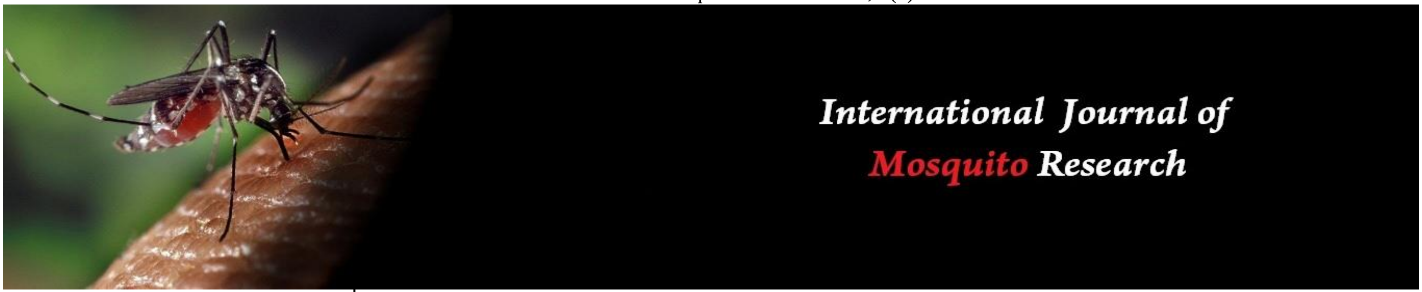

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Current sensitivity status of *Anopheles gambiae* (s.l.) (*Culicidae*) to DDT and pyrethroids in two agricultural sites of Mouila, Gabon

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Abstract
The present study was conducted during the rainy season of 2017 in Mboukou and Moutassou, two palm oil concessions located in the suburbs of Mouila city. It aimed at determining the susceptibility status of *Anopheles gambiae s.l.* to organochlorines and pyrethroids. The larvae and pupae of *An. gambiae s.l.* were collected from different breeding sites by the dipping method and reared to the adult stage in the field laboratory. The adult females were exposed to various insecticides: Organochlorine (DDT 4%) and Pyrethroids (Permethrin 0.75%, 3.75% and 7.75%; Deltamethrin 0.05%, 0.25% and 0.50%; Lambdacyhalothrin 0.05%, 0.25% and 0.50%). The sensitivity of *An. gambiae s.l.* populations to insecticides was assessed following WHO protocol. All tested mosquitoes were identified by PCR method. *An. gambiae s.s.* and *An. coluzzii* were the only anopheline species identified in all the prospected sites. The results of the susceptibility tests revealed that the malaria vectors of the surveyed sites were susceptible or resistant to the tested insecticides depending on the type and the dose of insecticide. These *Anopheles* were resistant to DDT 4% and low doses of pyrethroids (Permethrin 0.75%, Deltamethrin 0.05%, Lambdacyhalothrin 0.05%). But they were sensitive to all pyrethroids at high doses (98 to 100% mortality).

Keywords: sensitivity, *Anopheles gambiae s.l.*, DDT, pyrethroids, mouila, gabon

1. Introduction

For several decades, humanity has faced several dangerous diseases and one of them is malaria. Malaria is a parasitic disease caused by a protozoan parasites belonging to the genus *Plasmodium* and transmitted to humans via a bite of infected female anopheline mosquitoes [1]. It occupies a significant position in terms of endemic diseases [2, 3] and remains the major cause of morbidity and mortality in the world, particularly in Africa [4]. In 2016, we estimated about 216 million, the number of people living with the disease worldwide, of which 90% of malaria cases and 92% of malaria deaths occurred in sub-Saharan Africa [5]. Globally, this parasitosis caused about 445, 000 deaths in 2016, and the most affected being children aged under 5 years and pregnant women [6].

Faced with this major public health problem, the countries concerned are developing strategic plans for the prevention and the control of vectors as well as in the management of patient [7, 8]. The main entomological control methods against malaria transmission are the massive distribution of long-lasting insecticidal nets (LLINs) and the indoor residual spraying (IRS). However, the massive use of synthetic antimalarials (chloroquine, amodiaquine) and residual insecticides (DDT) after the Second World War led unfortunately to the development of antimalarial resistance and the rapid emergence of insecticide resistance [9]. It is therefore essential to know the efficacy of insecticides towards *Anopheles* malaria vector to better guide malaria control strategies [10-12].

In Gabon, a tropical African country, malaria is hyperendemic and mainly caused by *Plasmodium falciparum* and its major vectors consist of *Anopheles gambiae s.l.* and *Anopheles funestus s.l.* [13]. This disease continues to be the main cause of morbidity and mortality in the country. According to the National Malaria Control Program, nearly 45% of children and 71% of pregnant women consulted were hospitalized because of severe forms of malaria [14, 15]. To effectively roll back malaria, Gabon promote vector control based on the mass distribution of impregnated bed nets and indoor residual insecticide spraying and the management of environment as main control strategies [16]. However, insecticide resistance of *Anopheles gambiae* has been reported for a few years in Libreville and Port-Gentil by Pinto *et al*. (2006) [17] and Mourou *et al*. (2010) [14]. These resistance phenomena can seriously hinder control efforts.

In Mouila, Koumba *et al*. (2018) [16] revealed that in the dry season, the populations of *An. gambiae s.l.* in the agricultural areas of this region were resistant to 4% DDT, 0.05% Deltamethrin and 0.75% Permethrin. Also, in order to know the sensitivity status of *Anopheles gambiae s.l.* to Organochlorines and Pyrethroids of various doses, a study was conducted in the agricultural sites of Mouila in the rainy season. The aim of this study was to determine the susceptibility status of *An. gambiae* sensu lato populations to one organochlorine and different pyrethroids in Mouila.

2. Materials and Methods

2.1 Study area

The present study was conducted in the rainy season of 2017 in two agricultural sites, in the suburbs of Mouila city, notably Mboukou and Moutassou. (Fig. 1). The Mboukou site (1°39'06 "S and 10°49'42.6" E) is located in the Tsamba-Magotsi department about 35 kilometers from the town of Mouila. It is limited to the East by river Ngounié and Saint-Martin and Migabe villages and to the West by the Douya, Doubou, Mboukou and Rembo villages [18, 19]. The Moutassou site (1°59'33.8 "S and 11°02'25.2" E) is located in the Douya-Onoye division about 13 kilometers from the town of Mouila [20]. It is limited to the north by the city of Mouila, to the East by the Moulandoufouala, Mbengui and Mbadi villages then to the West by the Moutassou, Koumbanou, Ikolo-Ikolo and Digabosse villages. It is an ecosystem dominated by savannas, forests and forest-savanna mosaics occupying 75% of the exploitation permit [20].

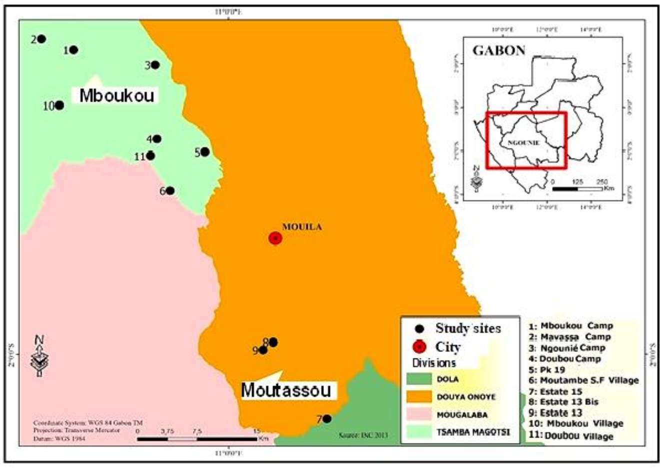

Fig 1: Geographical location of the study sites

2.2 Mosquito collection and rearing

For insecticide susceptibility tests, individuals of *Anopheles gambiae s.l.* population were collected at their larval stage from natural (ponds) and artificial (water reservoirs, pits and tires) breeding sites present in 11 sampling stations, distributed all over the study area. These stations were represented by Pk19, Moutassou camp (Estate 13, Estate 13Bis, Estate 15), Moutambe Sane Foumou village, Doubou camp, Mboukou village, Doubou village, Mavassa camp, Ngounié camp and Mboukou camp. Anopheline larvae and pupae were collected from breeding sites [21] using the dipping method [22], transferred to trays using pipettes and transported to the field laboratory for rearing [23]. These larvae were reared in water from the respective breeding sites and fed every second day with ornamental fish meal (*Sera Vipagran*) [24]. The adults obtained were fed using 10% sugar solution *ad libitum*. They were identified morphologically using the identification key of Baldacchino & Paupy (2010) [25] and the females were subjected to sensitivity tests.

2.3 Adult sensitivity tests

The WHO sensitivity tests kits (WHO tubes and accessories) and insecticide impregnated papers (DDT 4%, Permethrin 0.75%, 3.75% and 7.75%, Deltamethrin 0.05%, 0.25% and 0.50%, Lambdacyhalothrin 0.05%, 0.25% and 0.50%) were provided by WHO country office Gabon (Table 1).

Table 1: Insecticides used for ssensitivity test

Insecticide family	Insecticide	Tested concentration (%)
Pyrethroids	Permethrin	0.75 – 3.75 – 7.75
	Deltamethrin	0.05 -0.25 – 0.50
	Lambdacyhalothrin	0.05 – 0.25 – 0.50
Organochlorines	DDT	4

Bioassays were carried out using WHO test kits for adult mosquitoes and WHO procedures [26]. The insecticide susceptibility tests were conducted using 2-4 days old, glucose-fed but non-blood-fed female Anopheles mosquitoes, from larvae collected from the various breeding sites of the study sites. Each test on a batch of 25 mosquitoes was replicated four times. They were exposed to impregnated papers with different insecticides for 60 minutes. Controls made up of wild mosquitoes were exposed to paper treated with olive oil only. The number of Knocked-down mosquitoes was first recorded every 5 minutes during the first 20 minutes of exposure and then every 10 minutes until 60 minutes of exposure. After 1 hour of exposure, the mosquitoes were transferred into WHO observation insecticide free tubes and maintained on sugar solution (10%). Mortality was recorded 24 hours post-exposure. In addition, all insecticide-impregnated papers were pre-tested with adults of Kisumu, reference susceptible strain to confirm their efficacy on the susceptible strain [27]. Sensitivity tests were performed under laboratory conditions of 26-29°C temperature and 74-82% relative humidity.

At the end of the tests, all tested *Anopheles gambiae* (dead and survivor) from each site were stored in 1.5 ml Eppendorf tubes containing silica gel for PCR analysis. These *Anopheles* were then packaged separately in groups: the dead ones formed one group and the survivor another. These two groups of specimens were used for molecular identification of mosquito species [27, 28].

2.4 Molecular identification of members of the *Anopheles gambiae* complex
The mosquitoes from sensitivity tests were identified by the Polymerase Chain Reaction (PCR) using genomic DNA extracted from these insects individually. Thus, the genomic DNA of the mosquitoes was extracted using a protocol slightly modified from Collins *et al*. (1987) [29] and a 1/15 dilution was made using sterile water. The molecular identification of members of the *An. gambiae s.l.* was performed using the Restriction Fragment Length Polymorphism (PCR-RFLP) via the method described by Favia *et al*. (1997) [30]. This involved amplification of a 1500 bp fragment of the gene (with primers A.O and A1.3) and subsequent digestion of the amplicons by Hin61 or Tru9 restriction endonucleases, making it possible to identify species of *Anopheles gambiae* complex as *An. coluzzii* and *An. gambiae s.s.*

2.5 Data analysis
The KDTs (knock-down times) were determined as the necessary time for obtaining 50% (KDT50) and 95% (KDT95) adult female mosquitoes ‘knocked down’ after 1 hour of contact with an insecticide. These KDTs were estimated from the probit function while mortality rates were calculated by dividing the number of mosquitoes killed by the total number of mosquitoes tested for each insecticide [31, 32]. The sensitivity test results were interpreted according to criteria defined by WHO [26]. Abbott's formula was used to correct the mortality observed in adult susceptibility tests and this occurred when the mortality in the control group was between 5% and 20% [33].

3. Results
3.1 Mortality of *Anopheles gambiae s.l.*
The results of insecticide susceptibility tests of *Anopheles gambiae s.l.* are shown in tables 2 and 3. These results revealed that populations of *Anopheles gambiae s.l.* from Mboukou and those from Moutassou were resistant to organochlorines (DDT 4%) and low doses of pyrethroids (Permethrin 0.75%, Deltamethrin 0.05% and Lambdacyhalothrin 0.05%), with mortality rates observed after 24 h between 0 and 86% in Mboukou, and between 12 and 66% in Moutassou. However, they were sensitive to pyrethroids at high doses in both study sites (Tables 2 and 3). Overall, among the 10 doses of insecticides tested against *Anopheles* in the study sites, it was observed that they were resistant to 4 insecticide concentrations and susceptible to 6 others (Tables 2 and 3).

Table 2: Mortality of *Anopheles gambiae s.l.* after 24 h of observation in Mboukou

Insecticide family	Tested insecticide	Number	Mortality (%)	Status
Pyrethroids	Permethrin 0.75%	100	12	Resistant
	Permethrin 3.75%	100	100	Sensitive
	Permethrin 7.75%	100	100	Sensitive
	Deltamethrin 0.05%	100	86	Resistant
	Deltamethrin 0.25%	100	100	Sensitive
	Deltamethrin 0.50%	100	100	Sensitive
	Lambdacyhalothrin 0.05%	100	28	Resistant
	Lambdacyhalothrin 0.25%	100	100	Sensitive
	Lambdacyhalothrin 0.50%	100	100	Sensitive
Organochlorines	DDT 4%	100	0	Resistant

Table 3: Mortality of *Anopheles gambiae s.l.* after 24 h of observation in Moutassou

Insecticide family	Tested insecticide	Number	Mortality (%)	Status
Pyrethroids	Permethrin 0.75%	100	12	Resistant
	Permethrin 3.75%	100	98	Sensitive
	Permethrin 7.75%	100	100	Sensitive
	Deltamethrin 0.05%	100	66	Resistant
	Deltamethrin 0.25%	100	100	Sensitive
	Deltamethrin 0.50%	100	100	Sensitive
	Lambdacyhalothrin 0.05%	100	56	Resistant
	Lambdacyhalothrin 0.25%	100	100	Sensitive
	Lambdacyhalothrin 0.50%	100	100	Sensitive
Organochlorines	DDT 4%	100	28	Resistant

3.2 Knock down time (KDT50 and KDT95)
The results of the evaluation of the knock down of *Anopheles* exposed to 10 different doses of insecticides are presented in table 4. These results indicated that Deltamethrin 0.5% had the lowest KDT50 and KDT95 values with 10.99 and 27.15 minutes in Mboukou and, 10.06 and 19.75 minutes in Moutassou. So, Deltamethrin 0.5% appeared as the insecticide which had the highest knock-down potential on wild mosquito populations. On the other hand, DDT 4% and Permethrin 0.75% showed a total loss of Knock-down effect (No Kd) in both study sites. Finally, Permethrin 3.75% had the highest KDT50 and KDT95 values, with 83.55 and 134.84 minutes in Mboukou and 59.1 and 287 minutes in Moutassou. Of all the pyrethroids, the fast knocking-down insecticides were those with high concentrations (Permethrin 7.75%, Deltamethrin 0.25% and 0.50%, Lambdacyhalothrin 0.25% and 0.50%) (Table 4).

Table 4: Knock down Time of *An. gambiae s.l.* exposed to different doses of insecticides after 60 minutes (IC at 95%).

Insecticides	Mboukou		Moutassou	
	KDT50 (IC)	KDT 95 (IC)	KDT 50 (IC)	KDT 95 (IC)
Permethrin 0.75%	No Kd	No Kd	No Kd	No Kd
Permethrin 3.75%	83.55 (70.59 – 111.86)	134.84 (108–196.11)	59.1 (48.6-80.5)	287 (173 -709)
Permethrin 7.75%	27.07 (17.64 –36.45)	60.85 (47.71 – 96.64)	24.6 (22.4-27.2)	81.3 (65.5 – 99.6)
Deltamethrin 0.05%	25.84 (24.34– 27.39)	46.09(41.64–52.69)	21.15 (18.62– 23.76)	46.99 (39.61– 60.48)
Deltamethrin 0.25%	13.89 (11.63-46.11)	37.80 (30.85-51.21)	11.56 (5.78-16.71)	39.67 (25.66-129.49)
Deltamethrin 0.5%	10.99 (8.53-13.27)	27.15 (21.42-41.03)	10.06 (8.37-11.59)	19.75 (16.56-26.53)
Lambdacyhalothrin 0.05%	63.36 (53.36– 88.35)	102.19 (80.79 – 165.75)	43.20 (38.61– 48.82)	70.94 (62.43– 85.52)
Lambdacyhalothrin 0.25%	17.05 (11.89-22.25)	46.09 (33.05-93.29)	25.10 (16.13– 33.88)	52.73 (41.52– 82.88)
Lambdacyhalothrin 0.5%	10.21 (2.94-16.49)	49.99 (28.54-352.84)	10.27 (3.96-15.75)	44.29 (26.94-188.50)
DDT 4%	No Kd	No Kd	No Kd	No Kd

No Kd: Total loss of Knock-down effect (less than 10% of mosquitoes knocked out after 60 minutes of exposure); IC: 95% Confidence Interval; KDT50 and KDT95: Knock-down time or necessary time for obtaining 50% and 95% of adult mosquitoes knocked down after 60 minutes of contact with an insecticide.

3.3 Molecular composition of *Anopheles gambiae s.l.*

All adult *Anopheles* obtained from the laboratory were species of the *An. gambiae* complex (Table 5). The identification of the members of the *An. gambiae* complex was performed using the PCR technique. The results of the PCR revealed the presence of *An. gambiae s.s.* and *An. Coluzzii*. In the study sites, *An. gambiae s.s.* was most predominant, with 93.14% than *An. coluzzii* (6.86%).

Table 5: Species composition of the *An. gambiae* complex in the study area

	An. gambiae complex	
Species	*An. gambiae s.s.*	*An. coluzzii*
Percentage	93,14% (n=394)	6,86% (n=29)

4. Discussion

The results of the present study contributed to the knowledge of the level of sensitivity and resistance of *Anopheles*, malaria vectors, to certain insecticides recommended by the WHO in the Mouila area. Biomolecular analyses revealed the presence of two sibling species of *Anopheles* (*An. gambiae s.s.* and *An. coluzzii*) considered as major vectors of malaria in Gabon [14, 17]. *An. gambiae s.s.* was more frequent and more abundant in the prospected sites. The presence of *An. coluzzii* observed in the rainy season could be explained by the abundance of breeding sites and the existence of some polluted breeding sites [34]. The predominance of *An. gambiae s.s.*, a major vector-species of malaria, is probably related to the abundance of sunny and unpolluted water collections [35]. An analysis of the level of pollution of *Anopheles gambiae s.l.* breeding sites in the study area should nevertheless be performed to obtain the TDS values.

The insecticide susceptibility tests carried out on mosquitoes collected during the rainy season made it possible to determine the sensitivity of *Anopheles gambiae s.l.* to DDT 4%, Permethrin (0.75%, 3.75%, 7.75%), Deltamethrin (0.05%, 0.25%, 0.5%) and Lambdacyhalothrin (0.05%, 0.25%, 0.5%). The results of these tests indicate that the populations of *Anopheles gambiae s.l.* were resistant to pyrethroids, at low doses and DDT, in the study sites [16]. From the results of the present study, low doses of Permethrin, Deltamethrin, Lambdacyhalothrin and DDT cannot kill *Anopheles gambiae s.l.* So, it would be better to use higher doses of these insecticides. In fact, the results of the sensitivity tests revealed that *Anopheles gambiae s.l.* populations were sensitive to Permethrin 3.75% and 7.75%, Deltamethrin (0.25% and 0.5%) and Lambdacyhalothrin (0.25% and 0.5%), with mortality rates of 98-100%.

The resistance to low concentrations of pyrethroids and DDT has already been observed in the Mouila area by Koumba *et al*. (2018) [16] in the dry season. This resistance profile has also been described by Pinto *et al*. (2006) [17] and Mourou *et al*. (2012) [36] who worked on *Anopheles gambiae s.l.* populations from Libreville (Estuaire). This resistance is likely due to the past widespread use of DDT and the use of insecticides by local populations in agriculture and public health [16, 37]. It has been recognized that prior exposure of mosquitoes to insecticides may induce selection pressures [38]. Aerosol pyrethroids are used in the study area for the control of mosquitoes and domestic pests. This could contribute to the development of resistance as reported elsewhere [34, 36]. According to the work conducted by Chandre *et al*. (1999, 2000) [39, 40], agriculture appears to be one of the main factors responsible for high insecticide resistance in *An. gambiae s.l.* in Africa.

The observed resistance of *An. gambiae s.l.* populations from the Mouila area to Permethrin 0.75%, Deltamethrin 0.05% and DDT 4%, corroborate the results observed by Toto *et al*. (2011) [41] in Lobito (Angola). These authors showed that *An. gambiae s.l.* populations in Lobito are resistant to these insecticides. Other studies conducted in Benin, Ivory Coast and Burkina Faso reported a general decrease in the sensitivity of *An. gambiae s.l.* populations to Lambdacyhalothrin 0.05% [42].

With high doses of pyrethroids, wild *Anopheles gambiae s.l.* populations were susceptible, and the time required to kill 50% and 95% of adult mosquitoes (KDT50 and KDT95) was very low. In contrast, low doses of pyrethroids revealed a high KDT50 and KDT95. This lengthening of the KDTs could be linked to the resistance developed by the *Anopheles* from study sites to insecticides. These results are similar to those obtained by Chandre *et al*. (1999) [39] who also observed an increase in KDT50 and KDT90 in the insecticide resistant samples. Finally, with DDT 4% and Permethrin 0.75%, there was a total loss in the knock-down effect. This observation may be explained by the fact that local populations of *Anopheles gambiae s.l.* are highly resistant to both insecticides used in the vector control in the study area.

This decrease in mortality observed with low doses of pyrethroids and DDT could be related to the resistance mechanisms developed by mosquitoes. According to Koumba *et al*. (2018) [43], the resistance of *Anopheles gambiae s.l.* from the Mouila area to these two insecticides families is due to kdr-west and kdr-east mutations. However, some studies have shown the importance of metabolic resistance in *Anopheles* [44, 45]. It would be good to carry out a biochemical analysis to find the presence of this mechanism in these *Anopheles* populations.

5. Conclusion

The *Anopheles gambiae s.l.* populations from the prospected sites were resistant to DDT and low doses of pyrethroids, which are insecticides used in public health. However, these *Anopheles* were sensitive to high doses of pyrethroids. These pyrethroids could be used as alternatives in malaria control to overcome the problem of resistance observed recently in *An. gambiae s.l.* to low doses of pyrethroids in the Mouila area. Also, it is essential to monitor the sensitivity of *Anopheles* vectors of malaria to insecticides for the success of the fight against this parasitosis and against the vectors of the pathogens. The results obtained during the present study, thus constitute vital information for the orientation of the choice of imagocidal insecticides, commonly used for mosquito nets impregnation and indoor residual spray during vector control campaigns in different zones where these vectors are present. In addition, the monitoring of resistance level by taking into account the molecular biology and biochemical techniques, would also be necessary in order to better oriented vector control strategies by focusing on the allelic frequency of genetic mutations and their distribution all the study area over.

6. Declaration of interest

The authors declare that they have no competing interest in relation to this article. All the authors read and approved the final version.

7. Acknowledgment

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