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Impacts of agricultural insecticide pressure on the frequency of L1014F and G119S mutations in *Anopheles gambiae* SL. in Benin, A public health problem in Sub-Saharan Africa

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Abstract

The aim of this study was to assess the impacts of insecticidal pressure in agricultural environments on the frequency of L1014F and G119S mutations in *Anopheles gambiae* s.l. in Benin. Three to five-day-old adult females from larval collections in areas with high and low chemical pesticide use against crop pests were exposed to papers impregnated with permethrin (0.75%), deltamethrin (0.05%), DDT (4%) and bendiocarb (0.1%) according to the WHO protocol. Survivors were stored at -20 degrees Celsius for the detection of L1014F and G119S mutations in *Anopheles gambiae*. The Kisumu strain was used as a control for comparison and areas without any insecticide input were chosen as control areas. Results from this study show that *An. gambiae* has developed a resistance to pyrethroids and organochlorine but susceptible to carbamate. The L1014F mutation was found with an average of 0.9 and 0.85 frequency in areas of high and low pesticide use, respectively. However, the G119S mutation was found but at low frequency (0.05) regardless of the mosquito's collection. These findings suggested that insecticides pressure against crop pests seem to be one of the factors responsible for the emergence of resistance in *An. gambiae* populations. This would result in the development of high frequencies of the L1014F gene.

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Introduction

Malaria is one of the main causes of mortality and morbidity in infants and pregnant women (WHO, 2019). Many initiatives have been taken to combat this disease while waiting for an effective vaccine, the most important of which is vector control through the use of long-lasting insecticide-treated mosquito nets (LLINs) (Kanyangarara *et al.,* 2016). Between 1990-2020, the introduction of long-lasting insecticide-treated mosquito nets as a prevention tool against mosquito bites resulted in a reduction of malaria morbidity, which ranged from 40% to 63% in children under five (Owusu-Addo *et al.,* 2014; UNICEF, 2021).

According to Akogbéto *et al.* (2018) the burden of malaria in sub-Saharan Africa has been significantly reduced by a combination of several control measures. The results of this research indicate that in many parts of Sub-saharan Africa, the simultaneous use of Indoor Residual Spraying (IRS), LLINs and Artemisinin-based Combination Therapy (ACT) led to a significant reduction in the positivity of anopheles to *Plasmodium falciparum circumsporozoite* antigen (Akogbéto *et al.,* 2018). These results show that the burden of malaria is decreasing in Africa due to the use of impregnated mosquito nets and indoor residual spraying, and therefore offer hope for the fight against malaria. However, a major problem limiting their effectiveness is the emergence of resistance in malaria vectors to pyrethroids, the main insecticides used for net impregnation and indoor residual spraying (Salako *et al.,* 2018).

Over the last decade, the emergence of resistance of *Anopheles gambiae sensu lato* (S.L.) to different insecticides used in public health has been reported in several African countries such as Côte d'Ivoire (Tia *et al.,* 2017), Uganda (Tukei *et al.,* 2017), Burkina Faso (Namountougou *et al.,* 2019), Nigeria (Ibrahim *et al.,* 2019), Togo (Ketoh *et al.,* 2018), Benin (Kpanou *et al.,* 2022). For many actors, the emergence of resistance within *An. gambiae* S.L. populations could be explained by the multiple insecticide use in agricultural environments which might have led to their resistance.

Nanmoutougou *et al.* (2019) in Burkina Faso and Yadouleton *et al.* (2011) reported a relatively high frequency of knock down resistance (*kdr)* mutations in *A. gambiae* collected in cotton farms under massive insecticide treatment compared to farms with no pesticide utilization. Indeed, the multiple insecticide treatments in agricultural settings has led to the widespread distribution of acethlycholynesterase (ace-1^R), the kdr-west (L1014F) and the kdr-east (L1014S) mutations in *An. gambiae* S.L and was reported by many scientists in sub-Saharan Africa (Namountougou *et al.*, 2012; Antonio-Nkondjio *et al.,* 2017). Moreover, an investigation study on the use of insecticides in many agricultural settings in Benin conducted by Yadouleton *et al.* (2018) showed that many pesticides were used but few were registered for pest control in agriculture. It is possible that the massive use of various insecticides contributes to the emergence of kdr and *ace-1*^{R} mutations among the natural populations of *An. gambiae* S.L in different agricultural settings in Benin.

This study proposes to assess the impacts of agricultural insecticide pressure on the frequency of L1014F and G119S mutations in *Anopheles gambiae* S.L. in different agricultural settings in Benin where insecticides have been used for both public health and agricultural purposes.

Materials and methods

Study area

In order to know the impacts of agricultural pesticides on the susceptibility of *An. gambiae* S.L., areas with high and low pesticide use by farmers to control crop pests were selected from the south to north of Benin.

Areas of high agricultural pesticide use

Three vegetable farming sites were chosen, two in the south of Benin (Houeyiho in Cotonou, Acron in Porto-Novo) and one in the north (Azèrèkè in Parakou), and four cotton growing sites (Parakou, N'dali, Kandi and Banikoara) in the departments of Borgou and Alibori (Fig. 1). In the cotton growing areas of Parakou, Kandi and Banikoara, a cotton field with a scheduled treatment (a treatment that follows a fixed schedule in which farmers apply 6 high-dose insecticide treatments during the cotton cycle) was chosen in each of these localities. In addition, in Kandi and Banikoara, an organic cotton field (without insecticide treatment and without chemical fertiliser) was chosen. Targeted Staged Control (TSC) cotton fields were chosen in N'dali, Kandi and Banikoara. The TSC method consists in treating the fields with a low dose of insecticide only when the threshold of attack of the cotton plant by pests is reached (5 *Helicoverpa armigera* larvae sampled on 50 cotton plants). In this work, the areas with high pesticide use are the cotton calendar treatment sites, the TSC sites and the vegetable farming where farmers apply between 4 and 6 treatments of cyfluthrin and alpha-cypermethrin at 250g/ha/year before harvest.

Areas with low agricultural pesticide use

In the framework of our study, these areas are defined as those where farmers apply at most two pre-harvest treatments at a dose of 10g/ha/year. The rice-growing area of Malanville in the north of Benin in the Alibori department was chosen for investigation. This rice-growing area is a perimeter of 70 hectares. Insecticide use remains low in this locality.

Control areas

Cereal growing areas were chosen as control areas. Cereal growing areas were chosen because cereals are grown without the use of chemical fertilizers and pesticides therefore, selective pressure from the use of insecticides is very low, which could make mosquitoes in these areas susceptible to insecticides. Three locations (Comè, Kétou and Séhouè) in southern Benin were selected. The choice of these locations surveyed took into account climatic conditions, the usual practices of protection against mosquito bites observed by farmers, and farmers' practices in controlling crop pests.

Insecticide susceptibility tests

Larvae collection

Larval collection was conducted at the various sites during the rainy season. Larvae collected were brought to the insectary and reared to the adult stage

Insecticide susceptibility tests

Females aged 3-5 days were selected for susceptible testing. The tests were performed with papers impregnated with deltamethrin at the diagnostic dose of 0.05%. The choice of diagnostic doses of the impregnated papers was based on the WHO recommendations for such tests (WHO, 2013). The susceptibility of mosquitoes to this product (deltamethrin) was compared to another pyrethroid, permethrin 0.75% and an organochlorine, DDT 4% (diagnostic dose). DDT was tested for cross-resistance between pyrethroids and organochlorines. Mosquitoes were also subjected to bendiocarb 0.1% and testing was performed according to the standard WHO protocol in a cylindrical tubes. The exposure time of the mosquitoes to the impregnated papers was 60 minutes and the observation time before reading the results was 24 hours. Upon exposure of the mosquitoes to the insecticide, the number of "knocked-down" mosquitoes (kd), i.e. mosquitoes that fall down to the bottom of the WHO tubes, were recorded after 10, 15, 20, 30, 45, 60 minutes. After testing, dead and a' live mosquitoes were kept separately on silica gel in Eppendorf tubes and stored at -20°C (freezer) for further research.

In accordance with the WHO protocol (WHO, 1986), the population is considered to be susceptible when the mortality rate is greater than or equal to 97%. When mortality is less than 90%, the population is considered resistant. Between the two values, we suspect resistance.

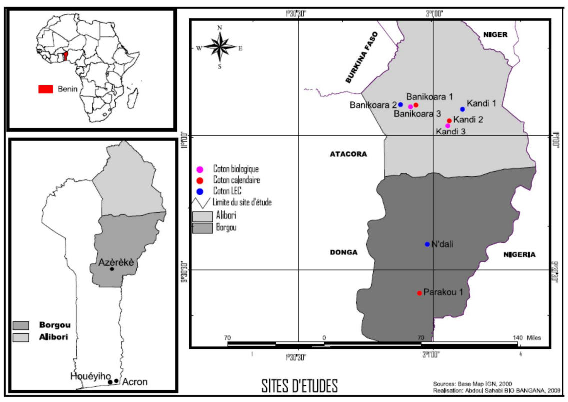

Fig. 1. Map of Benin showing the different study sites.

Identification of Anopheles species and resistance mechanisms

Species identification

All anophelines collected from the different study sites were subsequently identified morphologically based on the protocol described by Gillies and Cootzee (WHO, 1986; Gillies & Coetzee, 1987)

Detection of kdr and ace-1 *mutations*

Genomic DNA of 100 mosquitoes from each study site extracted was used to set the PCRs for the species within the *A. gambiae* complex based on the protocol of Santalomazza *et al.* (2008) but also for the detection of the L1014S *kdr* and *G119S Ace-1* mutations following the protocols of Martinez-Torres *et al.* (1998), and Weill *et al.* (2004), respectively.

Statistical analysis

Mortality rates were corrected using Abbott formula when mortality rates registered among control populations were above 5%.

Moreover, in accordance with the WHO protocol (WHO, 1986), the population is considered to be susceptible when the mortality rate is greater than or equal to 97%. When mortality is less than 90%, the population is considered resistant. Between the two values, we suspect resistance. The allele frequency at the *kdr* and *ace-1* resistance locus was calculated using the Genepop software (version 3.3)

Results

Level of resistance of Anopheles populations to insecticides

From the total 3,000.0 *A. gambiae* populations tested with the impregnated papers cited above, it appears that all the populations of Anopheles *gambiae* S.L. from the various study sites showed high sensitivity to bendiocarb. However, with pyrethroids and organochlorine, the situation is different. Indeed, in vegetable farming (Houéyiho, Acron and Azèrèkè), 600 *A. gambiae* S.L. females were tested (200 per site) with permethrin impregnated papers. The percentage of mortality observed was 32%, 33% and 35% in Houeyiho, Acron and Azèrèkè, respectively. These percentages indicate a strong resistance of *An. gambiae* S.L. populations from vegetable farming to permethrin (Fig. 2). In the cotton-growing area (Parakou, N'dali, Kandi, and Banikoara) with treatment calendar, out of 200 mosquitoes tested in each locality with permethrin, a mortality percentage of 22% on average in each of these three localities was recorded. These percentages indicate a high level of resistance of *An. gambiae* S.L. populations from the cotton areas to treatment calendar (Fig. 2). The same observation was made in the TSC treated cotton fields where *A. gambiae* populations from all the sites chosen developed resistance to permethrin with an average of 25% mortality. On the other hand, *An. gambiae* S.L. populations from cotton fields with an organic programme developed a suspicion of resistance with an average of 94% mortality.

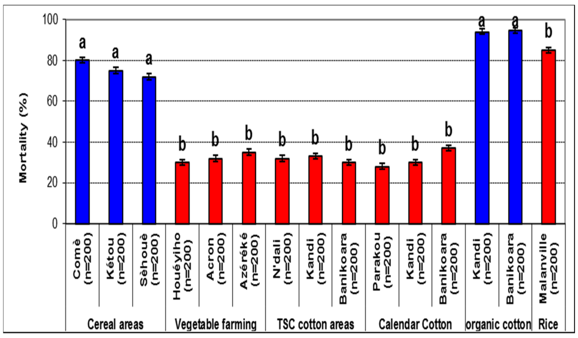

Fig. 2. Mortality observed after exposure of populations of *A. gambiae* S.L. to permethrin. (Mortality rates with the same superscript letter are not significantly different P>0.05).

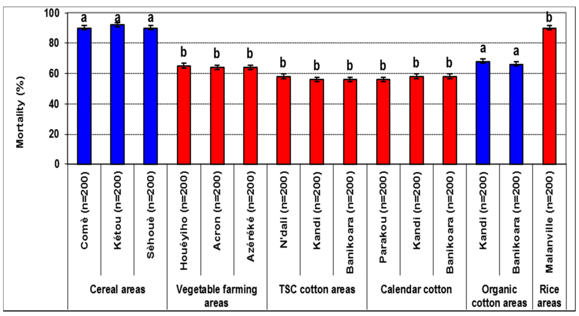

Fig. 3. Mortality observed after exposure of populations of *A. gambiae* S.L. to deltamethrin.

In the rice-growing area, *A. gambiae* S.L. populations from the Malanville rice-growing area were resistant to permethrin. Indeed, out of 200 *A. gambiae* S.L.

females subjected to permethrin, a mortality rate of 85% was observed with permethrin. Moreover, Populations of *A. gambiae* S.L. from cereal-growing areas were also resistant (79% mortality) to permethrin (Fig. 2). The same trend was observed with the deltamethrin insecticide (Fig. 3).

Regarding DDT, the level of resistance is very high. It varies from 10% to 13% in all of the three market gardening sites, the cotton calendar treatment sites and the TSC cotton sites (Fig. 4).

The resistance was also observed from biologically treated cotton sites with mortality rates varying between 55% and 65% (Fig. 4). The same scheme of resistance was observed in the rice-growing and cereal-growing areas, with respective mortality rates of 54 and 48% (Fig. 4).

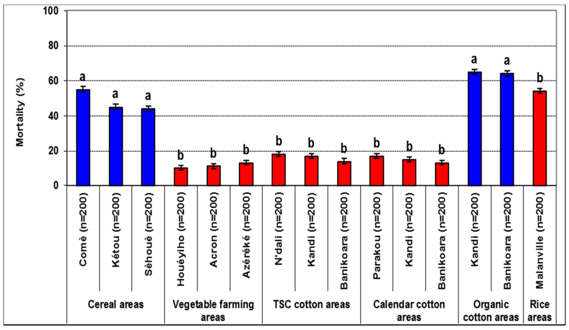

Fig. 4. Mortality observed after exposure of populations of *A. gambiae* S.L. to DDT.

Mechanism of resistance: PCR species, Kdr and Ace-1

In each locality, 200 mosquitoes were analysed for different species of the complex of *A. gambiae* and Kdr and Ace-l resistance mechanisms. In vegetable farming (Acron and Houeyiho), cereal (Kétou; Comè and Sèhouè) and rice production area (Malanville) sites, all the mosquitoes analysed by the "Polymerase Chain Reaction (PCR)" were *Anopheles coluzzii* species. On the other hand, the populations of *A. gambiae* S.L. from the vegetable farming site of Azèrèkè after screening were composed of *A. coluzzii* (65%) and *Anopheles arabiensis* (35%). In the cotton sites, *A. coluzzii* represented an average of 75% against 25% of *A. arabiensis* regardless of the phytosanitary protection strategy (Table 1)

Concerning the mechanism of resistance, the Kdr mutation seems to be the main mechanism of resistance observed within these populations of *A. gambiae* S.L. with a high frequency (Table I) in mosquitoes from cotton sites with treatment calendar (an average of 0.9), TSC (0.85) and in vegetable farming sites (0.9). However, an average of 0.45 frequencies of this mutation was recorded in *A. gambiae* populations from rice, cereal and organic cotton growing sites where no insecticide was requested for crop protection.

The *Ace-1R* mutation was detected but at very low frequencies ranging from 0.00 to 0.06.

Table 1. Distribution of species, the Kdr and Ace-1 frequencies of *A. gambiae* S.L. from the study areas.

		Species PCR		*Kdr_Pcr*	Pcr *Ace-1*
	Areas	% Aa	% AC	Frequency	Frequency
Market gardening areas	Houeyiho	0	100	0,81**	0,002
	Acron	0	100	0,80**	0,001
	Azèrèkè	35	65	0,88**	0,004
Cotton calendar treatment areas	Parakou	22	78	0,92**	0
	Banikoara	3	97	0,91**	0,05
	Kandi	5	95	0,92**	0,06
LEC cotton areas	N'dali	30	70	0,84**	0,02
	Kandi	4	96	0,87**	0,02
	Banikoara	2	98	0,86**	0,03
Organic cotton areas	Kandi	5	95	0,56*	0
	Banikoara	8	92	0,52*	0
Rice-growing areas	Malanville	0	100	0,4*	0
	Comè	0	100	0,54*	0
Cereal areas	Sèhouè	0	100	0,56*	0
	Kétou	0	100	0,55*	0

Aa= An. arabiensis; Ag= An. gambiae

(**) The frequency of the Kdr and Ace-1 gene in cotton and vegetable growing areas is much higher than that (*) in growing areas without insecticides (P <0.05).

Discussion and conclusion

The main malaria vector in Africa, *An. gambiae* S.L., has developed strong resistance to insecticides in areas with high and low agricultural insecticide use (cotton, vegetable farming, rice and cereal growing areas) in Benin. This resistance is mainly observed with pyrethroids and DDT. Contrary to what we were expecting, this resistance was observed not only in urban but also in rural areas with traditional cereal farming which does not require agricultural insecticides or fertilizers. The cross-resistance to pyrethroids and DDT observed in cotton, vegetable farming areas (except for organically treated areas), cereal and rice cultivation areas is due to the intense use of pesticides against crop pests in these agricultural settings. Indeed, farmers' practices in the use of insecticides in cotton growing areas to control *Helicoverpa armigera* (the main pest of cotton) and in vegetable farming areas especially and *Plutella xylostella* (the main pest of cabbage) seem to be a factor in the selection of resistant insects not only in crop pests but also in malaria vectors (Akogbeto *et al.,* 2005). After insecticide treatments, pesticide particles come into contact with the breeding sites. These particles exert either a lethal action on the larvae of certain insect populations or a selective pressure that progressively leads to the selection of resistance to insecticides in certain mosquito populations, particularly in *A. gambiae* (Yadouleton *et al.,* 2009, 2018). These authors showed that the massive use of pesticides in agriculture in Benin is due to the liberalisation of the input sector in Benin. In the 1960s, the selective role of organochlorine (OC) agricultural treatments on the resistance of *An. gambiae* s.l was observed in Mali in areas that had never been subject to public health treatments but where these insecticides were widely used in agriculture. In Côte d'Ivoire and Burkina Faso, it was shown in the late 1990s that the level of resistance of *A. gambiae* S.L to pyrethroids increased during the cotton season (Namountougou *et al.,* 2019).

Anopheles is a mosquito that lays its eggs in small puddles. Populations are high during the rainy season. The presence of insecticide residues on the surface layer of cotton soils is often sufficient to contaminate the puddles and lead to the selection of mosquitoes during their larval life. It is established that pyrethroids which are the only insecticides recommended by the WHO for the impregnation of mosquito nets are also used in agriculture for pests control, which creates resistance problems in malaria vectors and agricultural pests, a situation which complicates malaria control. Permethrin resistance in *A. gambiae* S.L. populations in the organic program may be related to the fact that the current organic program sites were cotton treatment calendar plots ten years ago, where huge doses of insecticides were used to suppress cotton pests.

Moreover, the high frequency of the Kdr gene in the insecticide-treated sites compared to the control is similar to the results of Diabaté *et al.* (2002). These authors showed that the frequency of Kdr resistance genes in *A. gambiae* is higher in cotton-growing areas usually subjected to insecticide treatments than in rural areas where farmers only grow cereal crops. The Kdr mutation is thought to be responsible for the selection of *A. gambiae* resistance to DDT and pyrethroids in West Africa. This resistance is also enhanced by a high activity of detoxification enzymes. These few examples of resistance of *A. gambiae* populations in several agricultural settings confirmed the hypothesis of several authors on the contribution of agriculture in the selection of malaria vectors resistance to insecticides.

Kdr resistance is not the only resistance mechanism in southern Benin. Research conducted by Aikpon *et al.* (2014); Padonou *et al.* (2012) in southern Benin showed the existence of the *Ace-1* mutation, but at a very low frequency. The absence of phenotypic resistance to bendiocarb, manifested by low frequencies of *Ace-1* mutation is a hope for Benin, which is investing in the search for alternatives to pyrethroids in vector control based on Indoor Residual Spray with bendiocarb (Akogbéto *et al.,* 2010). However, it is recommended that the evolution of the situation be periodically monitored to detect the slightest change.

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Conflict of interest
No conflict of interest to declare.

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