ORIGINAL RESEARCH

Development of rice farming: a cause of the emergence of multiple insecticide resistance in populations of *Anopheles gambiae s.l* and its impact on human health in Malanville, Bénin

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Abstract

Aim

The rise in rice production in the district of Malanville, Northen Benin, is a present concern, as it has resulted in the widespread usage of pesticides for crop protection. This could impact human health but also life cycle of *Anopheles gambiae*, the main vector of malaria.

Methods

Therefore, insecticide susceptibility bioassays were carried out on populations of *An. gambiae* s.l aged to 3-5 days old (two from areas where insecticide is highly used and other two areas of low insecticide use) and subjected to insecticide-impregnated papers (Permethrin 0.75%; deltamethrin 0.05%; DDT 4% and bendiocarb 0.1%) following WHO protocol. Polymerase Chain Reactions (PCRs) were used for the detection of Acethlylcholinestrase (Ace-1) and the knock down resistance (kdr) L1014F mutations in *An. gambiae* populations. Finally, indirect bioassays were conducted for the investigating on the factors affecting the life cycle of *An. gambiae* due to the use of pesticides.

Results

An. gambiae from the four sites were resistant to DDT (6 to 8% and 10 to 14% respectively from areas of high and low dose), pyrethroids (22 to 26% and 30 to 36% for permethrin, from areas of high and low dose respectively and 66 to 70% and 72 to 80% for deltamethrin, from high and low dose) but susceptible to carbamate. The kdr L1014F mutation was detected in *An. gambiae* populations (0.88 to 0.90 and 0.84 to 0.88 from high and low dose, respectively). The ace-1 was detected at low frequencies (<0.002). Bioassays on the impacts of the use of pesticides in the life cycle of *An. gambiae* showed that soil substrates with pesticides residues have a negative impact on the life cycle eggs of *An. gambiae*.

Conclusion

These findings confirmed the negative impacts of pesticides use in rice farming and its impacts on the life cycle of *An. gambiae.*

Key words: Insecticides, Rice, Resistance, life cycle, *Anopheles gambiae*, Benin

Introduction

In recent years, there has been an increase in rice cultivation in Africa[1]. The reasons for this occurrence include low soil fertility due to many years of cultivated lands located far away from cities, rural exodus, unemployment, and changes in dietary habits. In Benin, the government's approach to fight food insecurity has relied on intensive lowland rice farming[2,3]. Since then, marshes have been built throughout the country for rice production. Indeed, this activity has become a new vocation for many people in the society: men, women, youth, unemployed graduates, and even civil workers benefit immensely from it[2]. According to Kondja et al[4], rice contributes more calories and protein than any other cereal in sub-Saharan Africa and as much as all root and tuber crops combined.

However, in terms of health, the expansion of lowlands for rice farming results in a huge number of trenches that retain rainwater. During the rainy season, these trenches are ideal mosquito breeding sites, particularly for *Anopheles gambiae*, Africa's primary malaria vector[5,6]. In addition to these areas, farmers construct water dams for agriculture irrigation. These uncovered water wells and pits are often favourable for eggs laying and the growth of *An. gambiae* larvae, the primary malaria vector[7,8]. Furthermore, rice cultivation is subject to attacks by a number of pests and pathogens, and farmers use a variety of insecticides, including pyretrinoids, to control them[5]. The weekly application of insecticides causes insecticide particles to come into contact with soil and breeding sites, and with mosquito larvae. The pressure exerted by these insecticides could lead to the development of resistance in mosquitoes, particularly *An. gambiae*, to these

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insecticides[8,9]. Moreover, a KAP (Knowledge, Attitude and Practices) study on the use of insecticides in rice growing areas conducted by Yadouleton et al.[10] have shown the presence of pyrethroids, carbamates and organophosphates classes of insecticides in rice cultivation areas for pest control.

Many scientists reported that the use of the same classes of pesticides in agriculture for pest control and in public health particularly on the impregnation and reimpregnation of long-lasting insecticidal nets (LLINs), in the various formulations for mosquito control could contribute to the increase in the allelic frequencies of acethylcholinesterase (G119S Ace-1) and the knock down resistance (kdr L1014F) in *An. gambiae*[11,12].

Therefore, the improper manner of these all classes of insecticides cited above in rice cultivation areas will contribute to the emergence of multiple insecticide resistance in An. gambiae population and latter would also have an impact on *An. gambiae's* development cycle. The current study aimed to understand the impacts of pesticides use in rice farming and its impacts on the life cycle, as well as the emergence of multiple insecticide resistance in *Anopheles gambiae* at Malanville, a district located in North-east of Benin.

Material and Methods

Study areas

The study was carried out in northern Benin in the district of Malanville from June to October 2022 in the rice-growing areas of Malanville. Two rice-growing areas with high insecticide use (Bodjekali A and Bodjekali B) and two other with low insecticide use (Tomboutou A and Tomboutou B) located in the district of Malanville were selected for data collection.

In fact, the selected rice-growing sites of Mounin A and Mounin B are characterised as sites with high pesticide use against rice pests, since rice farmers on these sites use several families of insecticides, especially those intended for cotton cultivation, including pyrethrinoids and carbamates. In addition, to fight crop pests, farmers at the Mounin A and Mounin B rice-growing sites apply four insecticide treatments (cyfluthrin + cyfluthrin cypermethrin + dimethoate) from the transplanting phase to the harvest phase at four different time intervals. In contrast, the rice-growing areas of Toumboutou A and Toumboutou B are characterised as areas of low pesticide use against rice crop pests, as farmers use a small quantity of insecticide (only deltamethrine) limited to just 2 treatments throughout the rice production cycle.

Malanville is located in the far north of Benin Republic. The climate is Sudanese-Sahelian type and characterised by a dry season from November to June. The average rainfall is 750 mm. The main vegetation is savannah with grassland. The main agricultural activity is rice cultivation.

Mosquito collection

Anopheles mosquito's larvae were collected from the four study sites (Bodjekali A, Bodjekali B, Toumboutou A and Toumboutou B) from July to October 2022. In each of the four experimental sites, five larval breeding sites were chosen for sampling twice a week in order to maximise the number of larvae. All *Anopheline* larvae collected from the four study sites were brought to the insectary and reared. Adults aged between 3-5 days were subjected to susceptibility tests.

Insecticide susceptibility tests

Susceptibility tests were performed using cylinders' tubes base on WHO protocol[13]. In this study the following insecticide-impregnated papers were tested: DDT (4%), permethrin (0.75%), deltamethrin (0.05) and bendiocarb (0.1%). Adult female mosquitoes aged between 3-5 days from each of the four study sites were exposed to each paper impregnated with insecticides cited above and repeated four times. Practically, 20 to 25 female adults were randomly chosen from each batch and exposed to impregnated papers for an hour. All knocked-down mosquitoes were recorded every 10 min and after one hour of exposure, the mosquitoes were transferred into observation tubes[13]. Mosquitoes that were not exposed to the insecticides were used as control subjects. 24h post exposure, mortality rate was estimated and interpreted using WHO's protocol as a reference[13].

At the end of the bioassay, both dead and alive mosquitoes were kept in Eppendorf tubes at -20^{o}C for species identification and determination of resistance mechanisms (kdr L1014F and G119S Ace-1) using the PCR technique. The reference strain Kisumu was used as a control susceptible strain in all bioassays.

Molecular analysis

Following the protocol described by Yahouedo et al.[14], Cetyl Trimethyl Ammonium Bromide (CTAB) 2% was used for DNA extraction Genomic from individual mosquitoes. The DNA extracted was used to detect the different species of the complex An. gambiae s.l but also the knock down resistance (L1014F kdr) and the acetylcholinesterase (ace-1R G119S) mutations.

Detection of kdr and ace-1 mutations

100 mosquitoes from the susceptibility tests were used for the kdr detection following the protocol described by Bass et al.[15] .Two primers, Agd1 (5'-ATA GAT TCC CCG ACC ATG-3') and Agd2 (5'-AGA CAA GGA TGA TGA ACC-3') were used to amplify the sequence of 293 base pairs (bp) in all An. gambiae (s.l.) mosquitoes. The resistant sequence of 195 bp and the sensitive sequence of 137 bp were amplified respectively using specific primers Agd3 (5'-AAT TTG CAT TAC TTA CGA CA-3') and Agd4 (5'-CTG TAG TGA TAG GAA ATT TA-3'). These three bands of different sizes are easily resolved on 1.5% agarose gel and thus enable the easy determination of the genotype of each mosquito. The G119S-ace1 mutation was also screened based on the protocol of Bass et al.[15]. The DNA was amplified with the primers Ex3Agdir (5'-GAT CGT GGA CAC CGT GTT CG-3') and Ex3Agrev (5'-AGG ATG GCC CG CTG GAA CAG-3') for an initial denaturation step of 3 min at 94 °C, followed by 35 cycles (94 °C for 30 s, 62 °C for 30 s and 72 °C for 20 s). After the final cycle, the products were extended for 5 min at 72 °C. The PCR fragments were then digested with AluI restriction enzyme and fractionated on a 2% agarose gel. Base on the protocol described by Bass et al.[15], the susceptible homozygous mosquitoes (SS), the homozygous resistant (RR) and the Heterozygous individuals (RS) were detected following the base pairs.

Impacts of pesticides on the development of An. gambiae larvae

The method used was not intended to be a direct determination of pesticide residues in the breeding sites. Instead, an indirect assessment was carried out to check for

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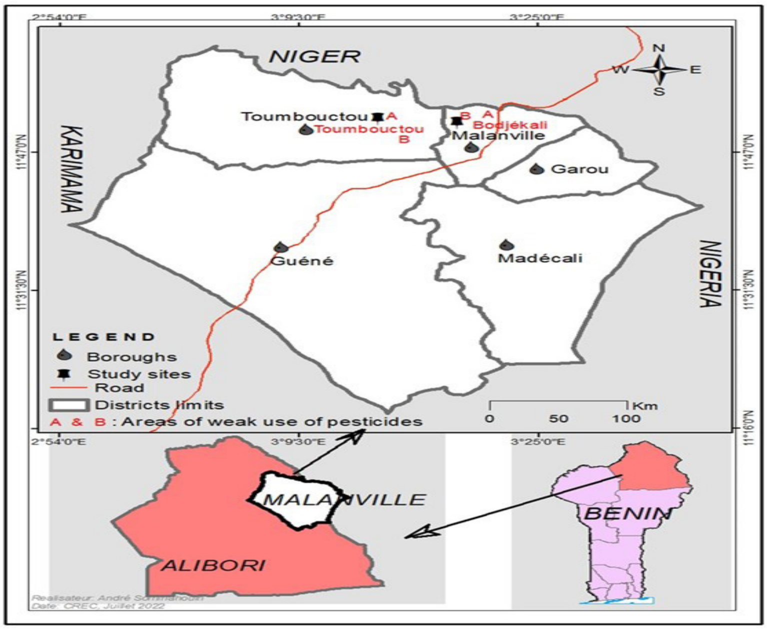

Figure 1. Map of Benin showing the study area

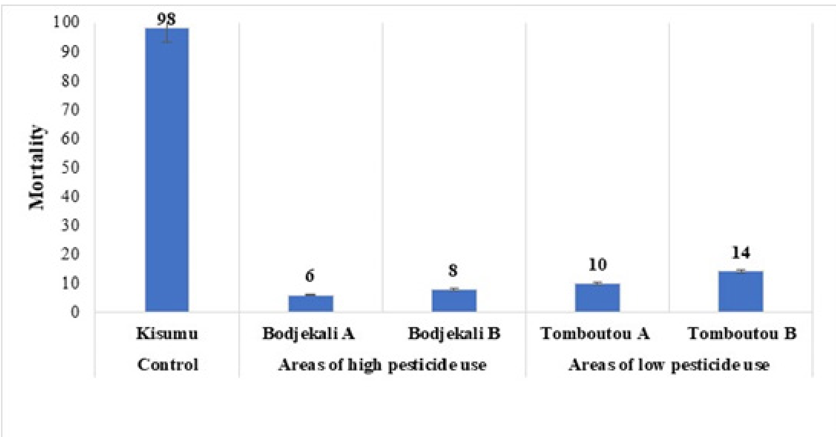

Figure 2: Mortality rates of *Anopheles gambiae s.l.* to DDT 4% using WHO bioassay tests

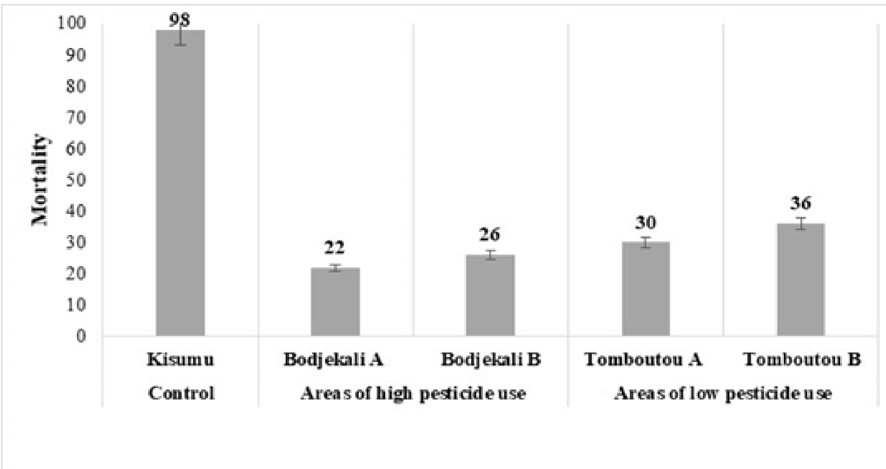

Figure 3: Mortality rates of Anopheles gambiae s.l. to permethrin 0.75% using WHO bioassay tests

the presence of negative factors that could inhibit the normal development of mosquito larvae in the breeding sites. We studied eggs hatch rate and larval development in *An. gambiae* from breeding sites made with soil substrates from insecticide-treated areas and tap water following the protocol described by Yadouleton et al.[16]. Results of this breeding sites were compared to those of a control (a mixture of tap water + soil from an area where no insecticide was used).

For this purpose, four soil substrates were used, two from an area with high insecticide use (Bodjikali A; Bodjikali B) and two from another rice-growing area with low insecticide treatment (Toumboutou A, Toumboutou B). In these breeding sites, we inoculated *An. gambiae* eggs to monitor the impacts of pesticides on the development of *An. gambiae* larvae. Results of these rearing environments were compared to those of a control environment (a mixture of tap water + soil from an area without any agricultural activity where no insecticides were used). Each breeding site consisted of 100 grams of soil mixed with 1000 ml of water. Approximately 200 *An. gambiae* eggs of the susceptible Kisumu strain were counted with a magnifying glass and transferred to each breeding site. The same procedure was carried out with mosquitoes from the resistant strain (VKPER).

Daily monitoring of the variation in egg hatch rate and the frequency of adult appearance was recorded. Data from the supposedly contaminated breeding sites were compared to those from the control to verify the possible existence of a factor inhibiting the development of the larvae.

Data analysis

Based on WHO protocol[13], *An. gambiae* population is considered as susceptible to the insecticide if the mortality rate is between 98-100%, but the resistance is suspected in the population of the mosquito when the mortality is between 90-97%. Below 90% mortality rate, the population is considered resistant. Genepop007[17] software was used to evaluated the allelic frequencies of kdr L1014F and G119S Ace-1.

Moreover, the Kruskal-Wallis test was used to compare the frequency between treatments and laboratory-susceptible mosquitoes (Kisumu).

The resistance allele frequency at the kdr and Ace- 1 locus was calculated using Genepop software (version 3.3) as described by Raymond and Rousset.

A Fisher's exact test was performed to compare the resistance allele frequency at the kdr and Ace- 1 among the mosquitoes from the different strategies.

An analysis of variance (ANOVA) was performed to compare the percentages of hatching eggs in the different treatments in order to know the impact of insecticide on the normal growth of mosquito larvae in cotton breeding sites.

Results

Results from this study showed that despite the use of insecticide, all *An. gambiae* from the four sites were resistant to DDT (6 to

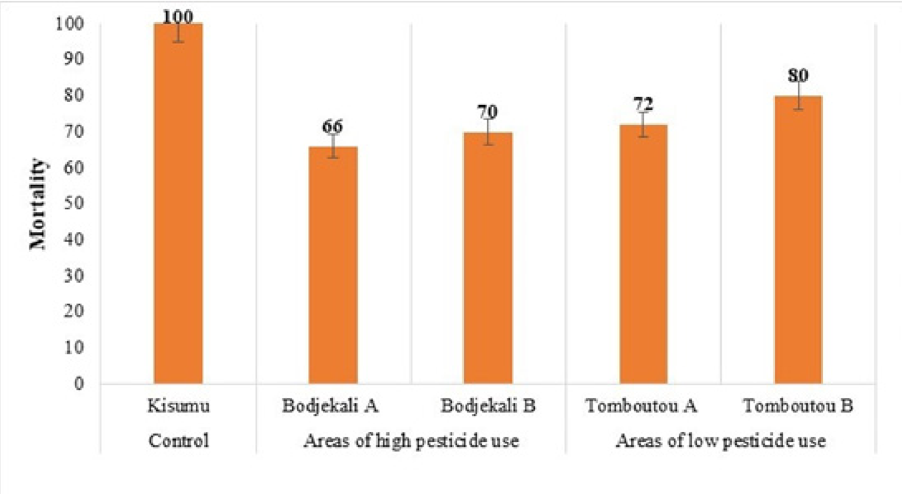

Figure 4: Mortality rates of Anopheles gambiae s.l. to deltamethrin 0.75% using WHO bioassay tests

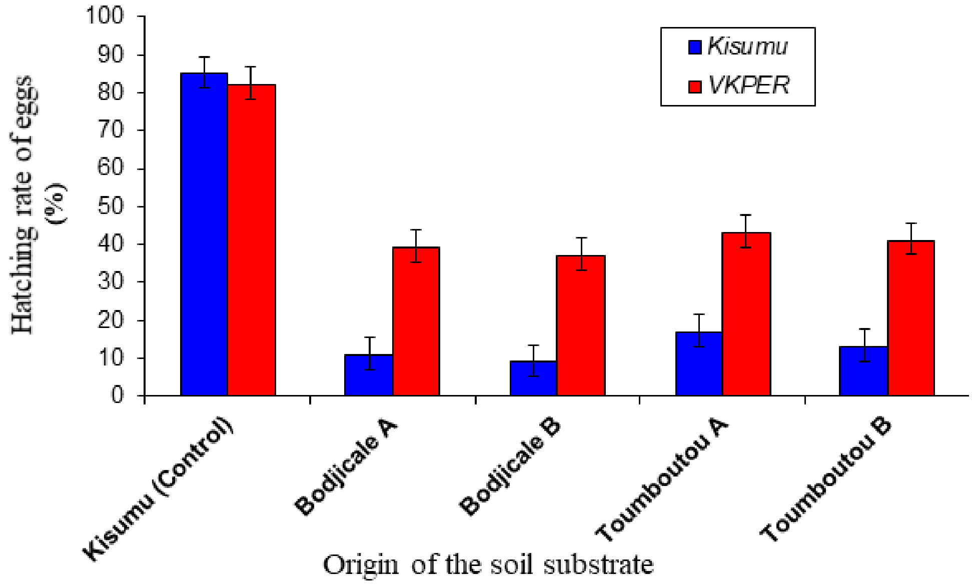

Figure 5: Hatching rate of Anopheles eggs from the different breeding sites simulations

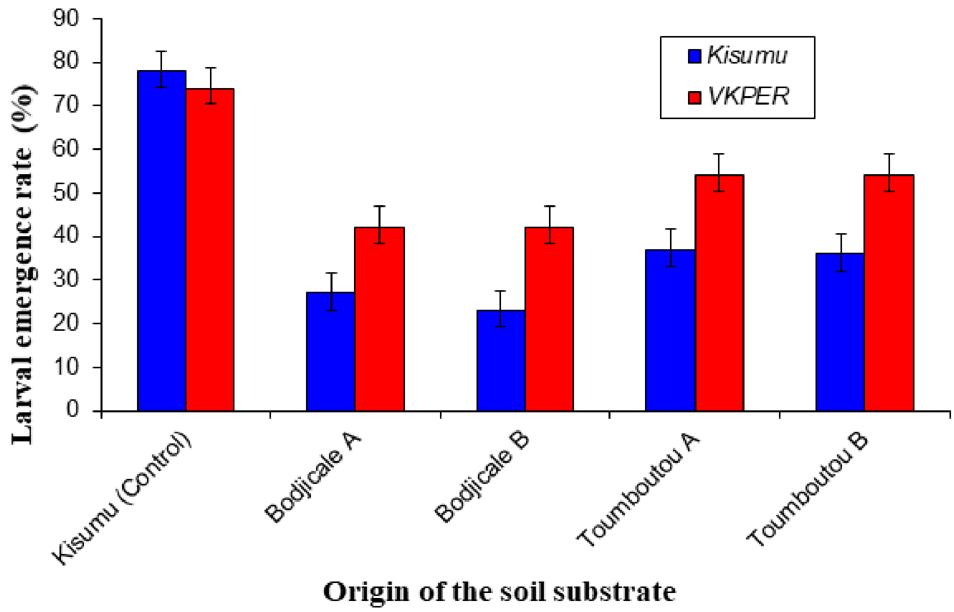

Figure 6: Effect of insecticides on the emergence of Anopheles larvae

8% and 10 to 14% respectively from areas of high and low dose), pyrethroids (22 to 26% and 30 to 36% for permethrin, from areas of high and low dose respectively and 66 to 70% and 72 to 80% for deltamethrin, from areas of high and low dose, respectively) but highly susceptible to carbamates in all sites.

Species identification and allelic frequencies of the kdr and ace-1 mutations. From the four experimental sites (Bodjikale A, Bodjikale B, Toumboutou A and Toumboutou B), 85% of the mosquitoes tested by Polymerase Chain Reaction (PCR) belonged to the species *An. arabiensis.* (Table 1). The Kdr gene appears to be the main resistance mechanism detected in these *An. gambiae* populations, with a high frequency irrespective of the use of the insecticide (0.88 to 0.90 and 0.84 to 0.88 from areas of high and low dose, respectively) (Table 1). The ace-1 mutations were also detected, but at low rates ranging from 0.02 to 0.04.

Impacts of agricultural insecticide application on An. gambiae eggs hatching

The tap water plus soil of the control provides relatively favourable conditions for hatching and larval development. Indeed, the average hatching rate (figure 5) of eggs from the control sites was 85% (n=200) for the Kisumu strain and 82% (n=200) for *An. gambiae* VKPER. However, when the control soil is replaced by soil substrates taken from the two rice-growing areas (Bodjikale A and B) where crops are grown with high use of insecticide, the average hatching rate from VKPER strain dropped to 38% but significantly higher than those from susceptible strain Kisumu which dropped to 10%. The same trend was observed (VKPER eggs (42%) and Kisumu eggs (15%) when the breeding sites were prepared with soil from Toumboutou (Toumboutou A and B) (figure 5).

Impacts of agricultural insecticide treatments on the development of An. gambiae larvae

Larval development was measured by considering the proportion of eggs that reached the pupal stage after being placed in water. This parameter is one of the indicators to evaluate the possible existence of a limiting factor which could maybe slow down larvae development of the larvae in the

Table I: Distribution of species and Kdr and Ace-1 frequencies of An. gambiae s.l from the study areas

SITES	Localities	PCR_Species		Pcr_Kdr	Pcr Ace-1
		% An. arabiensis (Aa)	%An. gambiae s.s. (Ag)	frequency	frequency
Rice growing areas with high insecticide use	Bodjikali A	86	14	0.88**	0.002
	Bodjikali A	84	16	0.90**	0.004
Rice-growing areas with low insecticide use	Toumboutou A	88	12	0.84**	0.002
	Toumboutou B	86	14	0.86**	0.001

Aa **:** *An. arabiensis*

Ag : *An. gambiae*. s.s

cottages. Certainly, this parameter takes into account the stay of the larvae in the cottages. Contrary to the very limited time that the egg takes to hatch in the water, the duration of this stay is sufficient for the larvae to develop.

In the control breeding sites, an average of 78% (Kisumu) and 74% (VKPER) of the larvae reached the adult stage (Figure 6). The breeding sites made from soil from the rice-growing areas of Bodjikale (Bodjikale A and Bodjikale B) led to lower larval development (an average of 25% in Kisumu and 42% in VKPER). There was a significant difference between the emergence rates of susceptible and resistant strains when the breeding sites was prepared from a soil sample from a treated environment ($P<0.05$). The same trend was observed when the breeding sites were made from soil from the rice-growing areas of Toumboutou (an average of 38% in Kisumu and 54% in VKPER).

Discussion

Malaria is a serious public health problem in many regions of the world, with Sub-Saharan Africa accounting for more than 90% of recorded cases[18]. Long-acting insecticide-treated nets (LLINs) are currently regarded as one of the most efficient mosquito vector protection methods, particularly in high-endemic areas[19]. However, N'Guessan et al. reported a reduction in the efficiency of LLINs against insecticide-resistant wild populations of *An. gambiae* in experimental huts in southern Benin[20]. This decrease in LLIN efficacy is assumed to be owing to the establishment of malaria vector resistance to pyrethroids, the principal insecticides used for both mosquito net impregnation and indoor spraying[21,22].

In the late 1980s, malaria vector resistance to insecticides was documented in Benin, along with resistance to organochlorines. The withdrawal of organochlorines in favour of pyrethroids was anticipated to alleviate some of the vector resistance issues. However, the misuse of pyrethroids in the agricultural environment, particularly in the rice-growing zone at high doses to control crop pests, has greatly contributed to the selection of resistant insects and pests that have developed multiple and cross-resistance to pyrethroids (PY) and DDT, as mentioned by several authors[23,24]. In our study, *An. gambiae* resistance to pesticides varies from one insecticide to the next in rice-growing areas. Our investigation on high and low pesticide use areas revealed that most *An. gambiae* s.l populations are resistant to DDT (organochlorine) and PY but still susceptible to carbamates. This phenotypic resistance of *An. gambiae* s.l. populations to DDT and PY observed in the four rice-growing sites is correlated with the frequency of the Kdr mutation, implying that the Kdr mutation is the major mechanism of cross-resistance to these two classes of insecticides (organochlorines and PYs) in *An. gambiae,* as suggested by many other studies[25,26]. Furthermore, the work of Nanmoutougou et al. in Burkina-Faso showed that in An. gambiae, the frequency of resistance genes (, Kdr) is higher in cotton-growing areas usually subjected to insecticide treatments than in rural areas where farmers only grow food crops for local consumption[27]. In Côte d'Ivoire, the Kdr mutation found in *An. gambiae* was probably selected by high dose of DDT and pyrethroids against cotton pests[28].

In addition to pyrethroid resistance, we observed low resistance of *An. gambiae* to carbamates (Ace-1R) in the four rice-growing sites, confirming the results of Ossé et al.[29] and Yadouleton et al.[30] in Benin. This identification of the Ace1R gene should be closely monitored, for some years in Benin, the National Malaria Control Programme has been using carbamates and organophosphates as indoor sprays as part of its malaria vector control strategies. This situation could increase the level of resistance of the Ace-1R gene in rice production in the coming years. Indeed, Yadouleton et al. demonstrated that in Benin, most farmers employ insecticides from the carbamate and PY families that are not approved for rice cultivation[31]. Continuous monitoring in rice-growing areas treated with these insecticides is therefore critical in order to track the evolution of the Ace-1R gene in relation to the original condition.

While it is clear that resistance to DDT and PY could have been selected by insecticide spray in agricultural areas, it is likely that resistance could have been selected by factors in the agricultural sector or in public health that could influence the life cycle of *An. gambiae* by inhibiting its development. Indeed, farmers' practices in the use of insecticides in agricultural areas, particularly in rice-growing areas, to control rice crop pests are a factor in the selection of resistant insects not only in crop pests but also in malaria vectors[31].

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 and progressively lead to the selection of insecticide resistance in certain mosquito populations, notably in An. gambiae[30]. Indeed, the presence of insecticide residues in the surface layer of soils in rice-growing areas is often sufficient to contaminate puddles and lead to mosquito selection during their larval life. Our results revealed a

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significant decline in the hatching rate and larval growth of the reference strain Kisumu when the soil of breeding site is from insecticide-treated rice-growing areas. In contrast, this decline is moderate with the resistance strain VKPER. It is likely that the multiple insecticide application in rice-growing areas have selected factors that are responsible for the low level of egg hatching on the one hand, but also for the low level of larval development on the other.

Overall, the results of this research have shown that although rice cultivation offers many economic advantages, it is also a source of intensive use of insecticides to control crop pests. This situation leads to resistance from malaria vectors, particularly An. gambiae, which complicates malaria control. As malaria is a public health disease that kills more children aged 0–5 and pregnant women, it is important to focus on pesticide management in rice-growing areas so that mosquito nets will play their role as a chemical barrier to limit malaria transmission

Conclusion

The findings of this study emphasize the effects of pesticide use in rice farming on the life cycle, as well as the emergence of multiple insecticide resistance in *An. gambiae* in Malanville. It would be important to consider a concerted rotation of insecticides in order to limit the spread of resistance genes and mechanisms to proportions where their use would no longer exert pressure of resistance on mosquito populations.

Conflict of Interest

The authors declare that there is no conflict of interest associated with this study.

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