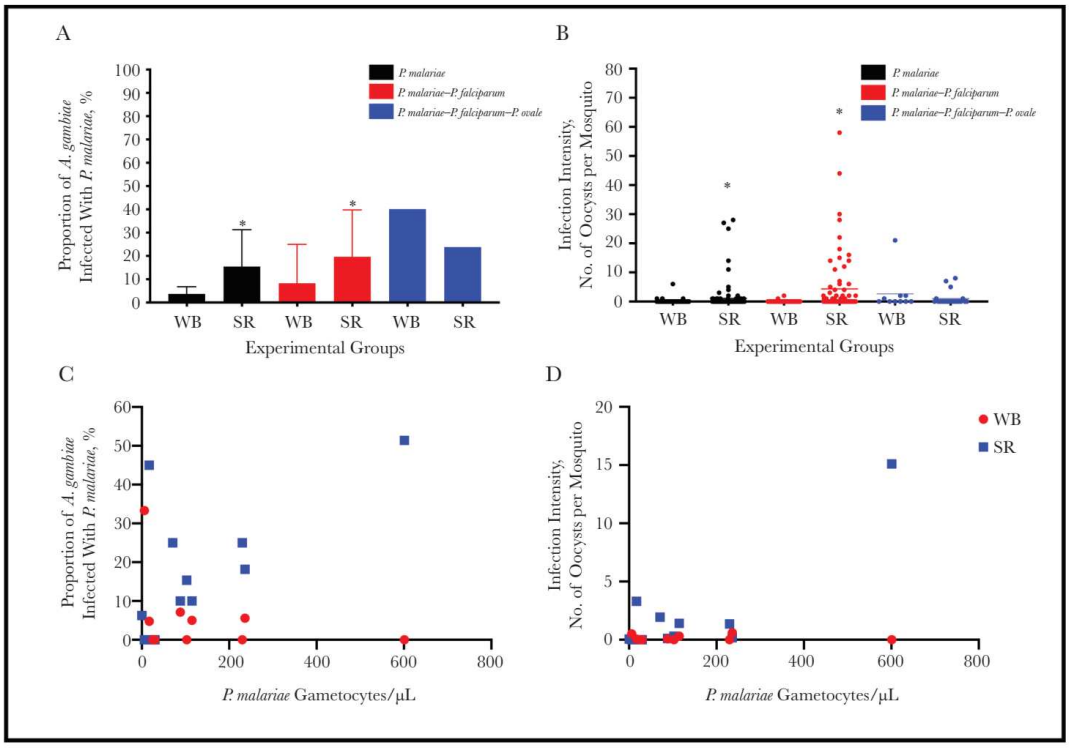

Figure 1. Infectivity of *Plasmodium malariae* isolates to *Anopheles gambiae* mosquitoes. Mosquitoes were fed with unmodified isolates (whole blood [WB]) or isolates after serum replacement (SR). *A,* Bar chart showing the proportion of infected mosquitoes per treatment group (7 *P. malariae* monoinfections, 5 *P. malariae–Plasmodium falciparum* coinfections, and 1 *P. malariae–P. falciparum–Plasmodium ovale* coinfection). Error bars indicate upper 95% confidence intervals. *B,* Dot plot of infection intensity, defined as number of oocysts per midgut per experimental group. *C,* Scatterplot of gametocyte density versus proportion of infected mosquitoes. Each dot represents an independent experiment. *D,* Scatterplot of gametocyte density versus infection intensity. *$P < .05$ for comparison between WB and SR groups.

and the intensity of infection (Pearson $r = 0.85$; $P < .001$) (Figure 1D) in mosquitoes fed with serum replacement isolates but not in those fed with whole-blood isolates ($r = -0.32$ [$P = .4$] and $r = -0.9$ [$P = .8$], respectively).

DISCUSSION

The recently published genome of *P. malariae* should prove to be a valuable resource [7] and stimulate research of this neglected parasite. As it stands, however, even basic characteristics, such as clinical burden, immunity, and transmission, remain ill defined. To start addressing some of those questions, we set out to establish a protocol for the experimental transmission of *P. malariae* in a colony of *A. gambiae* ss (Kisumu strain) mosquitoes. Our data demonstrate moderate transmission rates using fresh parasite isolates collected from asymptomatic carriers in an endemic area around Lambaréné, Gabon.

Previous reports have shown double-digit proportions of all *Plasmodium*-infected *Anopheles* mosquitoes to carry *P. malariae* in endemic areas in South America [4] and Africa [3], indicating that *P. malariae* is frequently and efficiently transmitted. However, experimental transmission of *P. malariae* isolates to *Anopheles* mosquitoes has only rarely been described. Studies have shown that mosquitoes could be infected by feeding on *P. malariae* Uganda strains from monkeys of the new world [10]. More recently, Woodford et al [11] demonstrated the infection of a single mosquito (infection rate, 2.9%) using the direct skin feeding assay from a volunteer inoculated with a *P. malariae* blood-stage isolate. Our results show a robust infectivity of *P. malariae* isolates to *A. gambiae* ss. The presence of *P. malariae* was confirmed by qPCR in whole mosquitoes as well as stained midguts in a subset of mosquitoes. The moderate infectivity rates in our study are most likely related to the low asexual parasite and gametocyte densities of the infecting isolates obtained from asymptomatic carriers. The observed transmission rates, however, are consistent with other *Plasmodium* species at low parasite densities and, more importantly, at low gametocyte numbers.

Infection rates in our study were similar to those described in *A. gambiae* infected by *P. falciparum* isolates from asymptomatic individuals in high-endemic settings in Burkina Faso and Ethiopia [12], and with *P. vivax* in Ethiopia and Thailand [13]. Our findings, though based on a small number of *P. malariae*–infected participants, are therefore in line with other findings from African settings. Mosquito infections can arise from asymptomatic infections, including asymptomatic qPCR-detected infections [12]. The low to moderate transmission rates in our and previous studies [12, 13] could be due to the fact that the employed Kisumu strain is from a different geographic area. Molina-Cruz et al [14] found that infections carried out with *Anopheles* and *P. falciparum* species from the same geographic areas resulted in higher infection rates. This possibility should be addressed in future studies on the transmission of *P. malariae*.

In agreement with previous studies with *P. falciparum*, the replacement of serum of malaria-infected individuals with AB serum from nonimmune donors before feeding resulted in higher mosquito infection rates compared with no-replacement controls. This is thought to be related to transmission-blocking activities in semi-immune serum, such as antibodies against antigens expressed by gametocytes [15].

To conclude, the present study demonstrated the successful use of a standard DMFA protocol for the experimental transmission of *P. malariae* to *A. gambiae* ss. The study is a step forward for studying parasite-vector interactions that determine the transmission of *P. malariae* in Africa.

Supplementary Data

Supplementary methods, supplementary Table 1 and supplementary information are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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SUPPLEMENTARY INFORMATION – DATA RAW

ID	Species qPCR	Parasites /ul	Gametocytes /ul	Infected mosquitoes /dissected mosquitoes		Infection rate (%)		Mean # oocysts per mosquito		Median # oocysts per mosquito		Range No. oocyst		# fed mosquitoes		# Dissected mosquitoes		# Dead mosquitoes		Mortality rate (%)	
				WB	SR	WB	SR	WB	SR	WB	SR	WB	SR	WB	SR	WB	SR	WB	SR	WB	SR
103	Pm	322	115	1/20	2/20	5	10	0.3	1.4	0	0	0-6	0-25	35	41	20	20	5	16	14.29	39.02
106	Pm	633	17	1/21	9/20	4.76	45	0.05	3.3	0	0	0-1	0-27	32	41	21	20	6	10	18.75	24.39
325	Pm	1203	71	-	4/16		25		1.94		0		0-28	0	25	0	16		2		8
379	Pm	805	30	0/10	0/14	0	0	0	0	0	0	0	0	27	33	10	14	12	12	44.44	39.39
416	Pm	181	25	0/19	0/20	0	0	0	0	0	0	0	0	29	48	19	20	5	18	17.24	37.5
568	Pm	1178	236	1/18	2/11	5.56	18.18	0.6	0.18	0	0	0-1	0-1	25	17	18	11	2	2	8	11.76
625	Pm	423	88	1/14	2/20	7.14	10	0.07	0.11	0	0	0-1	0-1	18	25	14	20	4	5	22.22	20

Supplementary material

METHODS

Study area and study population

The study was carried out from April to October 2019 in the Moyen-Ogooué Province of Gabon. Participants aged 3 years and who had no anti-malarial treatment for the last three months were included in the study after signing an informed consent form and legal representatives of children under the age of 11 were asked to give signed consent form. Children aged from 11 to 17 had to give their assent in addition to the signature of their legal representative. Because the study also involved collection of mosquitoes in households (not reported here), community consent was sought before individual household members were approached. Participants were screened during household surveys in communities located between 10 to 37 km from Lambaréné city alongside the road to Fougamou, a city located in the southern part of the country. We included individuals aged from 3 years old and above. Finger prick blood samples were collected from volunteers. These samples were used to perform a malaria rapid diagnostic test (RDT) SD Bioline Malaria AG P.f/Pan and to prepare thick and thin blood smears in the field by trained biomedical scientists to detect and quantify *Plasmodium* species using light microscopy.

Microscopic diagnosis

Thick blood smears were stained with 10% Giemsa solution for 45 min and examined microscopically by two qualified and experienced microscopists with a 1,000x magnification. Slide readings and calculations of the parasitaemia and the gametocytaemia were done according to the Lambaréné method as described previously [1, 2]. The following calculation was used: (number of counted parasites/number of investigated HPFs) X MF, where HPFs are the high-powered fields and MF the microscope factor. Slides were declared negative only after reading at least 100 HPFs.

P. malariae gametocyte carriers sampling

The following procedures were all carried out at dedicated facilities at CERMEL. A finger prick was taken from individuals found by microscopy to be infected with *P. malariae* either as mono-infection or as co-infection for directly determining the haemoglobin concentration (HemoCue Hb 201+). Individuals with more than 6 g/dL were invited to provide 5 mL of blood taken by venepuncture into a heparinised tube for direct membrane feeding assays (DMFA). The sample was immediately incubated at 37°C to prevent the exflagellation of male

gametocytes. All malaria slide-positive participants were treated accordingly with artemether-lumefantrine (Coartem®). From each whole blood sample, 500µl of blood were collected for qPCR parasite species identification and stored at -20°C in DNA/RNA Shield® (Zymo Research, Irvine, USA).

Plasmid cloning by PCR

The 18S rRNA gene target sequence was amplified using the forward primer (5' TTAAAATTGTTGCAGTAAAACGCTCGTA 3') and the reverse primer (5' GAAGTTTAAGGCAACAACAGG 3') and cloned using the manufacturer's recommended protocol (Thermo Scientific CloneJET PCR Cloning). Briefly, the DNA was isolated through gel purification and ligated into the pJET 1.2/blunt cloning vector and transformed into competent Escherichia coli cells. A colony PCR was performed to confirm the presence of the target sequence followed by an overnight bacterial culture. Plasmid DNA was isolated using the Thermo Scientific™ GeneJET™ Plasmid Miniprep Kit and DNA quantity was measured using a Nanodrop spectrophotometer. The number of plasmids was calculated using the following formula [3]:

$$\text{DNA (copy)} = \frac{6.02 \times 10^{23}\ \text{(copy/mol)} \times \text{DNA amount (g)}}{\text{DNA lenght (bp)} \div 650\ \text{(g/mol)/bp)}}$$

We then used serial dilutions of plasmids with known/calculated numbers of plasmids to determine the lower limit of detection of the DNA-based qPCR protocol (PMID: 29311086). This was established to be at least 100 copies per mL. We then tested whether our RNA-based assay performed better (i.e. yields a lower limit of detection) in comparison to the aforementioned DNA-based protocol (same primers, probes and cycle conditions) using diluted (1:10) co-infected samples using whole nucleic acid from a single extraction. Our RNA based protocol yielded Ct values with a delta of -5.6 (median; range, -5.5 - -5.7) compared to the DNA-based protocol. We thus calculated the limit of quantification for our assay at ≤10 parasites/mL, in the same range of the RNA-based *P. falciparum* assay [4].

Detection of *P. malariae* infections in human blood samples by qPCR

Total RNA extraction was performed with Quick-DNA/RNA™ Microprep Plus Kit (Zymo Research, Irvine, USA) according to manufacturer's instructions. Purified RNA samples were stored immediately at -20°C until use. qPCR assays were conducted in parallel to detect *P. malariae* with highest possible sensitivity in case of coinfections with *P. falciparum* or other

Plasmodium species (LightCycler 480, Roche, Mannheim, Germany). The first assay identified *P. malariae* using previously described primers and probes [4]. Each reaction consisted of 1µL RNA template, 5µL SensiFAST™ One-Step Mix (Bioline, London, UK), 0.1µL transkriptase, 0.2µL 10µM TaqMan probe, 0.4µL of each 10µM primer and 2.9µL water (10 µL total reaction volume).

The second assay distinguished between *P. falciparum* and other *Plasmodium* species including *P. malariae, P. vivax* and *P. ovale* as described previously [4-6]. Each reaction consisted of 1µL RNA template, 5µL SensiFAST™ One-Step Mix (Bioline, London, UK), 0.1µL transkriptase, 0.4µL of each 10µM TaqMan probe, 0.8µL of each 10µM primer and 1.5µL water. After an initial transcription step of 20 minutes at 45°C, followed by a hot-start step of 5 minutes at 95°C, cycling conditions were identical, allowing simultaneous runs in a single analysis step. The cycling conditions were 5 minutes at 95°C followed by 50 cycles of 93°C for 15 seconds and 60°C for 60 seconds. All samples were analysed in triplicates and each assay included a non-template control and a positive control in triplicates, respectively. All probes are listed in Supplementary Table 1.

Mosquito strain

Anopheles gambiae s.s. (Kisumu strain) mosquitoes were reared under standard laboratory conditions (27°C ± 1°C and 80% relative humidity) in climatic chambers (mytron, Heilbad Heiligenstadt, Germany) in the insectary of CERMEL under 12:12 day and night cycles. The larvae were fed on fish food (Vitakraft-flake mix) up to the pupal stage, after which they were transferred into cages. Adult mosquitoes were provided with a 10%-sugar solution. Three to six days old female adults were used for the experiments and starved for 12h before blood feeding.

***P. malariae* experimental transmission by direct membrane feeding assay (DMFA)**

DMFAs were performed in the negative air pressure, BSL-3 area of the insectary to prevent accidental release of infected mosquitoes. The DMFA procedure was performed as described elsewhere [7]. Fifty female mosquitoes were selected for each blood sample and put into secured paper cups. Immediately after blood collection, the mosquitoes were fed via an artificial membrane (Parafilm®) attached to a water-jacketed glass feeder (Zitt Thoma, Freiburg, Germany) maintained at 37°C. Two experimental groups were tested; one group was allowed to feed on 1ml of whole blood and the second group was fed after serum replacement

with 60% European naïve AB- human serum from the Centre for Clinical Transfusion Medicine Tübingen gGmbH (ZKT). We replaced the endogenous serum with naïve AB serum due to the potential transmission-blocking activities in sera from semi-immune donors [8-10]. For this, plasma was removed after centrifugation of the whole blood sample at 1,500g for 5 minutes at 37ºC to avoid exflagellation of male gametocytes, then the pellet was washed in 3mL of pre-warmed RPMI medium and the supernatant was removed. The pellet with parasitised red blood cells was added to pre-warmed AB serum to a haematocrit of 40% and a final volume of 1ml. Mosquitoes were fed for 30 minutes in the dark. Afterwards, unfed mosquitoes were removed and killed. Blood engorged mosquitoes were provided with a 10% sugar solution and kept inside the paper cups in a climatic chamber at standard insectary conditions.

Mosquito infection was determined 10-12 days after blood feeding [11]. At least 20 mosquito midguts were dissected using a stereomicroscope (ZEISS Stemi 508, Jena, Germany), stained with mercurochrome (0.2% in PBS) and oocysts were counted at 100x magnification under a light microscope (Leica DM750, Wetzlar, Germany). Additionally, 10 randomly selected fed mosquitoes per cup were stored in 500µl of RNAlater® at -20°C for molecular confirmation. After examination by microscopy, stained midguts were stored in 40µL PBS at −20°C following the procedure reported by Wang et al. [12]. Briefly, stained midguts were taken from slides previously flooded with PBS to allow easy removal of coverslips and the excess of mercurochrome was removed by dragging the midgut through a clean PBS droplet. Salivary glands from mosquitos dissected in the serum replacement with *P. malariae* mono-infections (n=2) and *P. malariae-P. falciparum* co-infections (n=2) were collected on day 21 into a 1.5 ml tube with 100 µl of RPMI/3%BSA on ice. After grinding mosquito salivary glands with a homogenizer (EPPI-Pistill) to release sporozoites from the salivary glands, the mixture was transferred to a haemocytometer for counting.

Detection of *P. malariae* in infected mosquitoes by qPCR

Confirmation of *Plasmodium* infections in *An. gambiae* mosquitoes fed with mono- or co-infected fresh isolates was carried out using a TaqMan assay described previously [5]. Of note, two TaqMan probes were used to discriminate *P. falciparum* (first probe) from *P. vivax, P. ovale* and *P. malariae* (second probe).

Data analysis

All statistical analyses were carried out using STATA 16 (StataCorp.,TX, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., CA, USA). The prevalence (% proportion of infected mosquitoes = 100 × number of mosquitoes with at least one oocyst / total number of dissected mosquitoes) and infection intensity (geometric mean number of oocysts per midgut) by day 12 post feeding were used as indicators of infectivity. Mosquito infection proportions were calculated using the Fisher's exact test and oocyst densities were compared using the Mann-Whitney U test. Pearson rank correlation coefficient (r) was calculated to study the relationship between non-parametric variables. The statistical significance level was set to 5%.

The output of ultrasensitive qPCR reactions was analysed by visual inspection and calculating the cycle threshold (Ct) using software LightCycler 480 Software (version 1.5.1.62) via the second derivative maximum method. A quantification cycle (Cq) threshold of 40 was used as a cutoff to define sample positivity (Cq < 40). In terms of the upper limit of Ct value (set at 40 cycles), we found 1 false positive signal in negative controls from 35 independent experiments, resulting in a low false positive rate of 2.9%.

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2.3 Chapter 3: *Anopheles gambiae s.s.* resistance to pyrethroids and DDT in semi-urban and rural areas of the Moyen-Ogooué Province, Gabon

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Anopheles gambiae s.s. resistance to pyrethroids and DDT in semi-urban and rural areas of the Moyen-Ogooué Province, Gabon

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Abstract

Background Pyrethroids are the main insecticides used in vector control for malaria. However, their extensive use in the impregnation of long-lasting insecticidal nets (LLINs) and indoor residual spraying has led to the development of resistance, threatening its success as a tool for malaria control. Baseline data prior to large scale distribution of LLINs are important for the implementation of efficient strategies. However, no data on the susceptibility of malaria vectors is available in the Moyen-Ogooué Province in Gabon. The aim of this study was to assess the susceptibility to pyrethroids and organochlorides of malaria vectors from a semi-urban and rural areas of the province and to determine the frequency of insecticide resistance genes.

Methods Larvae were collected from breeding sites in Lambaréné and Zilé and reared to adults. Three to five-day old female *Anopheles gambiae sensu lato* mosquitoes were used in cone tube assays following the WHO susceptibility tests protocol for adult mosquitoes. A subsample was molecularly identified using the *SINE200* protocol and the frequency of *Vgsc-1014 F* and *– 1014 S* mutations were determined.

Results *Anopheles gambiae sensu stricto* (*s.s.*) was the sole species present in both Lambaréné and Zilé. Mosquito populations from the two areas were resistant to pyrethroids and organochlorides. Resistance was more pronounced for permethrin and DDT with mortality lower than 7% for both insecticides in the two study areas. Mosquitoes were statistically more resistant ($P < 0.0001$) to deltamethrin in Lambaréné (51%) compared to Zilé (76%). All the mosquitoes tested were heterozygous or homozygous for the knockdown resistance (*Kdr*) mutations *Vgsc-L1014F* and *Vgsc-L1014S* with a higher proportion of *Vgsc-L1014F* homozygous in Lambaréné (76.7%) compared to Zilé (57.1%).

Conclusion This study provides evidence of widespread resistance to pyrethroids in *An. gambiae s.s.*, the main malaria vector in the Moyen-Ogooué Province. Further investigation of the mechanisms underlining the resistance of *An. gambiae s.s.* to pyrethroids is needed to implement appropriate insecticide resistance management strategies.

Keywords *Anopheles gambiae*, Pyrethroids, Organochlorides, Insecticide resistance, Moyen Ogooué Province, Gabon

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Background

Vector control has been pivotal for malaria control, with long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) being the two main strategies used. It is estimated that these intervention especially LLINs have averted 69% of the 663 million malaria cases [1]. Five classes of insecticides are used in vector control: (1) pyrethroids, (2) carbamates, (3) organophosphates, (4) organochlorides and (5) chlorfenapyr, a pyrrole which recently received an interim approval from the World Health Organization (WHO) to be used in insecticide-treated nets and in IRS [2].

However, up to now, insecticides of the pyrethroid class are mostly used for impregnating bed nets. This reliance on a sole class of insecticides has led to the development and spread of resistance in major *Anopheles* vectors in Africa [3–7], especially following mass distribution of LLINs [8]. Two mutations (*L1014F* and *L1014S*) at the domain II of the voltage-gated sodium channel gene (*Vgsc*) have been identified as providing cross-resistance to pyrethroids and DDT in *Anopheles gambiae sensu stricto* (*s.s.*) [9, 10]. Although the consequences of this resistance on vector control measures are not fully elucidated, some studies have shown a loss of efficacy of LLINs in areas with insecticide resistance [4, 11, 12].

Malaria remains a public health issue in Gabon where it is a primary reason for consultation. Malaria prevention in Gabon is based on the provision of intermittent preventive treatment with sulfadoxine-pyrimethamine (IPTp-SP) to pregnant women who are in addition provided LLINs which are also freely available to children under five [13]. Despite the absence of LLIN mass distribution, previous studies have reported high frequencies of the *Vgsc-L1014F* and *Vgsc-L1014S* mutations in *Anopheles* populations from Libreville, Port-Gentil, Mouila and in villages of the Moyen-Ogooué province (Bindo, Zilé and Nombakélé) and in a lower frequency in Benguia [14–18]. In fact, Libreville was the first coastal West African location where the presence of both *Vgsc-L1014F* and *Vgsc-L1014S* alleles was reported suggesting the presence of high level of resistance in this mosquito population [19]. This may need re-evaluation of the effectivity of LLINs for the control of malaria morbidity in the country. However, only a single study assessed the phenotypic resistance in mosquitoes populations in Gabon [17] and there is a lack of data on the susceptibility of malaria vectors to insecticides from many parts of the country.

The aim of this study was to assess the susceptibility to pyrethroids and organochlorides of malaria vectors from two different settings of the Province of Moyen-Ogooué and to determine the frequency of insecticide resistance genes.

Methods

Study areas

The study was conducted in Lambaréné and Zilé from November 2017 to February 2018 (Fig. 1). Lambaréné is a semi urban area and is the provincial capital of the Moyen-Ogooué Province while Zilé is a rural area located approximately 12 km from Lambaréné. Although the two areas are close, a previous study in the Zilé area has showed that malaria transmission in this area is perennial with a fixation of the *Vgsc-L1014F* and *Vgsc-L1014S* resistant alleles in *An. gambiae s.s.* populations [18]. In addition, Zilé has the particularity of housing a rubber plantations scheme with the potential use of insecticides to deal with plant pests (although not assessed here) potentially driving the resistance of *An. gambiae sensu lato (s.l.)* populations.

Mosquito collection

Open water bodies in the areas were explored for the presence of anophelinae larvae by dipping and larvae were collected from breeding sites with the presence of larvae in the two areas. The larvae were reared up to the adult stage at the Medical Entomology Laboratory of the Centre de Recherches Médicales de Lambaréné (CERMEL). Emerging mosquitoes were provided with 10% sugar solution until the day of the testing. Adult mosquitoes were identified using the morphological identification keys of Gillies and de Meillon [20] and Gillies and Coetzee [21].

WHO susceptibility assays

The tests were carried out using impregnated papers with the following diagnostics concentrations: deltamethrin (0.05%), permethrin (0.75%) and DDT (4%) produced by the Vector Control Research Unit of Sains Malaysia University (Penang, Malaysia) and kindly provided by the Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale (OCEAC). The impregnated papers were tested with the Kisumu strain reared at the CERMEL before the tests were conducted with field collected mosquitoes to assess their quality. The results from the tests with the Kisumu were also used as comparators to the knockdown times obtained with field populations.

The tests were carried out according to the WHO protocol [22]. Briefly, three-to-five-day old, starved *An. gambiae s.l.* mosquitoes were exposed in WHO susceptibility kits to impregnated papers with insecticides while controls mosquitoes were exposed to untreated filter papers. The number of mosquitoes knocked down was recorded at 5, 10, 15, 20, 30, 40, 50 and 60 min. After 1 h of exposure, mosquitoes were transferred to observation tubes and were maintained on a 10% sugar solution for 24 h.

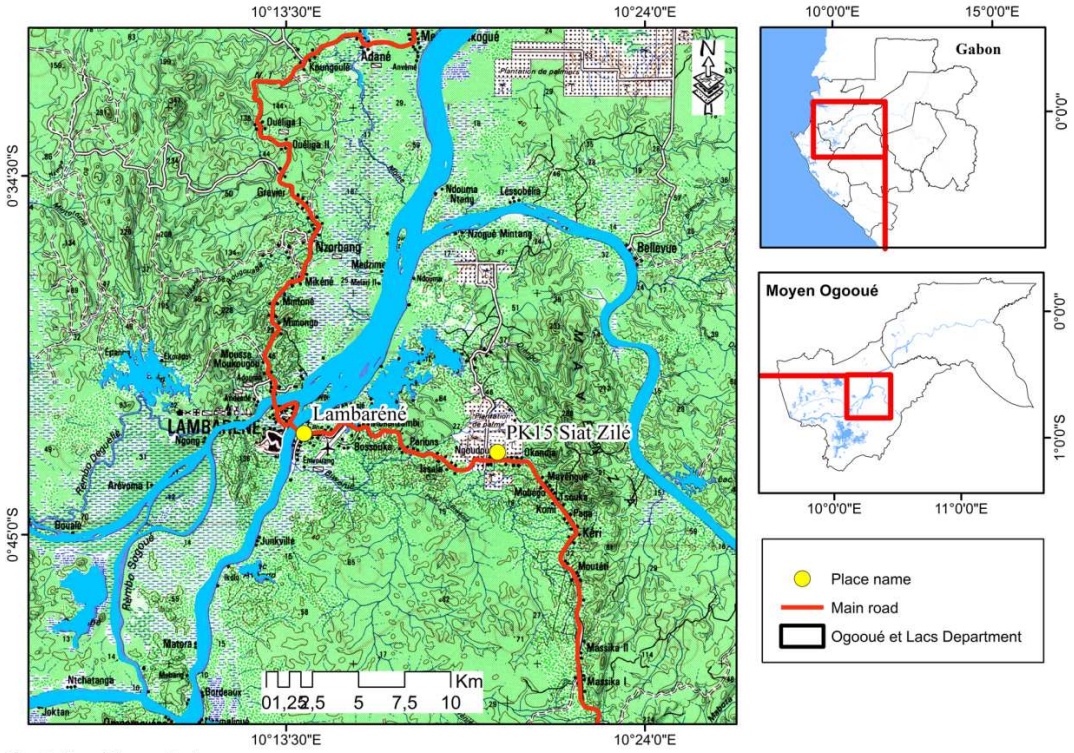

Fig. 1 Map of the studied areas

Mortality was recorded after a 24-hr recovery period. The mosquitoes were stored on silica gel for molecular assays.

Molecular identification

DNA was extracted from randomly selected control mosquitoes from Lambaréné and Zilé using the Livak protocol [23]. The mosquitoes were identified using the *SINE200* protocol [24]. This protocol allows for the simultaneous identification of members of the *An. gambiae* complex using the following set of primers: forward (5′-TCG CCT TAG ACC TTG CGT TA-3′) and reverse (5′-CGC TTC AAG AAT TCG AGA TAC-3′) primers. The cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, annealing at 54 °C for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR products were analysed on a 2% agarose gel.

kdr genotyping

A Taqman assay was used to determine the frequency of *kdr* mutations (*Vgsc-L1014F* and *L1014S*) in dead and alive mosquitoes based on the protocol from Bass et al. [25]. The protocol is based on the use of one set of primers (5′-CAT TTT TCT TGG CCA CTG TAG TGA T-3′; *kdr*-reverse: 5′-CGA TCT TGG TCC ATG TTA ATT TGC A-3) and three probes: one for the identification of the wild-type allele (5′-CTT ACG ACT AAA TTTC-3′) labelled with the HEX fluorophore, and the remaining two labelled with the FAM fluorophore for the identification of the *Vgsc-L1014F* mutation (5′-ACG ACA AAA TTT C-3′) and the *Vgsc-L1014S* mutation (5′-ACG ACT GAA TTT C-3′). The cycling conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s and 65 °C for 45 s.

Statistical analysis

The WIN DL (version 2.0, 1999) software was used to determine the different knockdown times for 50 and 95% tested samples knockdown times from each population (KDT_{50}, KDT_{95}) using a log-time probit model.

Mortality was calculated by dividing the proportion of dead mosquitoes after the 24 h recovery period to mosquitoes exposed to the insecticide. The results of the test

were interpreted based on the WHO guidelines [26] with mortality in the range 98–100% indicating susceptibility, while 90–97% mortality indicating potential resistance and a need to be further investigated. Mortality rates of less than 90% were interpreted as resistance.

The sample size of mosquitoes to be analysed for the molecular identification was based on an estimated proportion of *An. gambiae s.s.* equals to 98%. The following formula: $n = \varepsilon^2 [p (1-p)]/e^2$; with $\varepsilon = 1.96$ (alpha risk = 5%), e (precision) = 5% and p = expected prevalence; with the resulting n = 31 to be included from each site.

The differences in mortality and allelic frequencies in the mosquito populations from the two areas were compared using the Fisher's exact test with the R software v.3.2.5 [26]. In addition, the distribution of genotypes was also tested for conformity to Hardy Weinberg Equilibrium (HWE) within each site using a web-based tool (https://gene-calc.pl/hardy-weinberg-page).

Results

A total of 537 *An. gambiae s.l.* collected from Lambaréné and Zilé were tested in susceptibility assays. In Lambaréné, 85 mosquitoes were tested with permethrin, 130 mosquitoes with deltamethrin and 44 with DDT. Meanwhile in Zilé, 113 mosquitoes were tested with permethrin, 117 mosquitoes with deltamethrin and 99 with DDT.

Species composition

Out of the 116 *An. gambiae s.l.* that were identified molecularly, 54 out of 60 mosquitoes and all mosquitoes (56 mosquitoes) were successfully amplified in Lambaréné and Zilé, respectively. *Anopheles gambiae s.s.* was the sole species found in both Lambaréné and Zilé.

Knockdown times

The KDT_{50} and KDT_{95} could only be determined for deltamethrin while for permethrin and DDT, both were above 60 min. The KDT_{50} for deltamethrin were around 32 min and 30 min for *An. gambiae* from Lambaréné and Zilé, respectively (Table 1). When compared to the KDT_{50} of the susceptible Kisumu strain, these KDT_{50} represented a 3.4 and 3.1-fold increase in the amount of time required to knockdown 50% of the *An. gambiae s.s.* mosquitoes from Lambaréné and Zilé, respectively. Similarly, there was an increase in KDT_{95} (58 min in mosquitoes from Lambaréné and 48 min in Zilé) for deltamethrin when compared to the one from the susceptible Kisumu strain, representing a 2.6 and a 1.6-fold increase.

Insecticide susceptibility of *An. gambiae s.s.*

The mosquitoes from Lambaréné and Zilé were found to be resistant to permethrin, deltamethrin, and DDT but the resistance was more pronounced for permethrin and DDT (Fig. 2). The mortalities recorded after the 24 h recovery period with permethrin and DDT were below

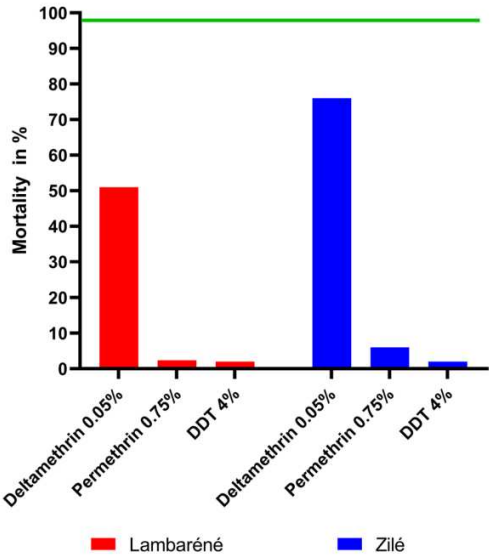

Fig. 2 Mortality rates of *An. gambiae s.s.* from Lambaréné and Zilé exposed to deltamethrin, permethrin and DDT. The green line represents the threshold for full susceptibility according to the WHO criteria

Table 1 Knockdown times and ratios of *An. gambiae s.s.* samples from Lambaréné and Zilé after insecticide susceptibility tests

Study sites	Insecticides tested	N	KD_{50}(min) [CI_{95}]	$Rtkd_{50}$ [CI_{95}]	KD_{95} (min) [CI_{95}]	$Rtkd_{95}$ [CI_{95}]	Status
Lambaréné	Del 0.05%	130	32.3 [30.8–33.7]	3.4	57.7 [54 -62.7]	2.6	Resistant
	Per 0.75%	85	> 60	NA	> 60	NA	Resistant
	DDT 4%	44	> 60	NA	> 60	NA	Resistant
Zilé	Del 0.05%	117	29.9 [28.6–31.1]	3.1	48.1 [45.4–51.7]	1.6	Resistant
	Per 0.75%	113	> 60	NA	> 60	NA	Resistant
	DDT 4%	99	> 60	NA	> 60	NA	Resistant

3% for both insecticides in Lambaréné. Whilst in Zilé, mortality with permethrin and DDT were 6% and 2%, respectively. The mortality rates for permethrin (Fisher's exact test, $P=0.30$) and DDT (Fisher's exact test, $P=1$) were comparable in both study areas. *Anopheles gambiae s.s.* mosquitoes from Lambaréné were significantly more resistant to deltamethrin (Fisher's exact test, $P<0.0001$) than those from Zilé, with mortalities of 51% and 76%, respectively.

Genotypic resistance markers

Out of the 118 mosquitoes that were randomly screened for *kdr* mutations, 116 (98%) were successfully amplified. All the mosquitoes were heterozygous (80.2%) or homozygous (19.8%) for *Vgsc-L1014F* and *-L1014S* which confer resistance to pyrethroids and DDT. The distribution of the resistant genotypes were similar in Lambaréné and Zilé (Fisher's exact test, $P=0.068$) with a higher proportion of homozygous *Vgsc-L1014F* (76.7% and 57.1%, respectively), followed by heterozygous individuals *Vgsc-L1014F/L1014S* (20% and 32.1%, respectively) with the rest made of homozygous *Vgsc-L1014S* (Table 2) (2% and 6%, respectively). However, the distribution of the genotypes were consistent with HWE in Lambaréné ($\chi^2=1.09$; $p=0.58$) and Zilé ($\chi^2=1.82$; $p=0.40$). None of the mosquitoes were found to carry the susceptible allele *Vgsc-L1014L*.

Discussion

Vector controls measures should be implemented based on the local epidemiological and entomological data. Thus, there is a need to determine the susceptibility of local vectors to the common types of insecticides before large vector control measures are deployed in an area. This report provides baseline data on the phenotypic susceptibility of malaria vectors in the Moyen-Ogooué Province. Results from the current study revealed a substantial increase of KDT_{50} and KDT_{95}, when compared to the Kisumu susceptible strain, beyond 60 min for permethrin and DDT. Whereas for deltamethrin, although KDT_{50} and KDT_{95} were below 60 min, it represented a 3-fold increase in KDT_{50} and up to a 2.6- fold increase in KDT_{95} when compared to the susceptible *An. gambiae s.s.* Kisumu strain. Similar increases in KDT_{50} were also recorded in susceptibility tests carried out in Mouila, the capital of the Ngounié province of Gabon [17]. This loss of susceptibility to the knockdown effect of pyrethroids may lead to the loss of the deterrence effect to this class of insecticide which are used in impregnating bed nets. This deterrence effect is of great importance in maintaining LLINs effectiveness especially when they are torn [11] as they prevent entry of mosquitoes. These results suggest that pyrethroid based insecticides are widely used in the local populations.

The mortality rates recorded for each of the insecticides tested are in line with the knockdown results suggesting *An. gambiae s.s.* from the area are highly resistant to pyrethroids and DDT. *Anopheles gambiae s.s.* populations were highly resistant to DDT and permethrin, a type I pyrethroid. Previous reports from other countries [5, 7, 27, 28] have shown a high resistance to permethrin, which is, together with deltamethrin, the main insecticides used in bed nets impregnation. Resistance to deltamethrin, a type II pyrethroid, was less pronounced than for permethrin thus LLINs impregnated with deltamethrin may be a better option for vector control in the Moyen-Ogooué Province and by extension to Gabon, as the current results are in line with those found in the Ngounié province for permethrin and deltamethrin [17].

The resistance in this study was more pronounced in the urban compared to the rural area despite the fact that the larval collection in the latter was carried out in an area surrounded with rubber and palm oil plantations where insecticides, such as pyrethroids, may be used for pest control as reported in agricultural settings in Burkina Faso [29, 30]. However, from discussions held with personnels from both rubber and palm oil plantations, insecticides are not used for pest controls with the plants only sprayed with the herbicide glyphosate which is not linked to pyrethroids resistance. Therefore, the personal use of pyrethroids based vector control tools in the form of insecticide sprays, mosquito coil, impregnated nets could play a major role in the selection pressure of local *Anopheles* spp

Table 2 Frequencies of *knock-down resistance* (*kdr*) alleles *An. gambiae s.s.* from Lambaréné and Zilé

	N	Genotypic frequencies n (%)			Allelic frequencies n (%)		HWE
		L1014F/L1014F	*L1014F/ L1014S*	*L1014S/L1014S*	*L1014F*	*L1014S*	
Lambaréné	60	46 (76.7)	12 (20)	2 (3.3)	52 (86.7)	8 (13.3)	0.58
Zilé	56	32 (57.1)	18 (32.1)	6 (10.8)	41 (73.2)	15 (26.8)	0.40
Total	116	78 (67.2)	30 (25.9)	8 (6.9)	93 (80.2)	23 (19.8)	0.13

p-values for chi-square test of Hardy Weinberg equilibrium

for reduced susceptibility to pyrethroids especially in urban areas. This high level of resistance could lead to the failure of control measures to reduce malaria transmission especially using bed nets that are impregnated with pyrethroids. These results point to the need for the implementation of mitigation strategies such as the use of LLINs impregnated with compounds such as piperonyl butoxide (PBO), which inhibits cytochrome P450, involved in metabolic resistance, and thereby can restore pyrethroid susceptibility.

Increases in KDT_{50} in field mosquito populations has been suggested to provide a sensitive indicator of the implication of *kdr* mutations in phenotypic resistance to pyrethroids [22, 31]. The *kdr* mutations in the local populations are fixed with all *An. gambiae s.s.* carrying either the *Vgsc-L1014F* or *Vgsc-L1014S* mutations which may explain the loss of knockdown effects of deltamethrin, permethrin and DDT. These results are in line with previous reports [14–17] from other parts of Gabon especially in Zilé [18], where high proportions of *An. gambiae* were carrying the *kdr* mutations. However, the fact that metabolic resistance was not assessed constitute a limitation to the present study as the differences in KDTs observed between the three insecticides (DDT, permethrin and deltamethrin) who have similar target sites, suggest the involvement of other resistance mechanisms.

Conclusion

The current study revealed a high level of resistance of *An. gambiae s.s.* in both Lambaréné and Zilé accompanied with a high frequency of the *Vgsc-L1014F* mutations. The resistance level was higher for permethrin and DDT compared to deltamethrin. Therefore, deltamethrin may be a better option for malaria vector control in the Moyen-Ogooué Province. However, the involvement of other resistance mechanisms should be further investigated for the introduction of control measures adapted to the local settings.

Abbreviations

WHO	World Health Organization
LLIN	Long-lasting insecticidal net
IRS	Indoor Residual Spraying
Vgsc	voltage-gated sodium channel IPTp-SP:intermittent preventive treatment with sulfadoxine-pyrimethamine
OCEAC	Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale
kdr	Knockdown resistance gene
PBO	piperonyl butoxide
CERMEL	Centre de Recherches Médicales de Lambaréné
DDT	dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
SINE200	Short INterspersed Element
KDT	Knockdown Time

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Author contributions
AAA, BM, PGK, PAA, HMK, SB, JCDA, TGW, JRE and STBS conceived and planned the study and its design. STBS, AGDN and BN performed the field activities. STBS, LNB, AGDN BN and TGW carried out the laboratory analysis of the samples. FM and STBS analysed the data. STBS drafted the manuscript. AAA, BM, PGK, PAA, HMK, SB, JCDA, TGW, JRE critically reviewed the manuscript. All authors made intellectual input to the study. All authors read and approved the final manuscript. STBS, AGDN, BN and AAA are members of CANTAM (EDCTP-CSA 2020 NOE-3100) networks.

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Raw data are archived and available on request from the corresponding author.

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Competing interests
The authors declare no competing interests.

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2.4 Chapter 4: Resistance of *Anopheles gambiae s.s.* against commonly used insecticides and implication of cytochrome P450 monooxygenase in resistance to pyrethroids in Lambaréné (Gabon)

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Resistance of *Anopheles gambiae* s.s. against commonly used insecticides and implication of cytochrome P450 monooxygenase in resistance to pyrethroids in Lambaréné (Gabon)

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Abstract

Background Insecticides are a crucial component of vector control. However, resistance constitute a threat on their efficacy and the gains obtained over the years through malaria vector control. In Gabon, little data on phenotypic insecticide resistance in *Anopheles* vectors are published, compromising the rational implementation of resistance management strategies. We assessed the susceptibility to pyrethroids, carbamates and organophosphates of *Anopheles gambiae sensu lato* (*s.l.*) and discuss the mechanisms involved in the pyrethroid resistance-phenotype.

Methods *A. gambiae s.l.* larvae were collected from breeding sites in Lambaréné. Emerging adults were used in WHO tube assays at an insecticide concentration that defines resistance (diagnostic concentration). Subsequently, deltamethrin and permethrin were used at 5x and 10x diagnostic concentrations and after preexposure with the cytochrome p450 (and glutathione S-transferase) inhibitor piperonyl butoxide (PBO). A subset of mosquitoes was typed by molecular methods and screened using Taqman assays for mutations conferring target site resistance at the Voltage-gated sodium channel 1014 (*Vgsc-1014*) locus and the acetylcholinesterase (*Ace-1*) gene.

Results All mosquitoes were *A. gambiae sensu stricto* (*s.s.*) and resistant to permethrin, deltamethrin and alphacypermethrin (mortality less than 98%). However, mosquitoes were susceptible to malathion but resistant to bendiocarb. The level of resistance was high for permethrin and at least moderate for deltamethrin. Pre-exposure to PBO significantly increased the mortality of resistant mosquitoes ($P < 0.0001$). They became fully susceptible to deltamethrin and permethrin-induced mortality increased 4-fold. The G119S *Ace-1* resistance allele, which confers resistance to both organophosphates and carbamates, was not present. All sampled mosquitoes were

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either homozygous for the *Vgsc-L1014F* or heterozygous for *Vgsc-L1014F/L1014S*, a marker for resistance to pyrethroids and organochlorides.

Conclusion These findings demonstrate a role of cytochrome P450 monooxygenases in the pyrethroid-resistance of *A. gambiae s.s.* from Lambaréné. Combining PBO with pyrethroids, as done in second generation bednets, may be used to revert resistance. In addition, malathion could also be used in combination with pyrethroids-based methods for resistance management.

Keywords *A. gambiae s.s.*, Pyrethroids, Resistance intensity, Cytochrome p450, Gabon

Introduction

Insecticide resistance is a looming threat on the success of malaria vector control measures. Although, a large share of the reduction in malaria cases has been attributed to vector control measures [1], a stagnation in malaria cases has been observed since 2016. From 2020, the number of cases even increased [2] due on one hand, to the disruptions in the delivery of medication and diagnostics during the COVID-19 pandemic [2] and on the other hand, to the reduced impact of vector control measures at further decreasing the incidence and mortality of malaria.

Malaria vector control in Gabon, a country located in Central Africa, is based on the free provision of Long Lasting Insecticidal Nets to pregnant women and children. Although previous reports have shown the spread of pyrethroid resistance in most sub-Saharan African countries [3–7], in Gabon the data are still sparse with few reports showing resistance to pyrethroid [8, 9] with low intensity resistance to permethrin, deltamethrin and lambdacyhalothrin in agricultural areas of Mouila [8]. However, the investigation of resistance mechanisms to insecticides in Gabon has been limited to genotyping *A. gambiae* populations for markers of target site resistance, which is a resistance mechanism where a modification of the site of action of an insecticide is changed such that it no longer binds effectively, resulting in the insect being unaffected or less affected by the insecticide [10]. Most reports in Gabon have shown a near fixation of the *Kdr* resistance alleles that confer resistance to pyrethroids and organochlorides [11–15]. In contrast, a glycine to serine (G119S) amino acid substitution (*ace-1*^{R}) in the mosquito's acetylcholinesterase, conferring resistance to both organophosphates and carbamates was reported only at a minor fraction of mosquitoes in Libreville [12]. Subsequent studies found no evidence of the allele in other parts of the country [12–15] with only one study reporting full susceptibility of *Anopheles* populations in Mouila to organophosphates and carbamates published, so far.

Metabolic resistance, the most common resistance mechanism in insects [10], is characterized by changes in a mosquito's enzyme system which result in a more rapid detoxification or catabolism of the insecticide, reducing the insecticide's concentration at its site of action [16]. In the case of malaria vectors, three enzyme families are known to be important insecticide metabolizers: cytochrome P450 monooxygenases, glutathione S-transferases and esterases. Resistant strains have been shown to possess higher levels or more efficient forms of these enzymes than susceptible counterparts [17] in Cameroon [18] and Tanzania [19]. Cytochrome P450 oxidases are most commonly involved in resistance, metabolizing a wide range of insecticides [17]. Cytochrome P450-mediated resistance mechanism have been reported in many *Anopheles* species across sub Saharan Africa (sSA) including *A. gambiae s.s.*, *A. coluzzii* and *A. funestus* [6, 20–25]. However, no reports have assessed metabolic resistance in *Anopheles* populations in Gabon. Gabon is planning to carry its first massive distributions of Long Lasting Insecticidal Nets (LLINs) in the coming years. In the context of reports of *Anopheles* resistance to insecticides at the molecular [11–15] and phenotypic [8, 9] levels, it is pivotal to collect data on the susceptibility of malaria vectors to pyrethroids and assess the effect of metabolism modifiers that can act synergistically with insecticides to revert resistance to pyrethroids as a potential resistance management strategy. The current study therefore aimed to evaluate the susceptibility profile of mosquitoes to pyrethroids, organophosphates and carbamates, to assess the level of resistance and the effect of piperonyl butoxide (PBO), a synergistic compound used to revert pyrethroid resistance.

Methodology

Study area and mosquito collections

The study was conducted in the second district of Lambaréné, the capital of the Moyen Ogooué province from December 2021 to May 2022 (Fig. 1). Lambaréné is a semi-urban settlement that is divided in three parts by the Ogooué River and is surrounded by forests. The city is also neighboured by two extensive agricultural schemes of palm and rubber trees. The second district is the most populated district of the city. Previous studies in the city and its surrounding villages have shown that *A.*

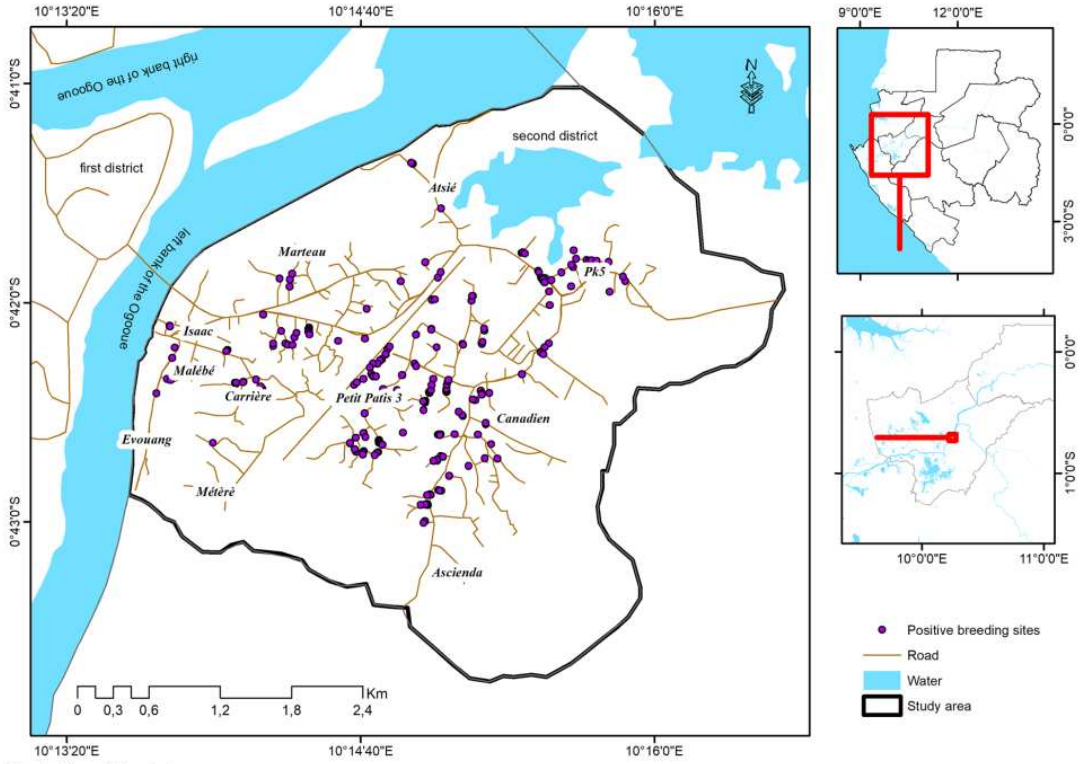

Fig. 1 Map of the study area

gambiae s.s. is the main malaria vector [15, 26, 27] with heterogenous malaria transmission intensity with perennial transmission in some areas while in others, no transmission was recorded during the dry season. In addition, previous reports have shown resistance of *A. gambiae s.s.* from this area to deltamethrin, permethrin and DDT [9].

Larvae were collected from breeding sites and reared up to the adult stage at 29 ± 1 °C under 12 h dark:12 h light cycle at the Medical Entomology Laboratory of the Centre de Recherches Médicales de Lambaréné (CERMEL). Emerging mosquitoes were provided with 10% sugar solution and kept at 26 °C ± 1 °C and 80 ± 10% relative humidity until the day of the test.

WHO susceptibility assays

The tests were carried out using impregnated papers with the following concentrations: deltamethrin (0.05%), permethrin (0.75%), alphacypermethrin (0.05%), bendiocarb 0.1% and malathion 5% following the WHO test tube protocol for adult mosquitoes [16]. These concentrations represent the threshold concentrations that discriminate the proportions of susceptible and resistant phenotypes in a sample of a mosquito population ("diagnostic concentration") [16]. We tested the impregnated papers with the Kisumu strain reared at the CERMEL before the tests with field collected mosquitoes to confirm the quality of the papers. All the tests were performed in controlled conditions at 26 °C ± 1 °C and 80% ± 10% relative humidity.

Briefly, three-to-five-day old unfed *Anopheles gambiae s.l.* mosquitoes were exposed in WHO susceptibility kits to impregnated papers with insecticides while controls mosquitoes were exposed to untreated filter papers. The number of mosquitoes knocked down was recorded at different time intervals (5, 10, 15, 20, 30, 40, 50 and 60 min). After 1 h of exposure, mosquitoes were transferred to observation tubes and were maintained on a 10% sugar solution for 24 h. Mortality was recorded after a 24-hour recovery period. The mosquitoes were stored on silica gel for molecular assays. Mortality in the field collected unexposed controls was less than 4%.

Assessment of resistance intensity

Based on the results from the susceptibility tests with standard diagnostic concentration, resistance intensity to pyrethroids was assessed using 5X and 10X the diagnostic concentrations for permethrin and 5X the diagnostic concentration for deltamethrin. The test procedure was the same as described above with knockdown recorded over a 1-hour period and mortality assessed after 24 h following WHO protocol [16] .

Piperonyl butoxide synergist tests

Resistance bioassays tests were performed with the PBO synergist which inhibits the activity of cytochrome P450 monooxygenases in order to assess the involvement of metabolic resistance. Briefly, three-to-five-day old, starved *Anopheles gambiae s.l.* mosquitoes were pre-exposed to 4% PBO for one hour and then exposed to 0.05% deltamethrin and 0.75% permethrin for another hour. The number of mosquitoes knocked down was recorded over a 1-hour period as described above. The mosquitoes were then transferred to observation tubes and maintained on a 10% sugar solution for 24 h. Mortality in the PBO-only exposure group was less than 4%.

Molecular identification

DNA was extracted from control for the screening of knockdown mutations which are already fixed in local anopheles populations [15] and from susceptible and resistant mosquitoes to bendiocarb using the Livak protocol [28]. The members of the *A. gambiae* complex were identified using the *SINE200* protocol [29]. Taqman assays were used to screen mosquitoes using previously published protocols for *Kdr* mutations (*Vgsc-L1014F* and *-L1014S*) [30] and *Ace-1* mutation [31]. For both Taqman assays, 1.0 µl of DNA template was amplified using 5 µl SensiFAST™ Probe No-ROX Kit (Meridian Bioscience Inc.), 0.8 µl forward (10 µM) and 0.8 µl reverse (10 µM) primers, 0.2 µl of each probe (10 µM) and 2 µL of nuclease free water to a final volume of 10 µl.

Statistical analysis

We used the WIN DL (version 2.0) software [32] to determine the different times required to knockdown 50% (KDT_{50}) and 95% (KDT_{95}) of the samples for each insecticide tested with a log-time probit model.

The results of the tests were interpreted based on the WHO guidelines [16] with mortality above 98% indicating susceptibility, while 90–97% mortality indicating potential resistance while mortality less than 90% interpreted as resistance. Low resistance intensity was defined as 98–100% mortality at the 5× concentration (but < 90% at 1×). Mortality < 98% at the 5 × concentration and 98–100% at the 10× concentration indicated moderate resistance intensity. Mortality < 98% at the 10× concentration indicated high resistance intensity [16].

The differences in mortality between insecticides tested were compared using the Fisher's exact and the chi-square tests. Graphical presentation of data was done using the GraphPad Prism Version 8.4.0 software (GraphPad Software Inc.). In addition, we also tested the distribution of genotypes for conformity to Hardy Weinberg Equilibrium (HWE).

Results

Species composition, genotyping of *Kdr* and *Ace-1* mutations

Out of the 140 *A. gambiae s.l* that were identified molecularly, 97.9% (137/140) were successfully typed as *A. gambiae s.s.* using the *Sine200* protocol.

All the 94 mosquitoes screened for the presence of the knockdown resistance genes were either homozygous (98.4%) for *Vgsc-L1014F* or heterozygous (1.6%) for *Vgsc-L1014F* and *Vgsc-L1014S*. The distribution of genotypes was in HWE equilibrium ($\chi^2 = 0.02$, df = 1, $P = 0.8764$). By contrast, all the mosquitoes screened for the *Ace1* mutation were carrying the G119 *Ace-1* susceptible allele of the gene.

Insecticide susceptibility

A. gambiae s.s. mosquitoes were resistant to 0.75% permethrin with a mortality of 11.4% (10/88) after the 24-hour observation period (Fig. 2B). There was a total loss of knockdown effect of permethrin on *A. gambiae s.s.* individuals tested; with only one (1) mosquito out of eighty-eight (88) knocked down after one hour exposure to the diagnostic concentration of permethrin (Fig. 2A). Therefore, no KDT_{50} nor KDT_{95} could be calculated after exposing the mosquitoes to the diagnostic concentration of permethrin.

Mosquitoes were more susceptible to deltamethrin in comparison to permethrin ($\chi^2 = 61.14$, df = 1, $P < 0.0001$). However, they were still resistant to deltamethrin with a 66.1% (76/115) mortality after 24 h (Fig. 3B). Although, a knockdown effect was observed with deltamethrin (Fig. 3A), there was still a 2.9-fold increase in the KDT_{50} of field populations (40.9 min) compared to the susceptible Kisumu strain (13.9 min) (Table S1) while the KDT_{95} was above 60 min.

A. gambiae s.s. were also resistant to 0.05% alpha-cypermethrin with a mortality of 45.8% (11/24) and both KDT_{50} and KDT_{95} above 1 h (Table 1). However, mosquitoes were fully susceptible to malathion with 100% (75/75) mortality whilst they were resistant to

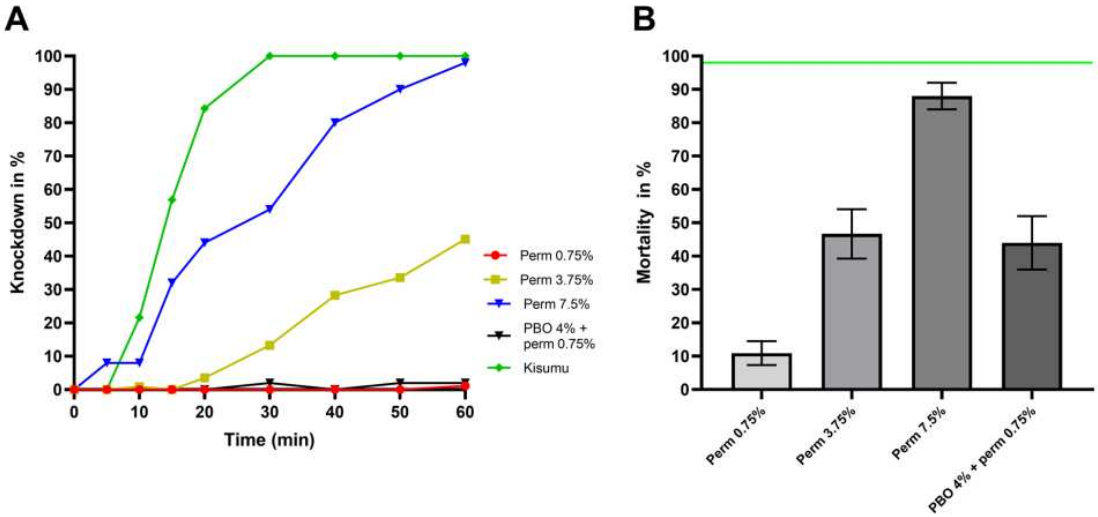

Fig. 2 Susceptibility profile of *A. gambiae s.s.* to permethrin only and after preexposure to PBO. **A** Proportion of mosquitoes knocked down after exposure to various concentrations. **B** Mean mortality after exposure to various concentrations. The horizontal green line represents the 98% WHO threshold for susceptibility. Error bars represent standard error of mean mortality after the 24h recovery period following insecticide exposure

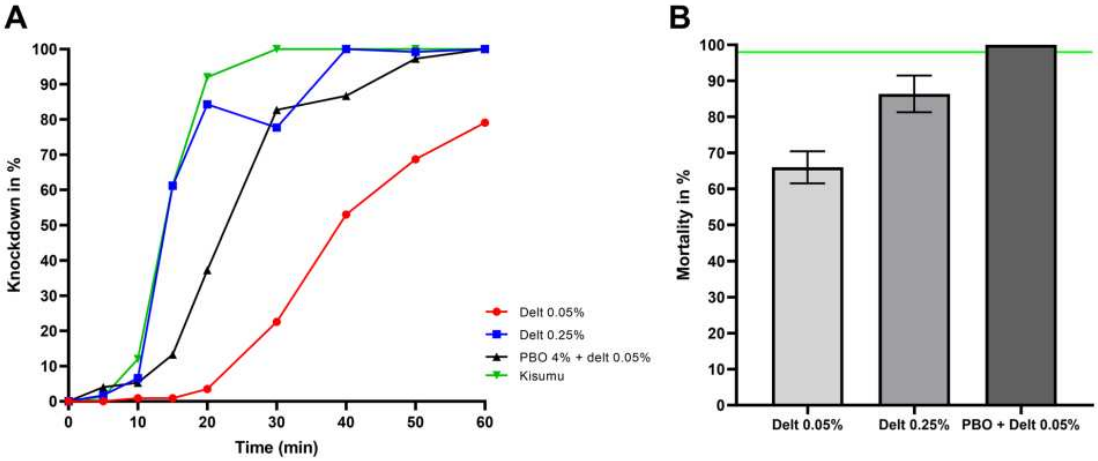

Fig. 3 Susceptibility profile of *A. gambiae s.s.* to deltamethrin only and after preexposure to PBO. **A** Proportion of mosquitoes knocked down after exposure to various concentrations. **B** Mean mortality after exposure to various concentrations. The horizontal green line represents the 98% WHO threshold for susceptibility. Error bars represent standard error of mean mortality after the 24h recovery period following insecticide exposure. Proportion of mosquitoes knocked down after exposure to various concentrations of deltamethrin

bendiocarb 0.1% with a mortality of 62.7% (47/75) after the 24-hour recovery period (Fig. 4).

Resistance intensity

Resistance intensity was only assessed for permethrin and deltamethrin. The resistance intensity to permethrin was high with mortalities below 98% at both 5×(47.7%, 53/111) and 10×(88%) the diagnostic concentrations (Fig. 2B). Although we observed an increased number of mosquitoes knocked down after one hour (51 out of 113), the resulting KDT_{50} and KDT_{95} at 5X the diagnostic concentration was above 60 min. Meanwhile, the KDT_{50} at 10X the diagnostic

Table 1 Knockdown times and mortality of A. gambiae s.s. from Lambaréné after insecticide susceptibility tests

Insecticides tested	*N*	KDT_{50}(min) [CI_{95}]	$Rtkd_{50}$ [CI_{95}]	KDT_{95} (min) [CI_{95}]	$Rtkd_{95}$ [CI_{95}]	Mortality (%)
Per 0.75%	88	No kd	NA	No Kd	NA	11.4
PBO + Per 0.75%	50	No Kd	NA	No Kd	NA	44
Per 3.75%	113	63.6 [57.4–73.2]	4.7	187.1 [143.4–278.6]	7.7	47.8
Per 7.5%	50	24.1 [20.7–27.2]	1.8	64.3 [54.5–81.8]	2.6	88
Del 0.05%	115	40.9 [38.9–43.1]	2.9	81.4 [73.5–93.0]	3.8	66.1
Del 0.25%	121	15.3 [9.6–19.7]	1.1	33.9 [25.3–76.4]	1.6	84.3
PBO + Del 0.05	75	23.2 [21.4–24.9]	1.7	44.2 [40.2–49.9]	2.1	100
Alpha 0.05	24	65.5 [55.2–97.2]	4.8	149.7 [99.7–463.1]	6.2	45.8

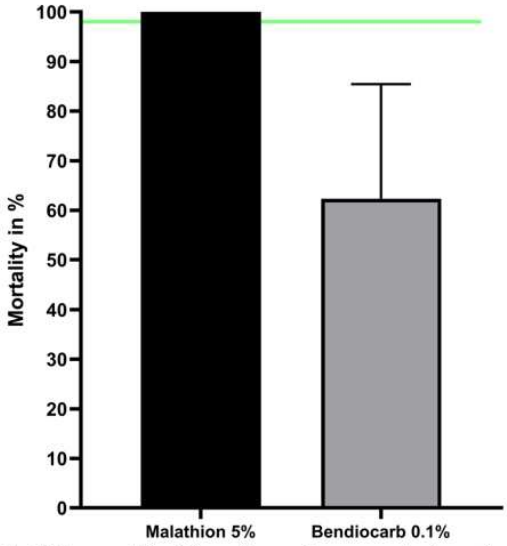

Fig. 4 Mean mortality of *A. gambiae* s.s. after exposure to diagnostic concentrations of malathion and bendiocarb. The horizontal green line represents the 98% WHO threshold for susceptibility. Error bars represent standard error of mean mortality after the 24h recovery period following insecticide exposure

concentration was 24.1 min, a 1.8-fold increase in comparison to the Kisumu strain. The KDT_{95} for the mosquitoes from Lambaréné was above one hour (Fig. 2A).

For deltamethrin tests were conducted with 5X the diagnostic concentration only because the number of available larvae was low. Mosquitoes displayed a moderate resistance intensity to deltamethrin with a mortality of 84.3% at 5X the diagnostic concentration (Fig. 3A). Both KDT_{50} (15.3 min) and KDT_{95} (33.9 min) with 0.25% deltamethrin were below 1 h with a 1.6-fold increase for the latter compared to the susceptible Kisumu strain (Table 1).

Synergist assays

Synergist assays were performed with both permethrin and deltamethrin. The mortality in the PBO only group was 4% (2/50). Preexposure to PBO led to a total recovery of the susceptibility of *A. gambiae s.s.* to deltamethrin (66.1% mortality with 0.05% deltamethrin vs. 100% mortality with PBO + 0.05% deltamethrin; Fisher's exact test $P < 0.0001$) (Fig. 3B). The recovery of full susceptibility following preexposure to deltamethrin was accompanied by a decrease in KDT_{50} (40.9 min for deltamethrin 0.05% vs. 23.2 min with PBO + deltamethrin 0.05%) and KDT_{95} (>1 h for deltamethrin 0.05% vs. 44.2 min with PBO + deltamethrin 0.05%) (Table 1). PBO and permethrin led to a 4-fold increase in mortality compared to permethrin alone (11% with 0.75% permethrin vs. 44% with PBO + permethrin, Fisher's exact test, $P < 0.0001$) (Fig. 2B). Interestingly, the knockdown capacity of the insecticide was not improved: only one (1) mosquito knocked down after exposure to this insecticide.

Discussion

Insecticide resistance has spread all over sSA and there is a need to monitor the pattern of its spread to ensure the implementation of suitable insecticide resistance management strategies.

From the current study, we found that *A. gambiae s.s* from Lambaréné were more resistant to permethrin than deltamethrin, with a loss of knockdown effect of permethrin and fold increases in knock down times for deltamethrin as similarly reported from previous studies in Gabon [8, 9]. However, mosquitoes were less resistant to deltamethrin when comparing the current results with previous ones from Lambaréné [9] which could be due to a reduction in the selection pressure resulting from the absence of large-scale deployment of vector control measures suspected to select for resistance in *Anopheles* mosquitoes [33, 34]. In addition, as Lambaréné is surrounded by two extensive agricultural schemes where pesticides are not used, suggest that the use of pesticides

by gardeners and the use of insecticides in households could be the main drivers of pyrethroid resistance. Mosquitoes were also resistant to alphacypermethrin, as also reported in other sSA countries [35, 36], and we report the first evidence of resistance to this insecticide in Gabon. The full susceptibility observed with malathion suggests that it could be used with pyrethroids in combination strategy. This strategy is based on the assumption that mosquitoes that will not be killed by one, will be killed by the other insecticide [37, 38]. However, the resistance observed to bendiocarb is worrying as it limits the pool of insecticides available for resistance management in Gabon.

Preexposure to PBO lead to fold increases in mortality of *A. gambiae s.s.*, as shown elsewhere [23–25], to both permethrin and deltamethrin with a full restoring of susceptibility to the latter. However, preexposure to PBO did not lead to the restoration of the knockdown effect of permethrin whilst a decrease in knockdown times was observed with deltamethrin. The knockdown effect in addition to the excito-repellency of pyrethroids are key features that make them suitable for bed nets impregnation, as they allow the nets to remain efficient even when they are torn [39]. The fact that preexposure to PBO increased the mortality of *A. gambiae s.s.* populations suggests that metabolic resistance, with an overexpression of P450 monooxygenases enzymes, is primarily responsible for the insecticide resistance phenotype [19, 40]. However, this does not exclude the involvement of other metabolic enzymes such as esterases and glutathione S-transferases. These results suggest that PBO LLINs may be a better option for mass distribution in Lambaréné and presumably in Gabon as those nets have been shown to provide superior protection in areas with pyrethroid resistant mosquitoes [41, 42].

There was a high resistance intensity to permethrin in *A. gambiae s.s.* and at least a moderate resistance intensity to deltamethrin contrary to results obtained in Mouila where a low resistance intensity to pyrethroids was recorded [8]. The difference with the aforementioned study points to the need for susceptibility testing in different regions of the country to have an overview of the resistance profile of malaria vectors in Gabon. However, the 5-year time gap between the two studies carried out respectively in 2017 and 2022 does not exclude an escalation of the resistance intensity in the meantime. According to the WHO criteria [16] our results point to the potential risk of operational failures of vector control measures based on the use of these two insecticides in Lambaréné, especially for permethrin. Current recommendations suggest that remedial action must be implemented in such cases with the use of synergists as a potential mitigation measure.

All the mosquitoes were carrying the knockdown resistance *Vgsc- 1014 F* and *−1014 S* alleles which is already fixed in the local *A. gambiae* population as previously reported in Gabon [11–15]. However, we found a lower proportion of the *Vgsc-1014 S* alleles compared to previous reports which could be due to a fitness cost associated with carrying this allele and to the fact that it may offer a lower protection against pyrethroids compared to the *Vgsc-1014 F* [43]. Despite the resistance to bendiocarb, no mosquitoes were found carrying the G119S *Ace-1* resistance allele in accordance with previous publications in Gabon where it was reported either absent [14, 15] or present at a low level [12] which points to the involvement of metabolic resistance to bendiocarb. Similar results were reported in Chad where despite high resistance to bendiocarb no G119S *Ace-1* mutation was found [44] contrary to results from Cameroon where resistance to this insecticide was strongly correlated to the presence of G119S Ace-1 mutation [45]. The main limitations of this study are the small sample size used for the tests with alphacypermethrin as well as the fact that we did not specifically determine the enzymes involved in metabolic resistance using transcriptional analyses.

Conclusion

A. gambiae s.s. populations from Lambaréné were resistant to permethrin, deltamethrin and alphacypermethrin. Here, we showed that mosquitoes were highly resistant to permethrin and at least moderately resistant to deltamethrin. This high level of resistance intensity especially for permethrin constitutes a serious threat for the mass distribution of LLINs. From our results, the combination of both PBO and deltamethrin should be considered for LLINs distribution in Lambaréné and the surrounding areas by the National Malaria Control Programme. The full susceptibility to malathion qualifies it as a welcome addition to the toolbox for the management of insecticide resistance in Lambaréné. It will be interesting to test its susceptibility pattern systematically in Gabon.

Abbreviations

Ace	acetylcholinesterase
CERMEL	Centre de Recherches Médicales de Lambaréné
kdr	Knockdown resistance gene
KdT	Knockdown time
LLINs	Long Lasting Insecticidal Nets
PBO	piperonyl butoxide
sSA	sub–Saharan Africa
Vgsc	Voltage-gated Sodium Channel

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
AAA, SB, BM, PGK, CSW, FN and STBS conceived and planned the study and its design. STBS, DY, AGDN, BN and RB performed the field activities and the susceptibility testing. STBS, DY, LNB, MFA, AGDN and BN carried out the laboratory analysis of the samples. STBS analysed the data and drafted the manuscript. DNN, RA, JDMN commented on and approved the manuscript. AAA, SB, BM, PGK, CSW, FN, FNN, RA, DY and AL critically reviewed the manuscript. All authors made intellectual input to the study. All authors read and approved the final manuscript. STBS, AGDN, BN and AAA are members of CANTAM (EDCTP-CSA 2020 NOE-3100) networks.

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Data availability
Raw data are archived and available on request from the corresponding author.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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Publisher's Note

Supplementary information

Table 1: Knockdown times of *An. gambiae s.s.* (Kisumu)

Insecticides tested	N	KDT_{50}(min) [CI_{95}]	KDT_{95} (min) [CI_{95}]	Status
Per 0.75%	51	13.7 [12.6 – 14.8]	24.3 [21.7 – 28.6]	Susceptible
Del 0.05%	75	13.9 [13.1 – 14.7]	21.3 [19.7 - 23.9]	Susceptible
Alpha 0.05%	51	13.7 [12.6 - 14.8]	24.3 [21.7 - 28.6]	Susceptible

3 Discussion

3.1 Assessment of malaria transmission intensity and insecticide resistance mechanisms in three rural areas of the Moyen Ogooué Province of Gabon

The mass distribution of LLINs has been shown to have a great impact on malaria transmission. The maintenance of the benefits obtained following mass distribution is of paramount importance to every control program in order to significantly and durably curb the transmission of malaria. The assessment of the effect of the distribution of LLINs entails that baseline information on the transmission and the susceptibility of malaria vectors be available in order to monitor their impact. Gabon has never carried out a mass distribution of LLINs and information on the transmission of malaria are scarce and outdated especially in the Moyen Ogooué Province. Data on the distribution of insecticide resistance genes in this area are inexistent. Thus, I aimed to fill this gap by identifying the vector species present, assessing the entomological inoculation rate (EIR) as a measure of malaria transmission, as well as the distribution of insecticide resistance genes in *Anopheles* mosquitoes in three rural areas of Moyen Ogooué Province [112].

Three groups of Anopheles species were identified morphologically namely *An. gambiae s.l.*, *An. moucheti* and *An. coustani* as further confirmed by sequencing of the ITS2 genes. *An. gambiae s.s.* and *An. coluzzii* were the two members of the *An. gambiae* complex with *An. gambiae s.s.* the predominant vector species making up to 98% of the mosquitoes identified by PCR in Bindo, Nombakélé and Zilé. These results are in accordance with previous ones from Gabon which also reported that *An. gambiae s.s.* was the dominant vector [41, 44, 113]. I also found one *An. gambiae s.s./An. coluzzii* hybrid, these two species recently raised to species level [49], were formerly known as *An. gambiae* M and S forms [114]. Hybrids of these two species are usually found at frequencies less than 1% [115]. *An. gambiae s.s*. is one of the major vectors in Africa and is a highly anthropophilic species. Despite the low proportion of *An. coluzzii,* the species composition of the *Anopheles gambiae* complex should be monitored as an increase in the proportion of *An. coluzzii* may have epidemiological consequences such as year round malaria transmission [116]. *An. coluzzii* prefers permanent breeding sites ensuing from anthropogenic activities [49].

The highest human biting rate was recorded in Zilé followed by Bindo and Nombakélé with these high HBRs expected from collections in rural areas. Previous reports have shown that

HBRs in rural areas tend to be higher compared to urban areas due to the availability of breeding sites. Zilé was home to permanent breeding sites from water leaks from taps that harboured *Anopheles* larvae which could explain the high HBR in this area. These high HBRs highlight the high human/vector contact in the area and therefore the high risk of malaria infections to people living in the study areas. The fact that Gabon has not yet embarked on a mass distribution campaign of LLINs, contrary to other countries [117–121], could also explain those high HBRs. These results point to the necessity of implementing comprehensive vector control measure in order to reduce the biting rates in those areas thus the risk of malaria infections.

There was a seasonal variation in the density of *Anopheles* species with significantly more mosquitoes collected during the rainy season than the dry season as more breeding sites are available during the former season. Densities of species like *An. gambiae s.s.* are especially affected by seasonal variations as they tend to breed in temporally variable and rain-dependent breeding sites [49]. My results clearly are in line with observations elsewhere in Gabon [41, 44] and in Africa [122–124].

An. gambiae s.l. were exophagic as a higher number of mosquitoes were collected biting outdoor than indoor in the three study areas as previously reported in Libreville [41]. This exophagic behaviour may have a negative impact on the efficacy of vector control measures such as LLINs and IRS, which are targeting indoor biting mosquitoes [125]. However, the fact that the peak biting times in the three areas were recorded during the second half of the night which corresponds to the time at which most people are indoor and asleep could still warrant the efficacy of LLINs. This late night/early biting behaviour is a common trait for *An. gambiae* that makes them suitable vectors of malaria [34, 126] but also suitable targets for LLINs which primary purpose is to act as physical barrier preventing human-vector contact. Notwithstanding, an increasing number of studies are showing a change in mosquito biting behaviour from indoor to outdoor biting following the deployment of vector control interventions [58]. Nevertheless in the absence of large scale deployment of vector control interventions in our study areas, this exophagic behaviour may be due to adaptations of the *Anopheles* populations to the sleeping behaviour of the population in combination with resistance to insecticides, which potentially exacerbate outdoor biting [127]. In addition, this exophagic behaviour may also result from an opportunistic biting behaviour as mosquitoes will tend to bite the first person they encounter which will be the collectors placed outdoor during human landing catches [122].

Anopheles gambiae s.s. was the only species infected with *Plasmodium* sp. with the most prevalent species being *P. falciparum.* Approximately 1/3 of the mosquitoes were infected with non-falciparum species. The second most prevalent species was *P. malariae* followed by *P. ovale curtisi* and *P. ovale wallikeri*, respectively. This species distribution was similar to that reported in humans from neighbouring rural areas [20], although contrary to this report, I did not find mosquitoes infected with more than one *Plasmodium* species. Indeed, high prevalence of coinfections was found in humans from neighbouring areas [20] which suggests that people living in this area are sequentially bitten by mosquitoes infected with different plasmodial species. Thus, mosquitoes from our study areas may be infected with only one *Plasmodium* species even after feeding on coinfected individuals. Our report is the first to investigate the distribution of *Plasmodium* species infecting mosquitoes as all the previous reports in Gabon were based on *P. falciparum* circumsporozoite protein determined by enzyme-linked immunosorbent assay [40, 41, 44, 128, 129]. The overall sporozoite rate was 2.3% with the highest sporozoite rate (13.8%) recorded in Zilé during the dry season. A similar occurrence was also reported in Thailand and was attributed to higher survival rates of *Anopheles* during the dry season [130].

The intensity of transmission was assessed by determining the EIR in each study area. The transmission of malaria was heterogenous in the three study areas with perennial transmission in Zilé and seasonal in Bindo and Nombakélé. The rainy period from October to December made a significant contribution to the overall malaria transmission burden in our study areas, as the highest EIRs in the three study areas were recorded during this period. The high annual average EIR recorded in Zilé could be associated with a high infection rate in humans. Beier et al. [131] showed that EIRs ≥ 200 are regularly associated with a > 80% prevalence of *P. falciparum* in humans and thus to a high burden of malaria in a community. Despite the lower average annual EIRs recorded in Bindo (80.2 ib/p/y) and Nombakélé (17 ib/p/y), these EIRs have been related to a *P. falciparum* prevalence in humans of at least 50% [131]. The high EIR recorded in Zilé, in addition to the availability of breeding sites, could also be due to the high prevalence of helminth infections such as *Schistosoma haematobium* and *Trichuris trichiura* in populations living in this area compared to those in Bindo and Nombakélé [132]. These helminths, particularly *S. haematobium*, have been associated with more *P. falciparum* infections in humans and higher transmission intensity either independently [133–135] or in combination with other helminths like *Trichuris trichiura* or hookworm [132].

The high genotypic and allelic frequencies of knockdown resistance mutations found in *An.*

gambiae populations in our study areas are similar to previous reports in Gabon, pointing to a high level of resistance to pyrethroids and DDT [41, 44, 99]. This resistance could be driven by the use of pesticides in agriculture [136, 137] as Gabon has not yet embarked on a mass distribution of LLINs, also known to select for resistance [118, 119]. However, the absence of the resistant allele to carbamates and organophosphates suggests that the mosquitoes may still be susceptible to insecticides from these classes. These results indicate that these insecticides could be used in a combination strategy to manage insecticide resistance in these areas whereby in addition to the distribution of LLINs, net wall hangings could be sprayed with carbamates and organophosphates. This strategy has shown its efficacy in experimental hut trials in Burkina Faso [138] and Tanzania [139].

This study has some limitations such as the low number of collectors used which could have not allowed me to have a better geographical coverage of the collection sites thus underestimating the number and the diversity of *Anopheles* mosquitoes present. Another limitation is the lack of insecticide bioassays to corroborate the genetic observations which are clearly suggesting high levels of resistance in *An. gambiae s.s.* populations from the study areas.

The results from this chapter point to the high level and the heterogeneity of malaria transmission in addition to the high frequency of insecticide resistance genes in the rural areas of the Moyen Ogooué province. These results should raise awareness on the potential adverse impacts on the local populations and necessitate the deployment of control measures to reduce the transmission of malaria in such areas. It also confirms the major contribution of *P. falciparum* while pointing also to the substantial contribution of non-falciparum species in the overall burden of malaria.

My results also raise more questions that could be answered by futures research projects such as:

- follow up studies in urban areas of the province to have a better overall picture of malaria transmission in the province as the current malaria transmission assessment I performed was limited to areas from where we recruited participants for a study that aimed to assess the effect of *S. haematobium* on the transmission of *P. falciparum.*
- an assessment of the multiplicity of infections to study the parasites genetic diversity in mosquitoes in comparison to humans
- experimental infections studies with field collected mosquitoes to test for the hypothesis that mosquitoes are only able to sustain a single plasmodial species infection

at a time even after feeding on individuals that are coinfected.

- assays to determine the phenotypic resistance of *An. gambiae s.s.* to insecticides used in malaria vector control programmes.

3.2 Experimental transmission of *Plasmodium malariae* to *Anopheles gambiae*

Malaria is caused by 5 *Plasmodium* species namely *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi.* However, the largest malaria burden is due to *P. falciparum* which is also the deadliest species. This has led to a negligence of the other plasmodial species which are less studied especially *P. malariae and P. ovale*. I aimed therefore in this chapter to establish the experimental infection of *P. malariae* to *An. gambiae s.s.* (Kisumu strain) [140].

Recent reports have demonstrated that about one third of the *Plasmodium* infections in both humans [20] and *Anopheles* mosquitoes [112] were due to other species than *P. falciparum*. Likewise, many studies have demonstrated prevalences of *P. malariae* ranging between 0 - 32% [15] while for *P. ovale* prevalence of less than 1% are reported. These results show that plasmodial species other than *P. falciparum* may be responsible for a non-negligible part in the overall burden of malaria especially with mounting reports of possible resistance to ACTs of *P. malariae* [141–143]. Although a handful of clinical trials are reported for non-falciparum species [144, 145], no studies have been carried out on the transmission blocking capacity of drugs on *P. malariae* and *P. ovale*. This is partly due to the lack of a continuous *in vitro* culture protocol for *P. malariae*. In addition, most previous experimental infections were carried out using monkeys infected with the Uganda I/CDC strain [146–148]. I successfully infected *An. gambiae s.s.* with field isolates of *P. malariae* in mono or co-infection with *P. falciparum.* Higher infection rates were achieved after serum replacement compared to whole blood using both *P. malariae* mono-infected ($P = 0.004$) and co-infected ($P = 0.001$) isolates. Similarly, higher infection intensities were recorded in mosquitoes fed after serum replacement compared to those fed with whole blood ($P < 0.001$). The higher infection rates and intensities obtained after serum replacement could be related to the presence of transmission blocking antibodies in the whole blood which are absent in the serum replacement [149] as the serum of the participants were replaced with AB serum from nonimmune donors.

These infections rates are 5.5-fold higher than those reported after direct skin feeding assay on a volunteer inoculated with a *P. malariae* isolate [150] and demonstrate the effectiveness of

our protocol at infecting mosquitoes. The differences between the aforementioned study and our may result from the fact that I used the Kisumu strain which is *An. gambiae s.s.* that is known to be the major malaria vector in Gabon [41, 44, 112, 151] together with local *P. malariae* isolates. Meanwhile in the study of Woodford *et al*. [150], they used a *P. malariae* isolate collected from a traveller returning from Guinea and *An. stephensi. An. stephensi* is a species that until recently was geographically restricted to the Indian subcontinent and the Persian Gulf [152] but with recent sightings in Africa specifically in Djibouti [153], Ethiopia [154], Sudan [155], Somalia [156], Eritrea [157], Kenya [158], Nigeria [157] and Ghana [157]. The importance of the geographical origin of both the *Anopheles* and *Plasmodium* species in experimental infection was demonstrated by Molina-Cruz *et al*. who achieved higher infections rates with *Anopheles* and *P. falciparum* from the similar geographic areas [159].

The low to moderate infection rates and intensities I obtained may be related to the low gametocyte densities of the isolates used. Notwithstanding, the infections rates obtained here are similar to previous reports of experimental infections done with *An. gambiae* using *P. falciparum* and *P. vivax* isolates collected from asymptomatic individuals in Ethiopia [160]. Since this set of experiments, I have been able to achieve higher infections rate and intensity (unpublished data) by introducing some changes to the protocol, e.g. reducing the number of washing steps from two to one. This change reduces the handling of the samples and shortens the time between blood draw and mosquito feeding. In addition, I also reinforced the sanitary measures to prevent and get rid of microsporidia infections in the *An. gambiae s.s.* colony.

The main limitation of this work is the low number of participants included in the DMFAs primarily due to the low prevalence of *P. malariae* infections and the failure to accurately diagnose this species using microscopy [16]. The difficult microscopic diagnosis of *P. malariae* is an important issue that leads to the underestimation of the contribution of this species in the burden of malaria. The ease of detection of *P. falciparum* and its regular control through treatment with ACTs combinations, may lead to the increase in cases due to other species. These species are often missed by microscopy and rapid diagnostics tests potentially undermining malaria control efforts.

In this chapter, I was able to establish reproducible and robust experimental infections of *An. gambiae s.s.* with local *P. malariae* isolates using a water-jacketed glass feeder system. I have been able to further extend this experimental infection setup to *P. ovale* in addition to the already established experimental infections with *P. falciparum* [161]. The establishment of this experimental infection platform for all three *Plasmodium* species endemic in Gabon, namely

P. falciparum, P. malariae and *P. ovale*, opens avenues for further research on the following:

- Identification of the molecular mechanisms involved in the susceptibility and refractoriness of *Anopheles* spp. to *P. malariae* and *P. ovale* infections
- Testing the efficacy of transmission blocking drugs and vaccines for species such as *P. malariae* and *P. ovale* through DMFAs
- Identification of the molecular mechanisms involved in the invasion of hepatocytes by *P. malariae* and *P. ovale* sporozoites
- The use of this system for by-bite controlled human malaria infection (CHMI) to speed up drug/vaccine testing and the identification of immune responses to species such as *P. malariae* and *P. ovale.*

3.3 *Anopheles gambiae s.s.* resistance to pyrethroids and DDT in semi-urban and rural areas of the Moyen-Ogooué Province, Gabon

Vector control of malaria through LLINs and IRS has been identified as the largest contributors in the aversion of over 600 million clinical cases between 2000 and 2015 [2]. However, the success of this control measure entails that baseline information on mosquito bionomics especially on their susceptibility to insecticides are available to inform the choice of the appropriate tools. In Gabon, data on the phenotypic susceptibility of malaria vectors are sparse and totally absent in the Moyen Ogooué Province. Therefore in this chapter I sought to assess the susceptibility to permethrin, deltamethrin and DDT and to determine the frequency of insecticide resistance genes of malaria vectors from two different settings of the Province of Moyen-Ogooué [162].

I first assessed the knockdown capacity of these three insecticides on local *Anopheles.* The knockdown capacity is an effect that makes pyrethroids suitable for LLINs impregnation as they allow the nets to remain effective even when they are torn [63]. From our results, permethrin and DDT have lost their knockdown efficiency as the time required to knock down 50% (KDT_{50}) and 95% (KDT_{95}) of the mosquitoes exposed to these insecticides were above 60 min when compared to the Kisumu susceptible strain. Meanwhile, deltamethrin knocked down mosquitoes but the KDT_{50} and KDT_{95} were 3-fold and 2.6-fold higher when compared to the Kisumu strain, respectively. These results are a clear indication that mosquitoes are resistant to pyrethroids and DDT. This is in accordance with data from susceptibility tests in Mouila [113] in Gabon and elsewhere in Africa [5, 93, 163]. The loss of knockdown effects of deltamethrin, permethrin, and DDT could be explained by the fixation in local *An. gambiae s.s.*

populations of knockdown resistance (*kdr*) gene mutations, with all mosquitoes carrying either the *Vgsc-L1014F* or *Vgsc-L1014S* mutations. This fixation of the *kdr* mutations aligns with previous reports [41, 44, 100, 101] from other areas of Gabon, particularly in Zilé [112], where a high proportion of *An. gambiae* were found to carry *kdr* mutations. This loss of knockdown in addition to the fixation of the *kdr* mutations are a threat to the efficacy of LLINs as they are still the main insecticides used in impregnating nets.

The mortality rates observed for each of the tested insecticides correlate with the knockdown results, indicating that *An. gambiae s.s.* from the region exhibit a high level of resistance to both pyrethroids and DDT. The *An. gambiae s.s.* populations showed significant resistance to DDT and permethrin, a type I pyrethroid, with mortalities of less than 10% for both insecticides, which is well below the 98% threshold that defines susceptibility. Resistance was less pronounced for deltamethrin with 51% and 76% in Lambaréné and Zilé, respectively. These results were similar to those in Ngounié Province where mosquitoes populations were more susceptible to deltamethrin than to permethrin [164]. These results suggest that deltamethrin may be the best choice for the impregnation of LLINs to be distributed in the study areas. However, the combined loss of knockdown and low mortality of *An. gambiae s.s.* to pyrethroids is of serious concern as it heralds the potential failure of pyrethroids vector control especially when the nets are torn. This result calls for the implementation of mitigation strategies such as the use of LLINs impregnated with compounds such as piperonyl butoxide (PBO) or with insecticides from other classes such as chlorfenapyr. PBO inhibits cytochrome P450 enzymes which are involved in metabolic resistance. An additional pre-emptive measure is the regular replacement of old and torn nets as despite the resistance to pyrethroids, the physical barrier provided by bednets will still prevent human vector contacts.

The use of pesticides in agriculture has been identified as one of the major drivers of insecticide resistance in *Anopheles spp*. Although the collection in Zilé were done in an area of intensive agriculture of rubber trees, I gathered from the workers that no pesticides were used on the rubber trees. Therefore, this high resistance in the absence of the use of pesticides in agriculture and of significant malaria vector control interventions known to select for resistance [118, 119, 165, 166], could be partly due to the personal use of pyrethroids based vector control tools in the form of insecticide sprays, mosquito coil, impregnated nets which could ultimately drive-up resistance to insecticides from this class. The fact that I did not assess metabolic resistance is the main limitation of this study as the results from this study clearly points to the involvement of other resistance mechanisms.

The results from this chapter portray a dire situation regarding the resistance of malaria vectors to permethrin and DDT in Gabon. However, mosquitoes are less resistant to deltamethrin making this insecticide the best option for malaria vector control in our study areas.
My results from this chapter call for additional research studies on:

- The investigation of the resistance of malaria vectors to insecticides from other areas of Gabon to have an overview of the resistance profile nationwide especially against compounds used in the latest generation of LLINs such as alphacypermethrin and chlorfenapyr to allow for an evidence-based selection of control measures by the National Malaria Control Programme
- The role of metabolic resistance mechanism in the resistance phenotype of *An. gambiae s.s.* in the study areas by performing tests with PBO
- The identification of drivers of insecticide resistance in Gabon

3.4 Resistance of *Anopheles gambiae s.s.* against commonly used insecticides and implication of cytochrome P450 monooxygenase in resistance to pyrethroids in Lambaréné (Gabon)

Insecticide resistance has been incriminated as one of the main reasons for the rebound and stagnation of the decrease in the number of cases and of deaths due to malaria. Recent reports from Gabon have shown that mosquitoes are resistant to pyrethroids. However, the investigation of the mechanisms driving this resistance in the country has been limited to screening the mosquitoes for target site resistance genes and none has explored the role of metabolic resistance. Therefore, I aimed to evaluate the susceptibility profile of mosquitoes to pyrethroids, organophosphates and carbamates, to assess the level of resistance and the effect of piperonyl butoxide (PBO), a synergistic compound used to revert pyrethroid resistance.

An. gambiae s.s. were resistant to both permethrin and deltamethrin accompanied by a total loss of knockdown effect of permethrin on mosquitoes and at least a 3-fold increase in knockdown times for deltamethrin as previously reported [162, 164]. However, mosquitoes were less resistant to deltamethrin than permethrin confirming our previous report [162] that the former insecticide may be the better choice for LLINs impregnation. Mosquitoes were also resistant to alphacypermethrin as also reported in Malawi [167] and Mali [168]. Alphacypermethrin is an insecticide used in third generation LLINs in combination with chlorfenapyr. This resistance against pyrethroids in the absence of large-scale distribution of malaria vector controls point to the role of pesticides used in agriculture especially by gardeners

as drivers of resistance. In addition, household use of insecticides could also contribute to this resistance. The observed resistance to bendiocarb is concerning, as it reduces the number of insecticides available for managing resistance in Gabon.

Notwithstanding, the full susceptibility of malaria vectors to malathion is a positive finding as it could be used in combination strategy for resistance management. This strategy consists of deploying two insecticides on the assumption that mosquitoes that are resistant to one insecticide will be killed by the one they are susceptible to [138]. In a study carried out in the Vallée de Kou in Burkina Faso, the combined use of organophosphate wall linings and LLINs was shown to possibly provide significant epidemiological benefits against a vector population resistant to pyrethroids but susceptible to organophosphates [138]. However, this strategy will be less useful in an area with multiple resistance as combination of organophosphate-treated wall linings and long-lasting insecticidal nets failed to provide additional control over long-lasting insecticidal nets alone against multiple insecticide-resistant *An. gambiae* in Côte d'Ivoire [107]. These results further emphasize the need for the determination of the local resistance profile prior to the deployment of LLINs and the introduction of resistance management strategies.

Resistance intensity of *An. gambiae s.s.* to permethrin was high with mortality at 10 times the diagnostic concentration still below the susceptibility threshold. Meanwhile, resistance to deltamethrin was at least moderate as there were not enough mosquitoes to test at 10 times the diagnostic concentration. Our results are in contradiction to those from Mouila [164] where low resistance intensity to pyrethroids was found. These divergent results from two cities from neighbouring regions of the country highlight the importance of conducting susceptibility tests across various regions to have a comprehensive overview of the resistance profile of malaria vectors in Gabon. However, the fact that the susceptibility testing in Mouila and Lambaréné were done five years apart (2017 and 2022, respectively) does not preclude an exacerbation of pyrethroid resistance in the meantime in Lambaréné and by extension in Gabon. Similar observations were reported in Uganda [7] and Burkina Faso [169] with increased resistance intensity leading to a loss of bednets efficacy in both cases. The results from our study strongly suggest that vector control based on the use of these insecticides especially permethrin may lead to control failure. The WHO recommends the implementation of remedial action in order to mitigate the impact of insecticide resistance on vector control programmes.

PBO is a synergist that acts by blocking the activation of cytochrome P450 enzymes that are involved in the detoxification of mosquitoes. In our study, preexposure to PBO led to significant increases in mortality of *An. gambiae s.s.* as previously reported in Côte d'Ivoire [91], Burkina Faso [92] and Nigeria [93]. I observed a full restoring of the susceptibility of *An. gambiae s.s.* to deltamethrin with all the mosquitoes killed after the 24h recovery period. This full susceptibility to the combination PBO-deltamethrin was also accompanied by a reduction in the knockdown time. These results suggest that the combination PBO-deltamethrin will be a more suitable choice for LLINs impregnation and distribution in our study areas. Although, there was a four-fold increase in mortality with permethrin (11% to 44%), the mortality induced by exposure to the combination PBO-permethrin was well below the susceptibility threshold. In addition, there was no restoration of the knockdown effect of permethrin after preexposure to PBO. The increase in mortality observed with preexposure to PBO implies that Cytochrome P450 monooxygenase enzymes accounts for most of the expression of the resistant phenotype to deltamethrin while they partially account for resistance to permethrin. Indeed, Cytochrome P450 monooxygenases catalyse the oxidation or reduction of compounds such as insecticides into less harmful compounds by converting non-polar xenobiotics into more polar and excretable forms [170, 171]. Cytochrome P450 enzymes including *CYP6P1, CYP9K1, CYP6P3, CYP6M2, CYP4H17, CYP6Z1* and *CYP6Z2* have been associated with insecticide resistance in *An. gambiae* [172–176]. Likewise, *CYP6P9a* and *CYP6P9b* play a significant role in resistance in *An. funestus* [6, 177–179]. However, the results with permethrin point to the involvement of other metabolic enzymes such as esterases and glutathione S-transferases in the resistance phenotype as previously reported in *An. gambiae* populations from multiple locations in sub-Saharan Africa [180].

As previously reported in Gabon [41, 44, 101, 112, 162], all the *An. gambiae s.s.* mosquitoes were either homozygous for *Vgsc-L1014F* or heterozygous for *Vgsc-L1014F* and *Vgsc-L1014S.* The current results are in line with previous studies which have demonstrated that these knockdown resistance genes are already fixed in the local *An. gambiae s.s.* across the country. Nevertheless, the lower frequency of *Vgsc-1014S* alleles observed in our study, compared to earlier reports, may be due to a fitness cost linked to this allele and its reduced ability to confer protection against pyrethroids compared to the *Vgsc-1014F* allele [181]. Although *An. gambiae s.s.* mosquitoes were resistant to bendiocarb, none of the mosquitoes was carrying the G119S *Ace-1* resistance allele that confers resistance to insecticides of the

organophosphate and carbamate classes. These results are contrary to other reports where resistance to organophosphates and carbamates were associated with the presence of the G119S *Ace-1* allele in *Anopheles* populations in Cameroon [105, 106, 182] and Côte d'Ivoire [183, 184]. Therefore, our results suggest that metabolic resistance mechanisms are driving resistance to bendiocarb in our study area.

The major limitations from this study are firstly the low number of mosquitoes that were used for the susceptibility tests with alphacypermethrin. Secondly, the major detoxification enzymes overexpressed in the pyrethroid- and carbamate-resistant *An. gambiae s.s.* populations were not identified by e.g. genome-wide transcriptional analyses. Thirdly, I did not perform synergist tests with 0.25% S,S,S-tributyl phosphorothioate (DEF) and 8% diethyl maleate (DEM) which are an inhibitors of esterases and glutathione S-transferase, respectively [185].

The results from this chapter confirm that *An. gambiae s.s.* are resistant to pyrethroid as reported in the previous chapter and allowed us to determine that metabolic resistance mediated by cytochrome P450 enzymes are crucial for the resistance phenotype observed. The use of permethrin to which mosquitoes are highly resistant will potentially lead to operational failures especially if the nets are not timely replaced when torn. However, despite moderate resistance to deltamethrin, the fact that preexposure to PBO leads to a full regain of susceptibility makes the combination PBO-deltamethrin the most effective choice for LLINs distribution in the study area. The full susceptibility to malathion offers the possibility of including it in a resistance management strategy. The divergent results observed between our results and those form neighbouring areas call for regular and countrywide susceptibility testing to have a clear picture of the resistance profile of mosquitoes.

My results from this chapter call for more studies on:

- The identification of specific genes that are overexpressed in resistant populations and the validation of their role in RNA interference experiments
- The susceptibility of *An. gambiae s.s.* to other classes of insecticides in other areas of Gabon
- The assessment of other resistance mechanisms such as cuticular resistance.

4 Summary

Malaria remains a major health concern in sub-Saharan Africa. Recent decline in malaria cases and deaths was largely driven by vector control through Long Lasting Insecticidal Nets (LLINs) distribution. However, since 2016, this decline has stalled, coinciding with growing reports of resistance to pyrethroids—the only class of insecticides used in LLINs. In Gabon, there is limited LLIN deployment and data on transmission are scarce especially in the Moyen Ogooué Province. In addition, the contribution of non-falciparum species to the overall burden of malaria is often overlooked. There are also knowledge gaps on the vector competency of *Anopheles* mosquitoes to *Plasmodium* species such as *P. malariae,* the second most common plasmodial species in Gabon. Therefore, there is a need to address gaps in entomological data on malaria transmission in the Moyen Ogooué Province of Gabon by investigating malaria vector bionomics and developing protocols to investigate the mosquito-stages of *P. malariae.*

In the first chapter of this thesis, I assessed malaria transmission and the distribution of insecticide resistance genes in *Anopheles* populations in three rural areas. *Anopheles gambiae sensu stricto (s.s.)* was the principal vector with other *Anopheles spp.* acting as secondary vectors. All *An. gambiae s.s.* except for one were either heterozygous and/or homozygous for the *L1014F* and *L1014S* resistance mutations indicating potentially high levels of resistance to pyrethroids. Malaria transmission was seasonal and heterogenous in the three study areas, with perennial transmission in Zilé and seasonal transmission in Bindo and Nombakélé. Although mosquitoes in the three areas were exophagic, the fact that the peak biting times in the three study areas was at times most people are sleeping argue for a positive effect of LLINs. The entomological inoculation rates (EIRs) recorded, have been previously associated with a high prevalence of *P. falciparum* in humans. Although most mosquitoes were infected with *P. falciparum,* one third of the mosquitoes were infected with other human-infecting species pointing to their important contribution to malaria. These results call for the deployment of tailored vector control measures adapted to the local setting.

In the second chapter, I established a protocol for the experimental infection of *An. gambiae s.s.* mosquitoes using *P. malariae* field isolates. I successfully infected *An. gambiae s.s.* mosquitoes with higher infection rates and infection intensities obtained after serum replacement compared to whole blood for *P. malariae* mono-infected isolates and coinfected *P. malariae–P. falciparum* isolates. The establishment of this platform will allow for the

assessment of transmission blocking interventions, the investigation of vector-pathogen interactions to identify new targets for the control of *P. malariae* and to fill the gaps in knowledge about its transmission.

In addition, I collected baseline data on the susceptibility of malaria vectors to pyrethroids and organochlorides. The principal malaria vector *An. gambiae s.s.* was resistant to both deltamethrin, permethrin and DDT in both Lambaréné and Zilé, with a more pronounced resistance to the two latter. The resistance to these three insecticides was accompanied with a loss of knockdown effect for both permethrin and DDT and fold increases in the knockdown time for deltamethrin in addition to all the mosquitoes carrying knockdown resistance mutations. The results from this study provide baseline data on the resistance of malaria vectors that will be important for the choice of LLINs to be deployed in our study areas.

Finally, I evaluated the susceptibility of mosquitoes to pyrethroids, organophosphates and carbamates, to assess the level of resistance and the effect of piperonyl butoxide (PBO). The mosquitoes were resistant to permethrin, deltamethrin, alphacypermethrin, bendiocarb and susceptible to malathion. The resistance was high for permethrin and at least moderate for deltamethrin. Preexposure to PBO restored the susceptibility of mosquitoes to deltamethrin while it increased the mortality to permethrin by 4-fold highlighting the implication of cytochrome P450 enzymes in the resistance phenotype to pyrethroids. All the mosquitoes were either homozygous or heterozygous for *Vgsc-L1014F* and *-L1014S* knockdown resistance mutations, however, despite resistance to bendiocarb no mosquito was carrying the *G119S Ace-1* resistance allele. The current results demonstrate the suitability of the PBO-Deltamethrin for the impregnation of LLINs to be distributed in Lambaréné.

This thesis provides a detailed picture of the high level of malaria transmission and calls for the deployment of tailored interventions to significantly reduce the level of transmission. In addition, the collection of baseline susceptibility data and the identification of the role of cytochrome P450 enzymes as the main driver of resistance allow us to suggest the use of the PBO-Deltamethrin combination for LLINs. This work calls for the investigation of additional resistance mechanisms for improved resistance management. The establishment of the experimental infection of *P. malariae* has filled a gap that had restricted research on this neglected plasmodial species that is increasingly reported as a major contributor to the overall burden of malaria.

5 German Summary

Malaria ist in Subsahara-Afrika nach wie vor ein großes Gesundheitsproblem. Der jüngste Rückgang der Malariafälle und -todesfälle ist vor allem auf die Vektorkontrolle durch die Verteilung von langlebigen insektizidbehandelten Moskitonetzen (LLINs) zurückzuführen. Seit 2016 ist dieser Rückgang jedoch zum Stillstand gekommen, gleichzeitig häufen sich die Berichte über Resistenzen gegen Pyrethroide – die einzige Klasse von Insektiziden, die in LLINs verwendet werden. In Gabun ist der Einsatz von LLINs begrenzt, und Daten zur Übertragung sind insbesondere in der Provinz Moyen Ogooué rar. Darüber hinaus wird der Beitrag von Nicht-Falciparum-Arten zur Gesamtbelastung durch Malaria oft übersehen. Zudem bestehen Wissenslücken hinsichtlich der Vektorkompetenz von Anopheles-Mücken gegenüber Plasmodium-Arten wie P. malariae, der zweithäufigsten Plasmodium-Art in Gabun. Daher besteht die Notwendigkeit, die Bionomie der Malariaüberträger zu untersuchen sowie Protokolle zur Untersuchung der Mückenstadien von P. malariae zu entwickeln, um die genannten Lücken in den entomologischen Daten zur Malariaübertragung in der Provinz Moyen Ogooué in Gabun zu schließen.

Im ersten Kapitel dieser Arbeit habe ich die Malariaübertragung und die Verteilung von Insektizidresistenzgenen in *Anopheles*-Populationen in drei ländlichen Gebieten untersucht. *Anopheles gambiae sensu stricto* (*s.s.*) war der Hauptvektor, während *An. moucheti* und *An. coustani* als Sekundärvektoren fungierten. Mit einer Ausnahme waren alle *An. gambiae s.s.* entweder heterozygot und/oder homozygot für die Resistenzmutationen *L1014F* und *L1014S*, was auf eine Resistenz gegen Pyrethroide hinweist. Die Malariaübertragung war in den drei Untersuchungsgebieten saisonal und heterogen, mit ganzjähriger Übertragung in Zilé und intermittierender Übertragung in Bindo und Nombakélé. Die Stechmücken in den drei Gebieten waren exophag und wiesen entomologische Inokulationsraten (EIR) auf, die zuvor mit einer hohen Prävalenz von *P. falciparum* beim Menschen in Verbindung gebracht wurden. *Plasmodium falciparum* war in etwa zwei Dritteln und andere *Plasmodium*-Arten in etwa einem Drittel der infizierten Mücken nachzuweisen. Die hohe Anzahl anderer *Plasmodium*-Arten war unerwartet. Möglicherweise wird deren Beitrag zur Malariamorbidität unterschätzt. Diese Ergebnisse zeigt die Relevanz von maßgeschneiderten Vektorkontrollmaßnahmen, die an die örtlichen Gegebenheiten angepasst sind.

Im zweiten Kapitel haben wir die experimentelle Infektion von *Anopheles gambiae s.s.* Mücken mit *P. malariae* Feldisolaten etabliert. Die Infektion von *An. gambiae s.s.* Mücken resultierte in höheren Infektionsraten und Infektionsintensitäten nach Serumersatz im Vergleich zu Vollblut für *P. malariae* monoinfizierte Isolate sowie für koinfizierte *P. malariae-P. falciparum* Isolate. Die Etablierung dieser Methode wird die Bewertung von Maßnahmen zur Blockierung der Übertragung und die Untersuchung von Wechselwirkungen zwischen Vektor und Erreger ermöglichen, um neue Interventionen zur Kontrolle von *P. malariae* zu entwickeln. Dies ist relevant, da *P. malariae* eine signifikante Krankheitslast verursacht.

Darüber hinaus haben wir Basisdaten zur Empfindlichkeit der Malaria-Vektoren gegenüber Pyrethroiden und Organochloriden gesammelt. Der wichtigste Malaria-Vektor, *An. gambiae s.s.,* war sowohl in Lambaréné als auch in Zilé gegen Deltamethrin, Permethrin und DDT resistent, wobei die Resistenz gegen die beiden letztgenannten Insektizide stärker ausgeprägt war. Die Resistenz gegen diese drei Insektizide ging mit einem Verlust der *Knockdown*-Wirkung von Permethrin und DDT einher, während sich die *Knockdown*-Zeit für Deltamethrin vervielfachte und alle Mücken *Knockdown*-Resistenzmutationen aufwiesen. Die Ergebnisse dieser Studie liefern Daten, die für die Auswahl der in unseren Untersuchungsgebieten einzusetzenden LLINs wichtig sind. Wir konnten nachweisen, dass die Mücken weitgehend resistent gegen Pyrethroide sind.

Schließlich haben wir die Anfälligkeit der Mücken gegenüber Pyrethroiden, Organophosphaten und Carbamaten untersucht, um den Grad der Resistenz und die Wirkung von Piperonylbutoxid (PBO) zu beurteilen. Die Stechmücken waren resistent gegen Permethrin, Deltamethrin, Alphacypermethrin und Bendiocarb und empfindlich gegen Malathion. Die Resistenz war hoch für Permethrin und zumindest mäßig für Deltamethrin. Eine Präexposition mit PBO stellte die Empfindlichkeit der Mücken gegenüber Deltamethrin wieder her. Auch die Mortalität gegenüber Permethrin war um das Vierfache erhöht, was die Bedeutung der Cytochrom-P450-Enzyme für den Resistenzphänotyp gegenüber Pyrethroiden verdeutlicht. Alle Mücken waren entweder homozygot oder heterozygot für *Vgsc-L1014F*- und *-L1014S-Knockdown*-Resistenzmutationen. Interessanterweise trug, trotz der Resistenz gegen Bendiocarb, keine Mücke das G119S-Ace-1-Resistenzallel. Die aktuellen Ergebnisse belegen die Eignung von PBO-Deltamethrin für die Imprägnierung von LLINs, die in Lambaréné verteilt werden sollen.

Diese Arbeit liefert ein detailliertes Bild des hohen Niveaus der Malariaübertragung im Studiengebiet und zeigt, dass maßgeschneiderter Maßnahmen nötig sind, um das Ausmaß der Übertragung deutlich zu reduzieren. Basierend auf den Daten zur grundlegenden Empfindlichkeit und der Rolle der Cytochrom-P450-Enzyme, die einen wichtigen Resistenzmechanismus darstellen, schlagen wir die Verwendung einer PBO-Deltamethrin-Kombination für LLINs vor. Diese Arbeit zeigt, dass die Untersuchung von Resistenzmechanismen zu einem verbessertes Resistenzmanagement führen können und sollten deshalb fortgesetzt werden. Außerdem konnte mit der Etablierung einer Methode zur reproduzierbaren Untersuchung der Transmission von *P. malariae* ein wesentlicher Beitrag zur Erforschung dieses vernachlässigten Malariaerregers geleistet werden.

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7 Declaration of contributions

The doctoral thesis entitled "Assessment of vectorial competency and resistance to insecticides of *Anopheles gambiae sensu stricto* in the Moyen Ogooué Province of Gabon" is based on four publications (Publication No. 1: Parasites & Vectors. 2022 Jun 20;15(1):217. PMID: 35725630; Publication No. 2: Journal of Infectious Diseases. 2021 Feb 13;223(3):522-526. PMID: 32621750; Publication No. 3: Malaria Journal. 2023 Dec 18;22(1):382. PMID: 38110952; Publication No. 4: BMC Infectious Diseases. 2024 Oct 30;24(1):1221. PMID: 39478447) with Mr. Stravensky Térence Boussougou Sambe as first author. We declare that Mr. Stravensky Térence Boussougou Sambe made significant contributions for all four manuscripts with his contribution and that of other authors listed below:

Contributions of PhD candidate and other co-authors:

Publication No. 1: Stravensky Térence Boussougou Sambe conceived, planned the study and its design, performed the field activities, performed the molecular analysis of the samples, analysed the data and drafted the manuscript. Ayôla A. Adégnika, Benjamin Mordmüller, Peter G. Kremsner, Jean Claude Dejon-Agobé, Ulysse Atéba-Ngoa, Tamirat Gebru Woldearegai conceived, planned the study and its design. Ange Gatien Doumba Ndalembouly and Barclaye Ngossanga contributed to the field activities with support from Jean Ronald Edoa, Jeannot F. Zinsou and Yabo J. Honkpehedji for the follow-up of the collectors. Tamirat Gebru Woldearegai performed the molecular analysis of the samples. Romuald Beh Mba analysed the data. Ayôla A. Adégnika, Benjamin Mordmüller, Peter G. Kremsner, Steffen Borrmann, Jean Claude Dejon-Agobé, Ulysse and Tamirat Gebru Woldearegai critically reviewed the manuscript.

Publication No. 2: Stravensky Térence Boussougou Sambe conceived, planned the study and its design, participated in the screening and recruitment of participants, performed the experimental infections and drafted the manuscript. Steffen Borrmann, Ayôla A Adegnika, Charles S. Wondji, Saadou Issifou, Luc Djogbénou, Francine Ntoumi, Yudi T Pinilla conceived, planned the study and its design. Yudi T Pinilla, Sarah Gräßle, Barclaye Ngossanga, Ange Gatien Doumba-Ndalembouly, Gedeon Bingoulou, Emma G Malinga participated in the screening and recruitment of participants. Yudi T Pinilla, Sarah Gräßle, Barclaye Ngossanga, Ange Gatien Doumba-Ndalembouly performed the experimental infections. Andrea Weierich performed the molecular analysis of the human samples. Daniel Nguiffo-Nguete performed the

molecular analysis of mosquito samples. Yudi T Pinilla and Sarah Gräßle drafted the manuscript. Steffen Borrmann, Ayôla A Adegnika, Charles S. Wondji, Saadou Issifou, Luc Djogbénou, Francine Ntoumi critically reviewed the manuscript.

Publication No. 3: Stravensky Térence Boussougou Sambe conceived, planned the study and its design, performed the field activities and insecticide susceptibility testing, performed the molecular analysis of the samples, analysed the data and drafted the manuscript. Ayôla A. Adégnika, Benjamin Mordmüller, Peter G. Kremsner, Parfait Awono-Ambene, Hilaire M Kenguele, Steffen Borrmann, Jean Claude Dejon-Agobé, Tamirat Gebru Woldearegai, Jean Ronald Edoa and Thierry Ndong Mba conceived, planned the study and its design and critically reviewed the manuscript. Ange Gatien Doumba Ndalembouly and Barclaye Ngossanga participated in the field activities and insecticide susceptibility testing. Lynda Nouage Boussougou, Ange Gatien Doumba Ndalembouly and Barclaye Ngossanga carried out the laboratory analysis of the samples. Fabrice Mougeni analysed the data.

Publication No. 4: Stravensky Térence Boussougou Sambe conceived, planned the study and its design, performed the field activities and insecticide susceptibility testing, performed the molecular analysis of the samples, analysed the data and drafted the manuscript. Ayôla A. Adégnika, Steffen Borrmann, Benjamin Mordmüller, Peter G. Kremsner, Charles S. Wondji, Francine Ntoumi, conceived, planned the study and its design and critically reviewed the manuscript. Ynous Djida, Ange Gatien Doumba Ndalembouly, Barclaye Ngossanga and Rodrigue Bikangui participated in the field activities and insecticide susceptibility testing. Ynous Djida, Lynda Nouage Boussougou, Maminirina Fidélis Ambinintsoa, Ange Gatien Doumba Ndalembouly and Barclaye Ngossanga carried out the laboratory analysis of the samples. Daniel Nguiffo-Nguete, Francis N Nkemngo, Romuald Agonhossou, Romaric Akoton commented on and approved the manuscript.

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