RESEARCH ARTICLE

REVISED Short-term metabolic resistance inductive effect of different agrochemical groups on *Anopheles gambiae* mosquitoes [version 2; peer review: 1 approved, 1 approved with reservations]

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Abstract

Background: In order to assess the impact of the different groups of agricultural pesticides used in Côte d'Ivoire on the increase of mosquitoes resistance to insecticides, the expression profiles of 7 P450 cytochromes and one GSTE2 of *Anopheles gambiae* involved in mosquito resistance to insecticides were studied. The goal of this study was to determine the effect of short exposure of mosquito larvae to different groups of agricultural pesticides on mosquito resistance.

Methods: Three groups of pesticides were selected: (i) agricultural insecticide solutions, (ii) non-insecticide pesticide solutions (a mixture of herbicides and fungicides), and (iii) a mixture of the first two. A fourth non-pesticide solution was used as a control. Four groups of each stage 2 larvae (strain Kisumu, male and female) were exposed to 20% concentrated solution for 24 hours. Susceptibility tests for dichlorodiphenyltrichloroethane (DDT) and Deltamethrin were carried out on adults aged 2-5 days. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed to quantify the expression of eight metabolic genes involved in mosquito resistance to insecticides.

Results: Susceptibility to DDT showed a similar increase in the time required to knock down 50% of mosquitoes (k_{dt50}) in I colonies exposed to insecticides and non-insecticides compared to the control colony. As for deltamethrin, k_{dt50} was higher in the colonies exposed to insecticides and the pesticide mixture compared to the colony

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exposed to non-insecticides. Of all the genes studied in all colonies, except for CYP6P1 induced only in the colony consisting of the pesticide mixture, no genes were induced.

Conclusions: This study confirmed that induction is influenced by the duration, the concentration of the solution and the type of xenobiotic used as an inducer. The overexpression of CYP6P1 confirmed the inductive effect that a short exposure of mosquito larvae to agricultural pesticides could have.

Keywords

Induction gene, *Anopheles gambiae*, cytochromes 450 and GSTE2, agricultural pesticides, Côte d'Ivoire

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REVISED Amendments from Version 1

In this manuscript, we have added the results of the mortality of the susceptibility tests. Also, we have corrected some sentences.

Any further responses from the reviewers can be found at the end of the article

Introduction

Vector control through the use of insecticides is one of the strategies put in place by the World Health Organisation (WHO) to fight malaria transmission. Despite the huge decrease in malaria cases and deaths between 2000 and 2015 following the implementation of this strategy (World Health Organization, 2015). Malaria in sub-Saharan Africa and is often linked to human activities and climatic conditions that contribute to the proliferation of larval deposits. Rice and vegetable fields create the most favorable biotopes for the proliferation of various malaria vector species in different major geographic regions (Dossou-Yovo *et al.*, 1998). The profound and permanent eco-system changes for irrigated rice fields establishment of favor the proliferation of *Anopheles* and impact malaria transmission (Koudou *et al.*, 2007; Robert *et al.*, 1991). One major consideration with two implications will further influence malaria transmission. Indeed, the food security which is one of the current priorities that the Food and Agriculture Organization of the United Nations (FAO) faces with the challenge of climate change will incite agricultural overproduction to meet the expectations of the world's future population. This overproduction suggests an expansion of rice growing areas, which as mentioned above, remain niches for the proliferation of malaria vectors, and an intensification of use of agricultural pesticides (Osta *et al.*, 2012). Intensification of use of agricultural pesticides can contribute to the development of insecticide resistance in the mosquitoes breeding in cultivation areas as molecules used are the same as those used for vector control. Field and laboratory studies have shown the involvement of pesticides used in agriculture in the development of mutational and metabolic resistance in mosquitoes (Fodjo *et al.*, 2018; Guy Bertrand, 2019; Nkya *et al.*, 2014). In Côte d'Ivoire, data on metabolic resistance in mosquitoes are scarce. Resistance studies are most heavily based on the *kdr* and *ace1* mutation genes and the use of synergists (Camara *et al.*, 2018; Zoh *et al.*, 2018) whereas an increase in the *kdr* gene frequency does not necessarily lead to the development of resistance (Sadia-Kacou *et al.*, 2017). Use of synergists has been described by Chouaïbou *et al.* (2014) as a simple and initial method to detect metabolic resistance in field population of mosquitoes. This method has been used to investigate the involvement of metabolic resistance in the Tiassale mosquito population from Cote d'Ivoire (Chouaïbou *et al.*, 2014); however, synergists do not specify the gene responsible for resistance but rather give the families of genes involved (Moore *et al.*, 2009). Data on the metabolic resistance is a prerequisite for the effective implementation of control interventions (Adolfi *et al.*, 2019). Furthermore, identifying the source of metabolic resistance is of the utmost importance to mitigate development and spread of this resistance. Côte d'Ivoire is one of the

10 African countries that import the most agricultural pesticides (Guy Bertrand, 2019). Recent studies have categorized their use according to crop type, the country, and their roles in increasing insecticide resistance in malaria vectors (Fodjo *et al.*, 2018). However, the studies carried out by these researchers do not described the insecticide resistance selective effect of pesticide use in Cote d'Ivoire. In the current study, we investigate the short term metabolic resistance inductive effect of non-insecticide- and insecticide-pesticides used in agriculture in Cote d'Ivoire. Seven P450 cytochromes and one Glutathion-S-Transferase known to be associated with mosquito resistance to insecticides were evaluated and their expression was characterized on *An. gambiae* Kisumu mosquito colonies at the larval stage. The study aimed to provide a better understanding of resistance and resistance source and provide data useful for planning and implementing a suitable vector control strategy.

Methods

Choice of agricultural pesticides and mosquito induction

The parental strain of mosquito used in this study was *Anopheles gambiae* originating from Kisumu, which is susceptible to insecticides used in public health. The 2–6 day old parental strain was fed with blood. The larvae from this laying were divided into four colonies of which three were exposed and one non exposed to serve as negative control. Among the exposed groups, one was exposed to insecticides, one to non-insecticides and the last one to a mixture of insecticides+non-insecticides. The choice of these pesticides was based on surveys of farmers in Côte d'Ivoire to identify the pesticides commonly used by farmers (Chouaïbou *et al.*, 2016). From this survey, two types of agrochemicals are used: agricultural insecticides (pyrethroids, organophosphates and carbamates) and non-insecticide products (herbicides and fungicides) (Table 1). Mosquitoes were reared (in september 2020) in Centre Suisse de Recherches Scientifiques laboratory in $27 \pm 2^\circ\text{C}$, $80 \pm 10\%$ relative humidity and photoperiod 12h/12 h light/dark,

Preparation of stock solution

A starting solution called stock solution was prepared according to the environmental doses of each chemical group recommended by the manufacturers and then combined together by group (e.g. insecticides group, non-insecticides group, and mixture group of insecticides+non-insecticides). For each group of pesticides, the environmental doses according to the manufacture is presented in Table 1.

Determination of lethal dose of 20% of population (ld_{20})

The determination of ld_{20} for each colony was done from the mother solution made of a combination of individual chemicals at environmental dose from each group of pesticides. For each colony, 100 Kisumu mosquito larvae were separated into 4 batches of 25 stage II larvae in plastic cups each containing 100 ml of water and cat food (Friskies®). This sample size was chosen because it is the one used in the susceptibility tests on adults. Several doses were used to expose the larvae. These were 0.01 μl , 0.02 μl , 0.05 μl , 0.07 μl for insecticides, 100 μl ,

Table 1. Groups, trading name, active substance, class, concentration of substance present in the different pesticides used in this study.

	Groups	Trading name	Active substance	Class	Concentration of substance	Environmental dose
Mixture	Insecticides	Furadan®	Carbofuran	Carbamates	50 g / kg	1 kg / 15 l of water
		K-optimal®	Acetamipride	Neonicotinoids	15 g / l	40 ml / 15 l of water
			Lambda-cyhalothrin	Pyrethroids	20 g / l	
		Legumax®	Deltamethrin	Pyrethroids	12 g / l	50 ml / 15 l of water
		Verso 480®	Chlorpyrifos ethyl	Organophosphates	480 g / l	15 ml / 10 l of water
	Non-insecticides	Banko Plus®	Chlorothalonil	Organochlorés	550 g / l	80 ml / 15 l of water
			Carbendazine	Carbamates	100 g / l	
		Garil®	Propanil	Amides	360 g / l	80 ml / 15 l of water
			Trichlopyr	Pyridines	72 g / l	
		Glyphader®	Glyphosate	Amino-phosphonates	360 g / l	80 ml / 15 l of water

250 µl, 500 µl, 750 µl and 1000 µl for non-insecticides and 1 µl, 3 µl, 5 µl and 7 µl for the mixture group. Each exposure per dose was done in four replicates and the exposure time of the larvae was 24 hours. The dl_{20} were obtained by counting live larvae, and the Polo Plus 1.0 (LeOra Software, 2002–2014) was used to estimate the exact concentration to be used later. In this study, ld_{20} was chosen in order to minimise the effects of the artificial selection that could occur if the amount of xenobiotic used is high. The AAT Bioquest LD50 Calculator is a free to use alternative software.

Larvae exposure

After determining the different ld_{20} , approximately 1000 stage 2nd larvae were exposed per colony for determination of the inductive effect. The inductive effect was evaluated by exposing 2nd instar larvae for 24 hours to a lethal dose of 20% (ld_{20}) of each pesticide group. After the 24 hours of exposure, the larvae were rinsed and then transferred to clean tap water. Then, they were fed with ground cat food and left to pupae. The pupae were transferred to the cages dedicated to each colony for breeding. The same selection process was carried out in parallel for all strains except the control colony.

Bioassays

Susceptibility tests in tubes were carried out with deltamethrin (0.05%) and dichlorodiphenyltrichloroethane (DDT) (4%)

in order to evaluate the level of phenotypic resistance of each selected colony. The protocol was conducted according to standard WHO bioassay protocol (World Health Organization, 2016). Females mosquitoes of each colony were exposed to impregnated paper for one hour. Controls included batches of mosquitoes from each site exposed to untreated papers. During the exposure time, the number knocked-down was recorded at different time intervals 5, 10, 15, 20, 30, 40, 50, and 60 minutes in order to calculate the time required to knock-down 20% and 50% of the mosquitoes (kdt_{20} and kdt_{50}) and after 60 min exposure mosquitoes were transferred into holding tubes and provided with cotton wools soaked with a 10% sucrose solution. Mortalities were recorded 24 hours post-exposure.

RNA extraction

The total RNA of the adult mosquitoes was extracted from four replicates per colony. Each replica contained 10 female control mosquitoes aged 2–5 days and non exposed to insecticides. The extraction kit was that of MagnaMedics with magnetic-bead (MagnaMedics GmbH, Aachen, Germany). It was carried out as follows: mosquitoes were crushed in 200 µl TE buffer. Then 150 µL lysis buffer was added to break up their cells. The mixture was vortexed for 30 seconds, incubated for ten minutes and centrifuged for 2 minutes at 16,000 (g) gravity. The supernatant was transferred to a new Eppendorf tube in which 20 µl magnetic beads and 440 µl binding buffer for the

electromagnetic effect were added. This new mixture was incubated for ten minutes. The supernatant was removed and 200 µl wash buffer 1 was added. Two hundred microliters of wash buffer 2 were added after vortexing the previous solution and removing the supernatant. The supernatant from the solution made with wash buffer 2 was removed after vortexing and 180 µL elution buffer was added to the new solution. This new solution was vortexed and incubated for 10 min at 50°C. The supernatant was collected in a new tube and stored for analysis.

Quantitative real-time PCR (qRT-PCR)

The Bio-Rad CFX 96™ device (Bio-Rad technologies, Hercules, Californie, USA) allowed to do the quantitative real-time

reverse-transcription PCR (qRT-PCR). The qRT-PCR was used to quantify the expression of eight genes (CYP6P3, CYP6M2, CYP9K1, CYP6P4, CYP6Z1, GSTE2, CYP6P1 and CYP4G16) involved in mosquito resistance to insecticides using RPS7 for normalization in each test. A total of four triplex detoxification assays (Detox (A)-Detox (D)) were designed using the three fluorophores, FAM (green), HEX (yellow) and Atto647N (red) of the TaqMan probe. Details of primers and probes are in Table 2. With RPS7, CYP6P3 and CYP6M2 compiled Detox (A), CYP9K1 and CYP6P4 Detox (B), CYP6Z1 and GSTE2 Detox (C) and CYP6P1 and CYP4G16 Detox (D). Reactions were performed in a total reaction volume of 10 µL. The heat cycle parameters were: 50°C for 15 min, 95°C for 3 min, and 40 cycles of 95°C for 3 s and 60°C for 30 s. The

Table 2. Gene sequence.

Reagent	Oligo_Name	Sequence	Assay Green: FAM, Yellow: HEX Red: Atto647N	10µM working stock needed for a total 10 µl reaction
Primer	RPS7_Fj	CCACCATCGAACACAAAGTTGA	S7-Detox (A-D)	0.1
Primer	RPS7_R	TGCTGCAAACTTCGGCTATTC	S7-Detox (A-D)	0.2
Probe	RPS7_P1	CCGTGACGTTACGTTCAATTCCCA	S7-Detox (A-D)	0.25
Primer	CYP6P3_Fj	ACAATGTGATTGACGAAACCCT	P3-Detox (A)	0.4
Primer	CYP6P3_R	GGATCACATGCTTTGTGCCG	P3-Detox (A)	0.5
Probe	CYP6P3_P1	ACCCGCGTACCGTCTGTGGACT	P3-Detox (A)	0.35
Primer	CYP6M2_F2	CTGGCGTTGAATCCAGAGGT	M2-Detox (A)	0.6
Primer	CYP6M2_Rj	GATACTTGCGCAGTGATTCATTAAG	M2-Detox (A)	0.4
Probe	CYP6M2_P	AGAGAAATCCTGCAAAAGCACACGGAGA	M2-Detox (A)	0.25
Primer	CYP9K1_F	CCGACACGTGGTGATGGATAC	K1-Detox (B)	0.2
Primer	CYP9K1_Rj	CGTCGTCGGTCCAGTCAAC	K1-Detox (B)	0.4
Probe	CYP9K1_P	CAATCTTCTGATGCAGGCCCGCAA	K1-Detox (B)	0.3
Primer	CYP6P4_Fj	CTGGACAACGTTATCAATGAAACC	P4-Detox (B)	0.4
Primer	CYP6P4_R	GCACGGTGTAATCACGCATC	P4-Detox (B)	0.5
Probe	CYP6P4_P	CCGATCGAGTCACTTTCGCGCG	P4-Detox (B)	0.3
Primer	CYP6Z1_Fj	CCCGCAACTGTATCGGTCTG	Z1-Detox (C)	0.1
Primer	CYP6Z1_R	TTCGGTGCCAGTGTGATTGA	Z1-Detox (C)	0.6
Probe	CYP6Z1_P1	TGATGCTGTCCCGATTAACTTTTCGGC	Z1-Detox (C)	0.25
Primer	GSTE2_Fj	CCGGAATTGTGAAGCTAAACC	GSTE2-Detox (C)	0.1
Primer	GSTE2_R	GCTTGACGGGGTCTTTCGG	GSTE2-Detox (C)	0.4
Probe	GSTE2_P	CGGTACGATCATACCGAGAGCCAC	GSTE2-Detox (C)	0.3
Primer	CYP6P1_Fj	ACAGGTGGTGAACGAAACCC	P1-Detox (D)	0.1
Primer	CYP6P1_R	GGTGAATCCTGTCCCGCAA	P1-Detox (D)	0.5
Probe	CYP6P1_P	CCGCTCGAAACGACGCTGCG	P1-Detox (D)	0.3
Primer	CYP4G16_Fj	GTCCAAGAAGTTGCGTCGGAC	G16-Detox (D)	0.2
Primer	CYP4G16_R	TCTTCGATTTGCGTTGACGTG	G16-Detox (D)	0.2
Probe	CYP4G16_P1	CTGCAGGCCGACATCATTTGAAGC	G16-Detox (D)	0.3

samples were amplified in two technical replicates for analysis of gene expression for each population. Each series always included one control. The real-time PCR system was used to calculate Ct values for each reaction, which were then used to calculate fold changes using the Pfaffl method (Pfaffl *et al.*, 2002).

Data analysis

The induction effect of the respective pesticides were assessed in term of knockdown effect in adult mosquitoes by comparing the time necessary for 20% and 50% of the population to be knocked-down (kdt_{20} and kdt_{50} respectively). The kdt_{20} and kdt_{50} were calculated using the PoloPlus software compared between exposed and non-exposed colony (control). The calculation of the 95% confidence intervals (CIs) and the significance was calculated with the Pfaffl method (Pfaffl *et al.*, 2002). This method was implemented in REST 2009 v2.013. It allows the entry of Ct values for the unexposed colony (control) and exposed strains for each gene with confidence intervals (CI) and 95% P values. The normaliser Ct values for the RPS7 gene and each target gene for each sample are jointly re-assigned to the exposed and unexposed strains and fold changes are calculated based on mean values after 2000 interactions. For correlation analysis between expression levels, these were calculated as relative quantification (RQ) units ($RQ = 2^{-\Delta Ct}$, where $\Delta Ct = Ct_{\text{target}} - Ct_{\text{normalise}}$). The significance level was set at $\alpha = 0.05$.

Results

Lethal dose for 20%

The lethal dose necessary to induce 20% of mortality in the population were 0.05 μl /100 ml for insecticides, 900 μl /100 ml for non-insecticides and 5 μl /100 ml for the mixture (Sadia *et al.*, 2021). These concentrations were used to assess the induction effect of the respective pesticide groups in adult mosquitoes. The mortality rate obtained 24 hours after exposure to deltamethrin and DDT was 100% in all colonies tested and control colony.

Knock down effect on adult mosquitoes

Exposure of mosquito larvae to a lethal dose of 20% resulted in a slight increase in the time required to knock down 20% and 50% of the adult mosquitoes in all colonies exposed to deltamethrin. In the colony exposed to the insecticides, the time required to knock down 20% (kdt_{20}) and 50% (kdt_{50}) of the mosquitoes at the adult stage was 13.147 and 18.187 minutes respectively. In the one exposed to non-insecticides, the kdt_{20} was 10,561 min and the kdt_{50} was 14,858 min. When exposed to deltamethrin in the mixture colony, the kdt_{20} was 16.399 min and the kdt_{50} was 18.501 min. In the control colony, the kdt_{20} was 6.578 min and the kdt_{50} 10.463 min (Table 3).

Concerning the exposure to the adult stage with DDT, in the control colony the kdt_{20} was 15.393 min and the kdt_{50} 21.665 min. In the colony exposed to insecticides the kdt_{20} was 16.803 min and the kdt_{50} 23.348 min. In the colony exposed to non-insecticides, the kdt_{20} and kdt_{50} were 18.771 minutes and 23.902 minutes respectively. The kdt_{20} was 16.925 and the kdt_{50} was 22.534 min in the mixture colony (Table 4).

Table 3. Different times (minutes) needed to knock down 20% and 50% (kdt_{20} and kdt_{50}) of mosquitoes exposed to different groups of agricultural pesticides (insecticides, non-insecticides and a mixture of all compounds). P value > 0.05 in all colonies.

	Control	Insecticides	None-insecticides	Mixture
	Delta	Delta	Delta	Delta
Kdt₂₀	6.578	13.147	10.561	16.399
Kdt₅₀	10.463	18.187	14.858	18.501

Table 4. Different times (minutes) needed to knock down 20% and 50% (kdt_{20} and kdt_{50}) of mosquitoes exposed to different groups of agricultural pesticides (insecticides, non-insecticides and a mixture of all compounds). P value > 0.05 in all colonies. DDT=dichlorodiphenyltrichloroethane.

	Control	Insecticides	None-insecticides	Mixture
	DDT	DDT	DDT	DDT
Kdt₂₀	15.393	16.803	18.771	16.925
Kdt₅₀	21.665	23.348	23.902	22.534

The kdt_{50} was similar in the colonies exposed to the insecticides and in the one exposed to the mixture when exposed to deltamethrin. On the contrary, when exposed to DDT, it was significantly similar in insecticides and non-insecticides colonies compared to the control colony (Figure 1).

Expression of the different genes studied

RT-qPCR analyses assessed the expression of several different genes in all exposed colonies compared to the control colony (Table 5). The RNAs of each colony included 10 mosquitoes per pool and four replicates per colony. From the eight genes studied, only the CYP6P1 gene in the mixture insecticides+non-insecticides was significantly increased in expression ($\alpha = 0.05$). The fold change of this gene was 3.144 suggesting that exposure of *An. gambiae* larvae to the pesticide mixture allowed induction of the CYP6P1 gene. Concerning the other genes, no significant increase was observed in the exposed colonies compared to the control colony. ($\alpha = 0.05$)

Discussion

In Côte d'Ivoire, it has been found several times that agriculture contributes to the development of resistance in mosquitoes (Camara *et al.*, 2018; Fodjo *et al.*, 2018; Mouhamadou *et al.*, 2019; Zoh *et al.*, 2018). The aim of this study was to assess the expression of selected cytochromes involved in insecticide resistance in susceptible mosquito larvae when they are exposed to different groups of agricultural pesticides used in Côte d'Ivoire at larval stage.

The bioassay results showed a slight increase variation of kdt_{50} to deltamethrin in colonies exposed to insecticides and

Figure 1. Number of mosquitoes knocked down as a function of time. The letters **a, b** and **c** represent the insecticides, non-insecticides and mixture colonies exposed to deltamethrin, respectively. The letters **d, e** and **f** represent the insecticides, non-insecticides and mixture colonies exposed to DDT (dichlorodiphenyltrichloroethane), respectively. The blue colour represents the control colony and the red colour represents the different colonies exposed to pesticides.

Table 5. Expression of genes in the different colonies exposed to the pesticides compared to the control colony. The number with the star is over-expressed with significant difference. CI=confidence interval.

	Insecticides			None-insecticides			Mixture		
	Ratio	P-value	95% CI	Ratio	P-value	95% CI	Ratio	P-value	95% CI
CYP6P3	0.127	0.147	0.004-2.069	0.169	0.186	0.003-8.277	0.002	0.134	0.00-1.628
CYP6M2	0.000	0.011	0.00-0.002	0.000	0.099	0.0-11621.9	0.000	0.003	0.00-0.002
CYP9K1	0.173	0.615	0.00-10.435	14.296	0.736	0.008-1003007	0.116	0.478	0.00-6.311
CYP6P4	1.350	0.745	0.082-10.426	1.699	0.575	0.164-693.519	0.420	0.595	0.005-9.153
CYP6Z1	1.070	0.664	0.271-3.583	1.417	0.451	0.377-3.476	1.292	0.548	0.409-3.631
GSTE2	0.314	0.000	0.099-0.746	0.906	0.853	0.269-2.457	0.258	0.059	0.067-1.170
CYP6P1	0.966	0.855	0.528-2.342	0.859	0.481	0.469-1.589	3.144 *	0.030	0.865-13.813
CYP4G16	0.654	0.218	0.316-1.714	0.745	0.351	0.397-1.817	0.354	0.053	0.100-1.069

mixture compared to unexposed colony. This could be explained by the short duration of exposure, which was one day, and also by the low dose of agricultural pesticides used, which was 20%. Indeed, Poupardin showed that pre-exposure of *Aedes* larvae to a sublethal dose of insecticide does not necessarily lead to increased tolerance to the insecticide. This may indicate that the dose or duration of exposure was not sufficient to obtain a significant toxicological effect (Poupardin *et al.*, 2008). In the colony exposed to non-insecticides, a slight increase in kdt_{50} was observed when adults of this colony were exposed to deltamethrin despite the lack of pyrethrinoids in the solution. Given the susceptibility of the parental colony, this slight variation could be due to the presence of organochlorine in the solution. Indeed, the organochlorines present in the solution could induce an action of the genes responsible for the cross-resistance of mosquitoes to organochlorines as well as pyrethrinoids, resulting in a slight increase in kdt_{50} and thus a cross-resistance between pyrethrinoids and DDT, even though deltamethrin is not used in the solution. This has been observed in several African countries where DDT resistant mosquitoes could easily be resistant to DDT with deltamethrin as both insecticides have the same sites of action and also have certain genes in common that may allow insecticide degradation even though there are no mutational genes (Orjuela *et al.*, 2019; Tchigossou *et al.*, 2018; Wanjala *et al.*, 2015). Also, the fact that the kdt_{50} of the colonies exposed with insecticides and with the mixture of pesticides is higher than the one in colony exposed with non-insecticides could suggest that the insecticides and mixture had a higher inductive effect than the non-insecticide colony. Indeed, the inductive effect may also be influenced by the nature and type of xenobiotic used as an inducer (Poupardin *et al.*, 2010).

The real-time qPCR allowed the expression of several different genes to be evaluated in all exposed colonies compared to the control colony. Eight genes (CYP6P3, CYP6M2, CYP9K1, CYP6P4, CYP6Z1, GSTE2, CYP6P1 and CYP4G16) related to insecticide resistance were characterised in this study. GSTE2 confers resistance to dichlorodiphenyltrichloroethane (DDT) and carbamates, CYP3P6 confers resistance to pyrethroids and carbamates, CYP6M2 confers resistance to pyrethroids (Adolfi *et al.*, 2019; Riveron *et al.*, 2014). CYP4G16 catalyses the biosynthesis of epicuticular hydrocarbons leading to cuticular resistance (Balabanidou *et al.*, 2016). CYP6Z1 metabolises bendiocarb, pyrethroids and DDT resistance (Chiu *et al.*, 2008; Ibrahim *et al.*, 2016). CYP6P4 is associated with resistance to alpha-cyano and non-alpha-cyano pyrethroids (Ibrahim *et al.*, 2016). CYP9K1 was found over-expressed in the deltamethrin-resistant Bioko population (Haruna *et al.*, 2020; Vontas *et al.*, 2018). CYP6P1 was over-expressed in mosquito populations in the Kou Valley resistant to DDT with permethrin, dieldrin and deltamethrin (Kwiatkowska *et al.*, 2013). Of the eight genes studied, only one was differentially over-expressed and one was under-expressed (Willoughby *et al.*, 2006). The other genes did not differ significantly. CYP6M2 was under-expressed in almost all the selected colonies with the exception of the non-insecticide colony where this gene was not significant. While CYP6M2 is responsible for the resistance of mosquitoes to

deltamethrin and DDT. This gene has been over-expressed in some cities in Côte d'Ivoire where agriculture is intensive and mosquitoes are resistant to several classes of insecticides used in public health (Edi *et al.*, 2014; Oumbouke *et al.*, 2020). This result could explain the low level of kdt_{50} observed in the different exposed colonies. The fact that this gene is under-expressed in these colonies could confirm the fact that the relationship between the capacity of an enzyme to metabolise an insecticide and its induction (induction in genetics is the set of cellular and molecular mechanisms leading to the triggering of the expression of a specific gene) by the insecticide is not always correlated (Willoughby *et al.*, 2006). Indeed, despite the presence of pyrethrinoids in these two colonies, the CYP6M2 gene was under-expressed in these two colonies, whereas this gene was several times over-expressed in mosquito populations under pyrethroid pressure (Djouaka *et al.*, 2008; Mitchell *et al.*, 2012; Stica *et al.*, 2019). This result may suggest that overexpression of this gene is a long process that takes place over several generations.

Our results also show that CYP6P1 was significantly induced in the mixture colony. Despite the involvement of this gene in the resistance of mosquitoes to DDT and pyrethroids, the tdk_{50} of this colony was not the highest of all selected colonies. This could imply that other genes not studied here may have been involved in increasing kdt_{50} in the other colonies. The high level of expression of CYP6P1 may indicate that this particular P450 plays a major role in the xenobiotic response during the adult stages in mosquitoes of this colony. The expression pattern observed at the CYP6P1 gene in the colony composed of the pesticide mixture could explain the kdt_{50} observed at the level of this colony when adults are exposed to deltamethrin. Also the overexpression of the CYP6P1 gene confirms the inductive effect that a short exposure of mosquito larvae to agricultural pesticides could have.

Conclusion

This study, which aimed to evaluate the expression level of certain cytochromes following the short term exposure of larvae to different groups of pesticides, confirmed that induction is influenced by the duration and concentration of exposure and the type of xenobiotic used as an inducer. With the exception of CYP6P1, which was induced in the strain composed of the pesticide mixture, all other genes studied were not induced suggesting that short-term exposure can still induce metabolic resistance. Other genes not studied in this study could contribute to induction. Therefore, it would be good to do a general mapping of all the genes involved in resistance by sequencing the exposed mosquitoes. Also, it would be ideal to see the effect of these agricultural pesticides on mosquitoes over several generations of selections.

Data availability

Underlying data

Zenodo: Short-term metabolic resistance inductive effect of different agrochemical groups on *Anopheles gambiae* mosquitoes. <https://zenodo.org/record/4791753#.YK0Ws6hKiUk> (Sadia *et al.*, 2021).

This project contains the following underlying data :

- susceptibility test and qRT-PCR.xlsx (The different tabs present the susceptibility tests carried out with DDT and deltamethrin in control, insecticide, none-insecticide and mixture colonies respectively. Following the susceptibility test tabs is the metabolic gene tab with detoxes A, B, C and D.)

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

Acknowledgments

We thank Centre Suisse de Recherches Scientifiques, Côte d'Ivoire laboratory staff.

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Rousseau Djouaka

International Institute of Tropical Agriculture, Cotonou, Benin

Minor comments:

Overall, I noticed that the comments were addressed in “Answer to reviewer’s comments” but not totally in the manuscript. So, we still have some concerns that need to be remediated before my full approval:

- ‘dl20’ should be changed to ‘DL20’ in the manuscript.
- Authors cited this paper in the manuscript, which should be corrected but this reference is still in the MS.
- The authors agreed to use ‘non-insecticide’ but the word ‘none-insecticide’ is still used in the MS.
- English editing: There’s still a lot of grammatical and typographical errors, words and punctuations misuse as well as several sentences that need to be recast/reviewed. I would recommend the authors conduct a thorough English language editing on the manuscript.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular Entomology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Version 1

Reviewer Report 08 July 2021

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Rousseau Djouaka

International Institute of Tropical Agriculture, Cotonou, Benin

Comments:

In this manuscript titled: "Short-term metabolic resistance inductive effect of different agrochemical groups on *Anopheles gambiae* mosquitoes", Sadia et al. emphasized the contribution of agrochemicals to insecticide resistance selection in *Anopheles gambiae*. They also highlighted the fact that known resistance-associated mutations like the Kdr and Ace-1 are getting fixed in *An.* mosquito populations and may not be possible to discriminate insecticide resistance, and that metabolic resistance may be driving insecticide resistance selection in agricultural areas. The authors then assessed the expression level of some selected cytochrome P450 and glutathione S-transferase genes in mosquitoes exposed to agro-insecticides solutions as compared to other agro-pesticides combinations.

This paper is of high public health importance, especially, with the emphasis on the contribution of human activities in the recent cases of malaria resurgence in several sub-Sahara Africa countries. However, in my opinion, the article is not good enough in the present state and some comments must be addressed for clarity and robust communication.

Major comments:

- I was expecting to see the mortality levels at larvae and adult stages after the assessment and not just the knockdown effect in adult mosquitoes. What was the mortality like after 24, 48, 72 hrs of larvae exposure and until adult emergence? Could there have been a greater effect on larvae than the adult with the short exposure of just 24 hours? Could it be that the short insecticide exposure has a knockdown effect and no effect on mortality? Why was the adult mortality not recorded after 24 hrs according to the WHO guidelines? Is there every likelihood that mosquito larvae that survived these insecticide solutions may develop resistance at the adult stage? These and other pertinent questions would have been answered if larvae and adult mortality were monitored and reported. I think this information should be added. At the present state of this manuscript, it may look more like preliminary information. If that was the intention, then the orientation of the title, discussion and conclusion should change. Also, the target-site mutations may have been screened if the exposure was long-term and monitored over generations since it will take a longer period for a mutation to be induced than enzymes being produced. Furthermore, since these targets cannot be assessed in this study, this reason should reflect as one of the study's limitations and be recommended for subsequent studies. It will be good to ascertain if mosquito's exposure to the selected agro-pesticides mixtures can induce known resistant mutations or probably the high kdr/ace-1 mutations reported in agricultural areas are from the migration of resistant mosquitoes.

- Also, the justification for choosing 100 larvae for your assessment cannot just be because that is the standard sample size for adult assessment as stated in the manuscript, there should be a more appropriate rationale. What if 20% and even more mosquitoes are lost at larvae stages during the experiment? It will be impossible to have the required 125-150 mosquitoes, including the control samples for the adult bioassay. This observation was also noted in the manuscript, how many mosquitoes survived into an adult in each colony? How many female mosquitoes emerged from the survivors? How many female mosquitoes were used for adult bioassay, considering that only 100 larvae were exposed to the chemical solutions? Where are the results of the other doses of agro-pesticides solutions used for the assessment as reported in the methodology? Authors should make sure this information is presented and make the methodology clearer than its present state.

Minor issues:

Introduction:

- "..... Indeed, the food security which is one of the current priorities that the Food and Agriculture Organization of the United Nations (FAO) faces with the challenge of climate change will incite agricultural overproduction to meet the expectations of the world's future population; this overproduction suggests an expansion of rice growing areas, which as mentioned above, remain niches for the proliferation of malaria vectors, and an intensification of use of agricultural pesticides (Osta *et al.*, 2012), which contribute to the development of insecticide resistance in the mosquitoes breeding in cultivation areas as molecules used are the same as those used for vector control.....". This statement is too long and should be broken into 2 to 3 sentences.
- "non-insecticides" and "none-insecticides" was used interchangeably throughout the manuscript. Authors should address this and be consistent with "non-insecticide" in the whole document.

Methodology:

- Preparation of mother solution? I guess you were referring to the stock solution, which I think is more appropriate. Please change this to "stock solution".
- "The determination of *dl*20 for each colony was done from the *mother solution*" change *dl*20 to LD20 in this sentence and other parts of the manuscript.
- Authors need to be consistent with the use of "non-exposed". Change "not-exposed" to "non-exposed".
- If LD20 was selected for this assessment, the authors should justify their decision. This is completely missing in the manuscript and should be addressed.

Results:

- "Lethal dose for 20%
The lethal dose necessary to induce 20% of mortality in the population were 0.05 µl /100 ml for insecticides, 900 µl /100 ml for none-insecticides and 5 µl /100 ml for the mixture (*Sadia*

et al., 2021). These concentrations were used to assess the induction effect of the respective pesticide groups in adult mosquitoes”.

It seems the authors cited this paper in the manuscript, if it is a mistake, they should check very well and correct it appropriately.

I was expecting that the authors will check for the significant difference of the kdt20 and kdt50 in the different colonies and establish if the observed differences in kdt are significant. The authors mentioned that some differences observed were significant but the P-value was not reported, which is required for this conclusion. This comment should be addressed so that the bioassay results can communicate better.

Discussion:

- The use of “indeed” is excessive in your discussion, you can use other adverbs to emphasize your points.
- “..... CYP6M2 was under-expressed in almost all the selected colonies with the exception of the none-insecticide colony where this gene was not significant. While CYP6M2 is responsible for the resistance of mosquitoes to deltamethrin and DDT. This gene has been over-expressed in some cities in Côte d’Ivoire where agriculture is intensive and mosquitoes are resistant to several classes of insecticides used in public health (Edi *et al.*, 2014; Oumbouke *et al.*, 2020). This result could explain the low level of kd50 observed in the different exposed colonies. The fact that this gene is under-expressed in these colonies could confirm the fact that the relationship between the capacity of an enzyme to metabolise an insecticide and its induction (induction in genetics is the set of cellular and molecular mechanisms leading to the triggering of the expression of a specific gene) by the insecticide is not always correlated (Willoughby *et al.*, 2006).....”

As much as I agree with the latter fact, there is no assessment in this study that suggests the involvement of CYP6M2 in the observed KD effect, hence no involvement in observed resistance under this circumstance. So, the above justification may not be very appropriate for this discussion. I would suggest that the authors should either remove this statement or recast/review it after addressing this comment.

English editing:

Overall, I observed a lot of grammatical and typographical errors, misuse of words and punctuations as well as several sentences that need to be recast/reviewed. I would recommend that the authors should conduct a thorough English language editing on the manuscript.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular Entomology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 20 Sep 2021

Gba Christabelle SADIA, Nangui Abrogoua University / Centre Suisse de Recherches Scientifiques, Cote d'Ivoire

RESPONSE TO ROUSSEAU DJOUAKA

Major comments

- What was the mortality like after 24, 48, 72 hrs of larvae exposure and until adult emergence?

The mortality rate obtained 24 hours after exposure to deltamethrin and DDT was 100% in all colonies tested and control colony.

- Also, the justification for choosing 100 larvae for your assessment cannot just be because that is the standard sample size for adult assessment as stated in the manuscript, there should be a more appropriate rationale.

The 100 larvae were used for the determination of the different dl_{20} in each group. After determination of the dl_{20} , more larvae were used for induction. About 1000 larvae per colony were used.

Minor issues

Introduction:

- This statement is too long and should be broken into 2 to 3 sentences

Indeed, the food security which is one of the current priorities that the Food and Agriculture Organization of the United Nations (FAO) faces with the challenge of climate

change will incite agricultural overproduction to meet the expectations of the world's future population. This overproduction suggests an expansion of rice growing areas, which as mentioned above, remain niches for the proliferation of malaria vectors, and an intensification of use of agricultural pesticides (Osta et al., 2012). Intensification of use of agricultural pesticides can contribute to the development of insecticide resistance in the mosquitoes breeding in cultivation areas as molecules used are the same as those used for vector control.

- non-insecticides" and "none-insecticides

I agree to use non-insecticide in this document.

Methodology:

- Preparation of mother solution? I guess you were referring to the stock solution, which I think is more appropriate. Please change this to "stock solution".

I agree stock solution

-The determination of dl_{20} for each colony was done from the *mother solution*"..... change dl_{20} to LD_{20} in this sentence and other parts of the manuscript.

I agree LD_{20}

- Authors need to be consistent with the use of "non-exposed". Change "not-exposed" to "non-exposed"

I agree non-exposed

- If LD_{20} was selected for this assessment, the authors should justify their decision. This is completely missing in the manuscript and should be addressed.

In this study, dl_{20} was chosen in order to minimise the effects of the artificial selection that could occur if the amount of xenobiotic used is high.

Results:

- It seems the authors cited this paper in the manuscript, if it is a mistake, they should check very well and correct it appropriately.

This citation was made by the newspaper. I think this is a mistake.

- P-value

Deltamethrin results

Control vs Insecticides colony : $P = 0.1$

Control vs Non-insecticides : $P = 0.35$

Control vs Mixture : $P = 0.11$

DDT results

Control vs Insecticides : $P = 0.45$

Control vs Non-insecticides : $P = 0.76$

Control vs Mixture : $P = 0.86$

Discussion:

we will revisit the CYP6M2 discussion.

RESPONSE TO THERESIA NKYA

- There is a need of justifying the use of 20% solution for the selection experiments.

In this study, dl_{20} was chosen in order to minimise the effects of the artificial selection that could occur if the amount of xenobiotic used is high.

- Why use only pyrethroids and OC and not other insecticides which are currently widely used for vector control like OP and Carbamates?

it was hoped to have a response with pyrethrinoids and OCs compared to carbamates and OPs. Indeed, resistance to carbamates and OPs is a process that takes place over several years. Therefore, we used the pyrethrinoids and OP insecticides.

Competing Interests: No competing interests were disclosed.

Reviewer Report 28 June 2021

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- Some of the references used are a bit old, there are newer reports from WHO in regards to current malaria status.
- There is a need of justifying the use of 20% solution for the selection experiments.

- Why use only pyrethroids and OC and not other insecticides which are currently widely used for vector control like OP and Carbamates?
- How about testing on the new classes of insecticides as they are now being pushed into the market?

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: insecticide resistance, vector biology, vector ecology, entomology, larviciding, integrated vector management, operational research

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.