Original Research Article

Anopheles gambiae larval development and toxicity's reduction of conventional agricultural insecticide in the laboratory conditions

Abstract

Background : Vector control of Malaria is mainly made by using impregnated bed nets and insecticides pulverizations indoor or/and outdoor. Besides, appearance and development of resistance's phenomenon among mosquito populations to insecticides, constitute a significant obstacle this fighting. ***Aims:*** To highlighting a neutralization phenomenon of three insecticides (methyl-parathion, dimethoate and cypermethrin) during development of the *Anopheles gambiae* s.s. larvae ***Methodology:*** Two setups followed one after the other were designed. In setup 1, four concentrations (with four replicates each) were freshly prepared and independently received a first batch of 100 first instars *An. gambiae* s.s. After emergence of adults from this first batch, the same test media were simply filtered and received a second batch of first instars larvae to make setup 2. Three endpoints were measured in this study: the duration of larval phase, the larval mortality, and the size of adults. ***Results:*** The development duration and mortality of larvae decreased significantly at setup 2 with cypermethrin and methyl-parathion. Thus, the duration of larval stage decreased from 10.18 days at setup 1 to 7.84 days at setup 2 for 0.010 µg/l (highest concentration) with cypermethrin and from 10.20 days at setup 1 to 8.27 days at setup 2 for 0.144 µg/l (highest concentration) with methyl-parathion. The larval mortality dropped from 79.32 % at setup 1 to 12.00 % at setup 2 for the highest concentration of cypermethrin and from 76.42 % at setup 1 to 12.50 % at setup 2 for the highest concentration of methyl-parathion. While adults size significantly increased in setup 2. For males, wing's length increased from 3.28 mm at setup 1 to 3.49 mm at setup 2 for the highest concentration of cypermethrin, from 3.31 mm at setup 1 to 3.49 mm at setup 2 for the highest concentration of methyl-parathion. In female, wing's length increased from 3.52 mm at setup 1 to 3.68 mm at setup 2 for the highest concentration of cypermethrin, from 3.49 mm at setup 1 to 3.68 mm at setup 2 for the highest concentration of methyl-parathion. ***Conclusion:*** This work shows that mosquito larvae, especially *An. gambiae* are able to modify breeding medium to improve its fitness during their development.

Key words: *Anopheles gambiae ss,* larvae, insecticide neutralization, mosquito resistance.

Introduction

Malaria control constitutes one of the priorities of the World Health Organization (WHO). In the current state of research, the lack of vaccine against this disease, makes vector control the only collective prevention method [1]. In this regard, use of insecticides is the most widespread approach. So, as a vector of malaria and of other dangerous diseases, mosquitoes are mainly controlled by long-lasting insecticidal nets and Indoor and outdoor residual spraying [2]. Besides, appearance and development of resistance's phenomenon among mosquito populations to insecticides, constitute a significant obstacle in vector control [3]. Indeed, The WHO *Global plan for insecticide resistance management in malaria vectors* (GPIRM) was launched in 2012 to provide a comprehensive approach to addressing this insecticide resistance phenomenon to malaria control and its elimination [4].

Mosquitoes reproduce in a wide variety of aquatic environments where larval stages develop. In agriculture, especially in market-gardening areas, water coming for example from watering, rains, drainage, and treatments of plants by insecticides, is collected in furrows and offers suitable breeding sites for mosquitoes [5]. Dilution of insecticides in these habitats, would put mosquito larvae in contact with active molecules of insecticides but would eliminate only susceptible individuals. Under effect of the selection pressure, resistant individuals develop adaptive mechanisms allowing to restore the balance of population [6]. Therefore, mosquitoes become increasingly resistant to insecticides as the same active molecules are used in both agricultural pest control and in public health. Many works in the world have reported the insecticide resistance among many species of malaria vectors [7, 8, 9, 10 et 11]. In Cameroun, the work carried out by [12] in many cities, highlighted resistance of some populations of *An. gambiae* s.l. to DDT and pyrethroids. Sites concerned by this study were market-gardening in towns Mbalmayo and Yaounde, the agro-industrial area in Foumbot, and cotton zones in Garoua and Pitoa (Northern Cameroun). All these cultural activities use insecticides for crops protection. According to some reports [13] and [14], the risk of resistance appearance is a result of a combination of specific insecticides factors, insect's biology, and factors related

to conditions of insecticides application. Thus, the origin of the resistance of anopheles' species that breed around or in agricultural areas, might be introduction of diluted insecticides in breeding sites via runoffs. This would be amplified by the misuse and/or overuse of insecticides in agricultural practices [15, 16]. Variations in initial conditions of breeding sites are determinant in comprehension of adaptive mechanisms of mosquito bodies [6]. For an effective strategy of prevention of malaria transmission and management of insecticides, it is relevant to know all biological, biochemical and even ecological mechanisms involved in insecticide resistance appearance and its evolution [13]. Moreover, [17, 18, 12, 19, 20 and 21] highlighted an enzymatic activity which is responsible of the resistance of *An. gambiae s.l.* in several insecticide families. This leads us to the idea that the origin of mosquito tolerance to insecticides might have as one possible explanation previous exposure to sublethal concentrations of runoffs from agricultural areas.

The present work aims to investigating the phenomenon of neutralization of insecticide by the mosquito organism of *Anopheles gambiae*.

Material and method

The work was conducted at the laboratory of the Biotechnology Centre of the University of Yaoundé I in Cameroon. Mosquito larvae used here came from a susceptible *An. gambiae* strain which was continuously bred in the laboratory for more than five years. Experimental conditions were: temperature between 26 and 30°C; relative humidity (RH) between 70 and 80%, and photoperiod L/D of 12/12.

Selection of insecticides

The insecticides used during this study belong to three families: carbamates (methyl-parathion), organophosphate (dimethoate), and pyrethroids (cypermethrin). Their selection was based on their common exploitation in market-gardening agriculture in Cameroon. Data about these insecticides are given in Table 1.

Preparation of test concentrations

We did not use the dilution method of insecticides suggested by the manufacturer for mosquito control because, the situation simulated here was a consequence of runoffs from agriculture treatments. So, stock solutions of the above selected insecticides were prepared by diluting 1µl rough insecticide in 0.5 litre of spring water. From this initial stock solution, we prepared test concentrations as indicated in table 2. These test concentrations were retained as a result of a screening test.

Test of insecticides stability under experimental conditions

The first test of this work concerning insecticide stability was very important because it permitted to gauge the influence of environmental conditions in the efficiency of insecticides on larvae of *An. gambiae* s.s. during the experiment.

To realise the test of insecticides stability, we prepared four replicates of the highest concentration of each insecticide in buckets of 30 cm diameter, and we added only food in each. These buckets were exposed as such during 14 days. Then on the 15th day, we prepared again four new replicates per insecticide and both new prepared milieu (new treated buckets) and old ones (old treated buckets) received 100 first instar larvae each. We compared the 24-hours mortality of first instar mosquito larvae between new and old exposed buckets.

Reduction of insecticides' toxicity by *An. gambiae* s.s larvae

Endpoints measured

Three endpoints were measured in this study: the duration of larval phase, the larval mortality, and the size of adults. These parameters are biological indicators of the harmonious development of mosquito larvae and are the first to be affected when environmental conditions deteriorate or improve [22, 23].

The duration of the larval phase corresponds to the time of transformation of the 2/3 individuals into nymphs [24].

The larval mortality was given by comparing the number of first instar larvae introduced into the test medium and the number of pupae obtained.

Size of adult mosquitoes was measured on 60 individuals (30 males and 30 females) randomly picked up in each test medium. The method applied for the size measurements was that of [25], using length and width of wings. Length of wings corresponds to the distance separating its insertion point to body with fringe of silks of the distal end; whereas width was taken on median of wing. Before measurements, wings were removed from anopheline body by using two needles and a magnifying glass equipped with an ocular micrometer. Mean values from the 120 individuals (60 males and 60 females) were expressed in millimetre and for each test medium.

Experimental design

- Setup 1: four replicates of all test concentrations (table 2) were prepared and 100 larvae of first instar *An. gambiae* (batch 1) were exposed in each of them till getting pupae. Dead larvae were daily removed from the breeding buckets and counted for assessing mortality. Besides, the duration of larval development and the size of adults were measured.
- Setup 2: after pupation of all larvae in Setup 1, all media were filtered (using a sieve with fine meshes of 0.1 mm in order to avoid the pollution due to organic matter overload) and received again another batch of also 100 first instar *An. gambiae* larvae (batch 2) in each replicate. Then, to assess the neutralization phenomenon of insecticides and the acquisition of tolerance by larvae, we compared the three endpoints (duration of larval development, larval mortality, and the size of adults) between the same test concentrations of setup 1 and setup 2.

Statistical analysis

The ANOVA test was performed to compare means of larval development duration and size of wings. If there was any difference, we realized the Tukey test for multiple comparison. We used also the Kruskal-Wallis in order to compare larvae mortality. If there was any difference, we realized the Wilcoxon test for multiple comparison. We also performed the Chi-square test to compare the data of the control with those of the tests' media. The software SPSS (Windows version 12.0) was used to perform the above-mentioned statistical analyses.

RESULTS

Insecticides stability under experimental conditions

The results showed that there was no significant difference (in larval mortality between old treated buckets and new treated ones (table 3) for cypermethrin ($p = 0.90$), dimethoate ($p = 0.89$) and methyl-parathion ($p = 0.90$) after 24 h.

Reduction of insecticides' toxicity by larvae of *Anopheles gambiae*

Comparison of the results of insecticides treatments showed similar observations for cypermethrin and methyl-parathion. Indeed, larvae of *An. gambiae* s.s were in general more susceptible to cypermethrin and methyl-parathion than to dimethoate (much higher concentrations used for dimethoate, but comparable effects

with the two other insecticides; Tables 4, 5, and 6). The duration of larval development was significantly higher in setup 1 than in setup 2 ($p < 0.0001$ for cypermethrin; $p < 0.0001$ for methyl-parathion, regardless of the concentration concerned (table 4 for cypermethrin and table 5 for methyl-parathion). The same trend was observed for larval mortality (table 4 for cypermethrin: $p < 0.0001$; table 5 for methyl-parathion: $p < 0.0001$). Besides, the size of the wings of the adults significantly increased in setup 2 in comparison to setup 1 in both cypermethrin ($p < 0.0001$ for males; $p < 0,0001$ for females) and methyl-parathion ($p < 0.0001$ for males; $p < 0.0001$ for females).

Furthermore, and in general, the duration of larval development and larval mortality significantly increased with insecticide's concentration within setup 1 (table 4 for cypermethrin and table 5 for methyl-parathion). In contrast, in setup 2 these two parameters no longer varied significantly with increased concentrations of insecticide increased. The wings size of individuals was significantly smaller in setup 1 than in setup 2 for all concentrations of the two insecticides; this observation was true for both males and females. Concerning the wings size of adults within setup 1, their length decreased when the concentration of insecticide increased (table 4 for cypermethrin and table 5 for methyl-parathion). However, in setup 2, no significant difference was observed for the length of wing size whatever the concentration for both males and females.

About dimethoate, the duration of larval development did not significantly change between setups 1 and 2 ($p = 0.24$); but within setups, it significantly increased with the increase of concentrations ($p < 0.0001$; table 6). Besides, the larval mortality significantly decreased from setup 1 to setup 2 ($p < 0.0001$; table 6) while, an increase was observed with the increase of concentrations within setups (table 6). So, in comparison with the two other insecticides (cypermethrin and methyl parathion), we observed in setup 2 a general concentration-dependent effect of the duration of larval development and larval mortality in dimethoate treatments (table 6). The size of male and female adults did not vary in general ($p = 0.41$ for males, $p = 0.39$ for females; table 6).

To conclude, the duration larval development and larval mortality were significantly higher in setup 1 (where freshly prepared test media received a first batch of *An. gambiae* s.s. larvae) than in setup 2 (where the same test media

received a second batch of *An. gambiae* s.s. larvae after pupation of the first batch) for cypermethrin and methyl parathion. Still for these two insecticides, the size of adults of *An. gambiae* s.s. was significantly higher in setup 2 than in setup 1. Concerning dimethoate, larval mortality followed similar trends than observations made for cypermethrin and methyl parathion between setup 1 and setup 2.

DISCUSSION

In the experiment about insecticides stability, the comparison of the results between old treated buckets (that received food and insecticides during fifteen days before receiving mosquito larvae) and new treated ones (that received food and larvae immediately after their preparation) showed no significant difference between both treatments for the larval mortality. This result revealed that the efficiency of insecticides in this study was not degraded neither by food, nor by the other environmental conditions during the test period (at least for two weeks). This is very important because it permitted to correlate any change in the effectiveness of insecticides with the presence of *An. gambiae* larvae in the environment for further work.

In the experiment about reduction insecticides' toxicity, the duration of the larval development and the larval mortality decreased while the size of adults increased in setup 2 compared to setup 1. The results of the stability experiment revealed that environmental conditions did not affect the efficiency of the tested insecticides on *An. gambiae* s.s. larvae. Therefore, the less susceptibility of larvae observed in setup 2 might be explained by the ability of the first batch of larvae of *An. gambiae* of setup 1 to have neutralized a part of active molecules of insecticides in test media through metabolic interactions, as described by [26]. Indeed, many studies have shown a metabolic resistance of mosquitoes induced by xenobiotics such as insecticides. For *An. gambiae* particularly, it was shown that CYP6Z1 [28], CYP6M2, CYP6P3, CYP6P4, CYP6Z3, CYP9K1, GSTD1-6, GSTD1-4 [29] are able to metabolize DDT; while CYP6P3 [30, 22], CYP6M2 [22], CYP6P4, CYP6Z3, CYP9K1, GSTD1-6, and GSTD1-4 [29] are involved in the metabolism of pyrethroids. Some authors [31, 32, 33] have even demonstrated that increased mosquito resistance to a specific insecticide, reflects a high activity of detoxification enzymes toward that insecticide. So, the metabolic capacity of insecticides by mosquitoes, as a

consequence of a detoxification activity of enzymes like monooxygenases (families of CYP4, CYP6, CYP9 genes with cytochrome P), glutathione-S-transferase (GSTs), and esterases [34, 18, 12, 35, 29, 36], might justify the neutralization of insecticides by mosquito larvae of setup 1, leading to better performances of larvae in setup 2. Thus, insecticides like permethrin, cypermethrin or DDT can induce overexpression of the CYPs genes [37, 38, 39, 40, 41, 19, 22, 42]. It is an example of intra-generational adaptive variation or phenotypical plasticity [6]. According to [43], the phenotype of an individual is optimal only for limited range of environmental conditions. In order to adapt to variations of their biotope, body is able to develop strategies to adjust their phenotype according to the new conditions.

Another result of the present study was the differential and pesticide-dependant responses of the duration of larval development and the size of adults to insecticides exposure. In fact, and only in cypermethrin and methyl-parathion treatments (on the contrary to dimethoate), a significant difference was observed for the two mentioned endpoints between setup 1 and setup 2. These two insecticides were also effective on larvae of *An. gambiae* s.s. at much lower concentrations than dimethoate. This means that the intensity of the enzymatic activity of detoxification would be proportional to the effectiveness of the insecticide.

Conclusion

This work shows that *An. gambiae* larvae are able to modify their living environment in the direction that is favourable to them. In the case of the present work, it is the presence of the insecticides that is the main disturbing element. Although we were not able to determine the concentrations of insecticides used before launching the setup 2, but we made an effort to find out the stability of these insecticides under the conditions and within the timeframe of our work. This is why we can affirm that the improvement of the life traits of *An. gambiae* observed in the second phase of our work is linked to the improvement of the development environment of the larvae.

DATA AVAILABILITY

The datasets used during the current study are available from the corresponding author on reasonable request.

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Table 1: Data about conventional agriculture insecticides selected

Families of Insecticides	Commercial names	Concentrations	Active molecules	Body diffusion
Carbamates	Pencap	240g/l	Methyl-parathion	Contact

organophosphates	Callidim	400g/l	dimethoate	Contanct and systemic
Pyrethroids	Cypercal	50g/l	cypermethrin	systemic

Table 2: Preparation of 0.5 l test concentrations of the three selected insecticides used in the experimental design

Insecticides (named by their active molecule)	test concentration to be prepared	Volume of the stock solution used
Cypermethrin	$2,5x10^{-3}$ µg/l	50 µl
	$5x10^{-3}$ µg/l	100 µl
	$7.5x10^{-3}$ µg/l	150 µl
	$1x10^{-2}$ µg/l	200 µl
Dimethoate	$1.2x10^{2}$ µg/l	300 ml
	$2x10^{2}$ µg/l	500 ml
	$2.8x10^{2}$ µg/l	700 ml
	$3.6x10^{2}$ µg/l	900 ml
Methyl-parathion	$8.4x10^{-2}$ µg/l	350 µl
	$1.08x10^{-1}$µg/l	450 µl
	$1.2x10^{-1}$ µg/l	500 µl
	$1.44x10^{-1}$ µg/l	600 µl

Table 3: Comparison of the 24-hours larval mortality of *An. gambiae* between new and old treated buckets with the highest concentrations of test insecticides; same letters in superscript means no significant difference between treatments and different letters expresses a significant difference with P<0.005?

	treatment type	Cypermethrine	Dimethoate	Methyl-parathion
Larval mortality (%)	New treated buckets	76.12 ± 11.03 [a]	85.93 ± 1.73 [b]	62.56 ±8.76[c]
	old treated buckets	77.75 ± 7.5 [a]	86.50 ± 1.73[b]	61.50 ± 6.65[c]

Table 4: Variation of the duration of larval development, larval mortality and the length of the wings of adults between setups 1 and 2 for *An. gambiae* s.s. in cypermethrin treatments; same letters in superscript means no significant difference between treatments and different letters expresses a significant difference with P<0.005?

Endpoints	Setups	Cypermethrin concentrations (µg/l)

	Control		0,0025	0,005	0,0075	0,01
Duration of larval development (days)	7.53 ± 0.12	1	9. 36 ± 0.09 a	9.77 ± 0.42 b	9.93 ± 0.30 b	10.18 ± 0.19 c
		2	7.80 ±0.16 d	7.81 ± 0.16 d	7.79 ± 0.21 d	7.80 ± 0.26 d
Larval mortality (%)	2.50 ± 0.01	1	27.50 ± 5.01 e	36.28 ± 4.23 f	42.21 ± 2.88 g	79.32 ± 9.03 h
		2	13.20 ± 3.14 i	13.41 ± 3.27 i	13.90 ± 4.20 i	14.09 ± 6.24 i
Male wing length (mm)	3,48 ± 0,08	1	3.26 ± 0,08 j	3.22 ± 0.10 k	3.21 ± 0.10 k	3.20 ± 0.07 k
		2	3.46 ± 0.06 l	3.46 ± 0.06 l	3.45 ± 0.07 l	3.44 ± 0.10 l
Female wing length (mm)	3,66 ± 0,08	1	3.55 ± 0.08 m	3.51 ± 0.14 n	3.49 ± 0.13 n	3.48 ± 0.10 n
		2	3.63 ± 0.07 p	3.61 ± 0.08 p	3.61 ± 0.09 p	3.60 ± 0.11 p

Table 5: Variation of the duration of larval development, larval mortality and the length of wings of the adults between setups 1 and 2 for *An. gambiae* s.s. in methyl-parathion treatments; same letters in superscript means no significant difference between treatments and different letters expresses a significant difference with P<0.005?

Endpoints		Setups	Methyl-parathion concentrations (µg/l)			
	Control		0,084	0,108	0,12	0,144
Duration of larval development (days)	7.53 ± 0.12	1	9.06 ± 0.12 a	9.16 ± 0.06 a	9.40 ± 0.06 b	10.20 ± 0.05 c
		2	8.18 ± 0.11 d	8.21 ± 2.84 d	8.20 ± 0.23 d	8.27 ± 0.22 d
Larval mortality (%)	2.50 ± 0.01	1	24.50 ± 4.43 e	33.20 ± 2.14 f	38.21 ± 5.87 g	76.42 ± 11.03 h
		2	11.00 ± 3.74 i	10.75 ± 4.78 i	12.97 ± 4.20 i	13.5 ± 6.24 i
Male wing length (mm)	3,48 ± 0,08	1	3.28 ± 0,09 j	3.26 ± 0.09 j	3.22 ± 0.10 k	3.21 ± 0.09 k
		2	3.48 ± 0.07 l	3.50 ± 0.09 l	3.48 ± 0.08 l	3.49 ± 0.11 l
Female wing length (mm)	3,66 ± 0,08	1	3.59 ± 0.11 m	3.56 ± 0.14 m	3.50 ± 0.13 n	3.49 ± 0.10 n
		2	3.63 ± 0.10 p	3.61 ± 0.07 p	3.62 ± 0.07	3.60 ± 0.06 p

p

Table 6: Variation of the duration of larval development, the larval mortality and the length of wings of the adults between setups 1 and 2 for *An. gambiae* s.s. in diméthoate treatment; same letters in superscript means no significant difference between treatments and different letters expresses a significant difference with P<0.005?

Endpoints		Setups	Dimethoate concentrations (µg/l)			
	Control		120	200	280	360
Duration of larval development (days)	7.53 ± 0.12	1	9.13 ± 0.36[a]	9.53 ± 0.37[b]	9.75 ± 0.15[c]	10.08 ± 0.11[c]
		2	9.08 ± 0.22[a]	9.49 ±0.19 [b]	9.71 ± 0.11[c]	10.03 ± 0.10[c]
Larval mortality (%)	2.50 ± 0.01	1	33.50 ± 3.69[g]	44,50 ± 8.18[h]	53.50 ± 8.34[j]	56.25 ±1.70[j]
		2	32.75 ± 4.52[g]	34.00 ± 3.56[k]	39.25 ± 5.90[k]	46.00 ± 5.71[l]
Male wing length (mm)	3,48 ± 0,08	1	3.26 ± 0.11[m]	3,24 ± 0.12[m]	3,22 ± 0.10[m]	3,22 ± 0.12[m]
		2	3.28 ± 0.12[m]	3.25 ± 0.10[m]	3.23 ± 0.09[m]	3.24 ± 0.09[m]
Female wing length (mm)	3,66 ± 0,08	1	3.55 ± 0.15[r]	3.54 ± 0.11[r]	3.53 ± 0.15[r]	3.54 ± 0.17[r]
		2	3.56 ± 0.15[r]	3.52 ± 0.10[r]	3.55 ± 0.14[r]	3.55 ± 0.18[r]