Original Article

Oviposition Behavior of *Culex quinquefasciatus* and *Anopheles coluzzii* Females According to the Ovitrap Color and Presence of Fertilizer in Breeding Sites

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Abstract

Malaria and lymphatic filariasis are still important public health problems in Cameroon and in most part of sub-Saharan Africa. However there is still not enough informations on the bionomic and oviposition behaviour of their respective vectors. The present study assesses the influence of color and organics pollutants presence on *Culex* and *Anopheles coluzzii*
females oviposition behaviour. Laboratory experiments using *Culex quinquefasciatus* and *Anopheles coluzzii* laboratory colonies were conducted. Gravid females were offered cups of different colors (red, green, yellow, black, purple and white) with water for oviposition. Experiments were also conducted with cups containing different concentration of NPK fertilizers (1%, 0.75%, 0.5% and 0%). The influence of NPK fertilizer on the larval development time was also assessed.

*Cx quinquefasciatus* and *Anopheles coluzzii* gravid females were found to be attracted by dark colors over 50% of eggs were laid in cups of black, red or green colors. The experiment was conducted with the mother and the F1 and F2 generations and similar oviposition preference were registered. When mosquitoes were offered cups containing different concentration of NPK for oviposition it appeared that females of the two species prefer laying their eggs in low concentration of NPK. Rearing of larvae in water containing NPK fertilizers was found to accelerate the larval development time of *Culex quinquefasciatus* but not of *Anopheles coluzzii*.

The study indicated that several factors could be influencing vector bionomic and need further understanding to improve the fight against vector populations.

**Keywords:** *Anopheles coluzzii*; Color; *Culex quinquefasciatus*; Experiments; NPK

**Introduction**

Mosquitoes are largely distributed worldwide [1,2]. They are also vectors of several diseases to human and animals. Anopheline species such as members of the *Anopheles gambiae* complex are efficient vectors of malaria and filariasis [3,4]. Malaria is still an important public health threat in Cameroon with the whole country exposed to the risk of transmission [5]. *Culex* mosquitoes are responsible for high nuisance and the transmission of several diseases such as arboviruses and filariasis particularly in East Africa [6,7]. These species preferentially breed in permanent and/or semi-permanent, organically polluted water collections [8]. Studies conducted so far have reported high implication of *Culex* species in the transmission of lymphatic filariasis [9], avian malaria [10], and arboviruses such as West Nile virus [11]. In Cameroon, Lymphatic filariasis is considered to be endemic with mean prevalence level (ICT >1%) estimated at 3.3% countrywide [12]. *Culex quinquefasciatus*, one of the members of the *Culex pipiens* complex is largely distributed in urban settings of Cameroon [8,13]. Although their direct implication in LF transmission in Cameroon is still not well documented [8], previous studies indicated frequent circulation of arboviruses in *Culex* mosquitoes species [14,15].

To improve vector borne disease surveillance it is important to monitor the evolution of both *Culex* species and *Anopheles gambiae* s.l species as these are largely distributed across the country [3,8]. Main control measures used to control these vectors include the use of long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) [16,17]. These tools between 2000 and 2015, significantly contributed to the decrease of malaria morbidity and mortality across the world [17]. However, their efficiency is now affected by the rapid expansion of insecticide resistance and changes in the biting and resting behavior of mosquitoes [8,18].

In this context, understanding vector behavior, in particular oviposition behavior and tolerance to
organic pollutants could provide key information that could be used to design new control strategies for better vector management. The information on vector population behavior could also be used for surveillance activities and to better appraise the impact of control interventions. Ovitraps have been widely used for monitoring mosquito populations and this sampling technique is considered as efficient, easy to use, cheap and sensitive [19]. In Cameroon, studies conducted so far in the city of Yaoundé indicated high adaptation of both anopheline and culex species to polluted habitats [20]. Further research on An. gambiae complex reported high tolerance of An. coluzzii to polluted habitats in urban area compare to An. gambiae s.s [21]. Yet so far there have not been many studies assessing the influence of pollution and color on anopheline or culex females choice of oviposition site. In urban areas mosquitoes are highly prevalent in agricultural cultivated sites because of the availability of standing water collections where fertilizers are commonly used by farmers [22] to enhance their productivity. These compounds are considered to alter the physical and chemical properties of aquatic habitats making them more suitable for mosquitoes. Many studies revealed that fertilizer used in rice fields are associated with a dramatic increase of mosquito larvae populations [23,24]. The influence of these compounds on vector bionomic and fitness of major vectors remain poorly understood.

In the present study, a series of laboratory experiments were conducted to assess the influence of color and presence of NPK fertilizer in breeding sites on the oviposition behavior of females Culex quinquefasciatus and An. coluzzii.

Materials and Methods

Establishment of laboratory colonies of Culex quinquefasciatus and Anopheles coluzzii

Immatures stages of Culex quinquefasciatus and Anopheles coluzzii mosquitoes were collected in eight districts of Yaoundé city using the deeping method [25] and were reared to the adult stage in OCEAC insectary under standard laboratory conditions (27-28°C temperature; 70-80% hygrometry). Pupae were collected daily and transferred in cages (30 × 30 × 30 cm). Adult mosquitoes were provided continuous access of 10% glucose solution. For blood meal, Culex were fed overnight (7 pm) on chicken whereas Anopheles were fed on rabbit. Culex species collected were all identified as Culex quinquefasciatus using the morphological identification keys of Jupp. Anopheline collected were confirmed as Anopheles coluzzii after molecular identification [26,27]. The identified Culex quinquefasciatus and Anopheles coluzzii were used to establish laboratory colonies.

Influence of ovitrap’s color on the oviposition behavior of Culex quinquefasciatus and Anopheles coluzzii females

In order to find out whether Culex quinquefasciatus females during oviposition are attracted by certain colors, six colored (black, green, yellow, purple, red and transparent) plastics cups of 50 ml capacity containing spring water and two leaves of Bermuda grass as an oviposition attractant for gravid Culex mosquitoes [28] were placed in a cage. The cups were placed according to a clockwise direction and the transparent cup was used as control (Figure 1).
One hundred of five to seven days old gravids females of *Culex quinquefasciatus* were introduced in cages to lay eggs. Every morning, the ovitraps were examined and eggs rafts laid in each cups were removed, counted and recorded. The dead females were removed and not replaced. To avoid bias, position of ovitraps was changed daily by circular rotation of cups. The experiment lasted 18 days for each mosquito generation with a variable number of replicates according to generation.

For experiments with *Anopheles coluzzii* only 35 gravid females were introduced per cage with seven colored cups (black, green, yellow, purple, red, white and transparent). The experiment was conducted similarly as the previous describe for *Culex* and was also performed with F\textsubscript{0}, F\textsubscript{1} and F\textsubscript{2} generations to check whether there was a change in the oviposition behavior from one generation to another.

**NPK fertilizers influence on the oviposition behavior of *Culex quinquefasciatus* and *Anopheles coluzzii***

NPK used in this study is a fertilizer containing the following components nitrogen (N), phosphorus (P) and potassium (K) at similar proportion [29]. Four different solutions of NPK at different concentrations (1%, 0.75%, 0.5% and 0%) were prepared. The 1% solution was obtained by adding 1mg of NPK in 100ml of distilled water. No NPK was added in the control cup. The cups were placed in a cage with *Culex quinquefasciatus* or *An. coluzzii* gravid females.

A total of 500 gravids *Cx. quinquefasciatus* and 175 gravids *An. coluzzii* females were distributed into 05 cages for eggs laying, 100 *Culex* and 35 anopheles females per cage were used. The ovitraps were rotated every two days to minimize a position bias. Dead females were removed and not replaced. The number of eggs laid in each cup were removed, counted using a stereomicroscope and recorded every morning.

**Effect of the NPK fertilizers on the fitness of *Culex quinquefasciatus* and Anopheles coluzzii larvae***

Eggs collected from each cup were reared in bigger containers. For each species, the first instar larvae (L\textsubscript{1}) obtained after eggs hatching were transferred in a container with a similar concentration of NPK as the cup with thirty five to sixty larvae/tray respectively. Larvae reared in clean water served as control. All larvae were fed with the same amount of food and were maintained under standard insectary conditions.
conditions. Dead larvae were not replaced and the time to pupation was monitored. The experiment was replicated 12 times.

Data analysis

Oviposition activity index

The oviposition activity index (OAI) was determined according to the Kramer and Muller formula (1979):

$$OAI = \frac{Number\ of\ eggs\ in\ test\ cup - number\ of\ eggs\ in\ control\ cup}{Number\ of\ eggs\ in\ test\ cup + number\ of\ eggs\ in\ control\ cup}$$

The index value varies from -1 to +1, with 0 indicating neutral response. Positives values are indicative of a positive attraction of the mosquito by a color compared to the control. A negative index is indicative of less attraction or deterrent/repellent effect of the oviposition cup.

The mean number of eggs per ovitrap and per concentration was calculated. A General Linear Model (GLM) using a correction of « quasipoisson » was used to evaluate the influence of the ovitrap’s color on the number of egg rafts and eggs laid by Culex and Anopheles coluzzii. The same analysis was carried out to evaluate the effect of fertilizer concentration on the above-mentioned parameters. A post-hoc Tukey-HSD (High Significance Difference) in the package « agricolae » [30] was used for pairwise comparisons. A GLM was also used to evaluate the influence of NPK fertilizers concentration on the duration of larval development of both species. Box-plots were drawn using the « ggplot » package. All the data were analyzed using R version 3.5.2 [31] and results with P < 0.05% was considered to be statistically significant.

Results

Influence of color on the oviposition behavior of Culex quinquefasciatus and Anopheles coluzzii gravid females

Out of the six color used for the experiment with Culex quinquefasciatus gravid females, it appeared that black color cups registered the highest eggs rafts compared to the others (F ratio = 10.264; P = 4.426e-07). The preference for dark colors particularly black color was similar for the different generations tested (Table 1). The average egg rafts in black color cups was $13.45 \pm 10.33$ eggs rafts while it was $1.90 \pm 2.38$ egg rafts for the purple which scored the lowest average egg rafts: (Table 1).
Table 1: Egg rafts of *Culex quinquefasciatus* distribution according to color during the F₀, F₁ and F₂ generations

| Cups colors | Black | Green | Red | Transparent (Control) | Yellow | Purple |
|-------------|-------|-------|-----|-----------------------|--------|--------|
| Eggs rafts means ± CI 95% | 21.60 ± 18.93 | 11.60 ± 10.16 | 9.40 ± 8.23 | 7.40 ± 6.48 | 6.80 ± 5.96 | 3.60 ± 3.15 |
| Number of replicates (F₀ generation) | 04 | 04 | 04 | 04 | 04 | 04 |
| Eggs raft mean ± CI 95% | 4 ± 4.52 | 2.0 ± 2.26 | 3.33 ± 4.04 | 1.33 ± 1.50 | 0 ± 0 | 0.33 ± 0.37 |
| Number of replicates (F₁ generation) | 03 | 03 | 03 | 03 | 03 | 03 |
| Eggs raft mean ± CI 95% | 9.33 ± 10.55 | 1 ± 1.13 | 2 ± 2.26 | 0.66 ± 0.74 | 0± 0 | 0.66 ± 0.74 |
| Number of replicates (F₂ generation) | 03 | 03 | 03 | 03 | 03 | 03 |

F = 6.789; P = 0.0004491

F = 1.111; P = 0.2065

F = 11.72; P = 0.0002789

**F** = F-ratio; **P** = P value; (*) Data followed by different letters are significantly different at the 5% level; **N** = number of replications

The oviposition activities index (OAI) values were positive and high for black color cups while these values were negative for the purple and yellow color cups who display a repellent effect (Figure 2).

The black color cups were also prefer by ovipositing gravid *Anopheles coluzzii* females (Table 2). A significantly, high number of eggs were recorded in black color ovitraps. This trend did not significantly vary between the three different mosquito generations monitored in the course of the study (P=4.524e-13; F=18.5008) (Table 2). Green cups also appeared to be highly preferred by *An. coluzzii* females for oviposition (Table 2).
Figure 2: Oviposition activity index of *Culex quinquefasciatus* for different colors

Table 2: Oviposition sites color preferred by *Anopheles coluzzii* gravid females

| Ovitraps' colors | Black  | Red    | Green  | Transparent (Control) | White  | Yellow | Purple |
|------------------|--------|--------|--------|-----------------------|--------|--------|--------|
| Number of replicates (F₀ Generation) | 04     | 04     | 04     | 04                    | 04     | 04     | 04     |
| Eggs means ± CI CI 95% | 156.50 ± 186.53 | 27 ± 44.20 | 102.25 ± 30.42 | 4.5 ± 8.81 | 10.5 ± 20.57 | 54.25 ± 72.99 | 3.25 ± 6.36 |
| F = 2.104; P = 0.09606 |

| Number of replicates (F₁ Generation) | 04     | 04     | 04     | 04                    | 04     | 04     | 04     |
| Eggs means ± CI CI 95% | 258.25 ± 113.26 | 53 ± 21.73 | 59.50 ± 51.32 | 4.25 ± 8.32 | 0.25 ± 0.48 | 16.75 ± 32.82 | 3 ± 5.87 |
| F = 13.37; P = 3.183e-06 |

| Number of replicates (F₂ Generation) | 04     | 04     | 04     | 04                    | 04     | 04     | 04     |
| Eggs means ± CI CI 95% | 243.75 ± 38.59 | 96.25 ± 59.73 | 105.25 ± 89.86 | 21.75 ± 14.84 | 25.50 ± 24.87 | 25.25 ± 24.87 | 31 ± 19.31 |
| F = 11.8; P = 8.449e-06 |

F = F-ratio; P = P value; (*) Data followed by differents letters are significantly differents at the 5% level; N = number of replicates
Influence of NPK presence on the oviposition behavior of gravid females of *Culex quinquefasciatus* and *Anopheles coluzzii*

In laboratory, *Cx. quinquefasciatus* was found to prefer laying eggs in ovitraps with no NPK fertilizer. Control cups with no fertilizer recorded the highest number of egg rafts (15.60 ± 6.42). Cups with different NPK concentrations had a similar attraction to gravid females with egg rafts varying from 3.80 ± 2.77 for the 0.5% NPK to 4 ± 1 for the cup with 1% NPK (Figure 3).

![Figure 3: Eggs rafts Culex quinquefasciatus distribution according to different NPK concentrations](image)

For gravid *Anopheles coluzzii* females it appeared that high density of eggs was recorded in the control cups and cups with a concentration of 0.75% of NPK (Figure 4).

![Figure 4: Number of eggs laid by Anopheles coluzzii females according to different concentrations of NPK fertilizers](image)
Assessment of the NPK fertilizer influence on the larval development of *Cx quinquefasciatus*

Table 3 shows the influence of fertilizer on *Culex* larval development. Significant variations between larval development time and pupae productivity in relation with NPK concentrations was recorded ($F=3; p<0.001$). Out of 3120 *Cx. quinquefasciatus* (L1) larvae reared in water with NPK fertilizer, only 935 (29.96%) succeeded to the pupae stage whereas in the control group, out of 1200 (L1) larvae, 850 (70.83%) emerged as pupae. Larval development time was found to be shorter (about 8 days) with high concentration of NPK and longer in low concentration (12 days) and control group (10.60 days) (Table 3).

**Table 3:** Influence of NPK fertilizers on *Culex quinquefasciatus* larvae development

| NPK Concentrations | Number of exposed larvae | N  | Development time from 1st instar to pupae (Min ± Max), Mean ± SD | pupae productivity (%) |
|--------------------|---------------------------|----|---------------------------------------------------------------|-------------------------|
| 0% (control)       | 1200                      | 20 | (16-24) 10.60 ± 3.73a                                         | 70.83%                  |
| 0.5%               | 1200                      | 20 | (6-23) 12.14 ± 3.86b                                          | 41.33%                  |
| 0.75%              | 1200                      | 20 | (6-14) 8.36 ± 2.01c                                           | 11.83%                  |
| 1%                 | 720                       | 12 | (6-13) 8.44 ± 1.99c                                           | 41.25%                  |

MLD = Mean larval development; N = Number of replications; Min = Minimal value; Max = Maximal value; SD = Standard deviation

The number of pupae obtained was very high in control sample compare to the other groups out of 1200 first instar larvae, 850 successfully arrived at the pupae stage. Within the other groups (0.5%; 0.75%; 1%), small differences of pupae production was observed.

**Effect of the presence of the chemical fertilizers (NPK) on larval development of *Anopheles coluzzii* larvae**

*Anopheles coluzzii* larvae were found to survive well to different NPK fertilizers concentrations ($F=3; P=0.09$). Whereas there was no significant difference on larval development time according to various concentrations of NPK fertilizers (Table 4). The proportion of first instar larvae reaching the pupae stage was high in the control group compare to the remaining groups. Also high concentration of NPK were found to induce a high mortality with only 43.2% of larvae succeeding to the pupae stage with NPK 1% (Table 4).
Table 4: Effect of exposure to NPK fertilizers on Anopheles coluzzii larvae

| NPK Concentrations | Number of exposed larvae | N | Development time from 1st instar to pupae (Min-Max), Mean ± SD | pupae productivity (%) |
|--------------------|--------------------------|---|---------------------------------------------------------------|------------------------|
| 0% (control)       | 560                      | 16 | (16-24) 10.43 ± 1.58a                                       | 80.17%                 |
| 0.5%               | 280                      | 08 | (6-23) 10.36 ± 1.32a                                       | 54.42%                 |
| 0.75%              | 280                      | 08 | (6-14) 10.89 ± 1.2a                                         | 48.21%                 |
| 1%                 | 175                      | 05 | (6-13) 10.88 ± 1.5a                                         | 43.42%                 |

N = Number of replicates; Min = Minimal value; Max = Maximal value; SD = Standard deviation

Despite similar lives span among larvae within each group, the control sample appeared to produce more pupae than the others.

Discussion

The study objectives were to assess the oviposition behaviour of both Anopheles coluzzii and Culex quinquefasciatus females. The study suggested that Culex quinquefasciatus and Anopheles coluzzii gravid females prefer laying eggs in black or dark colour cups. The attractiveness of Culex females by dark colour could be explain by the fact that the black color mimic shading water body such as dark gutter or pit latrine were Culex quinquefasciatus larvae are usually found [8]. Moreover, black containers are also known to absorb light across most of the visible spectrum than other colors and might reduce the exposition of larvae to sun shine effects [32,33]. Darkness and wetness were also identified as critical positive cues for Anopheles gambiae oviposition [32-34]. Females anopheline were also found to oviposit in different cups at a given time. This behavior also known as skip oviposition have been reported elsewhere [34] and could be part of an oviposition strategy which has an objective to disperse egg laid in order to give more chances to the progeny to grow to the adult stage. Many mosquitoes were reported to choose their oviposition site on the basis of the physico-chemical composition of the site this resulting in species-specific sites [35,36]. In the laboratory experiments conducted using different concentration of NPK fertilizers in cups no clear trend in the preference of Cx quinquefasciatus gravid females nor An. coluzzii females to specific concentration of the fertilizer was recorded. The results could derive from the limited number of replicates conducted. The presence of organic matters or microbial organisms in water collections was reported to release chemical cues that could be captured by gravid females to locate suitable oviposition sites [39,40]. During oviposition, females were reported to look for suitable breeding places by
analyzing specific cues called semio-chemicals signals such as volatile substances released from the breeding site and which are driving potential olfactory cues mediating oviposition [39,40]. Sites full of organic matters were reported to register high oviposition rate and high egg hatch and to provide enough food for mosquito larvae [41,42]. The fact that mosquito oviposition behavior was not affected by olfactic cues could derive from the low concentration of the NPK used or the fact that the compound were freshly prepared and the absence of bacteria in the milieu to break down organic particles.

Yet, sites full of organic matters while attracting females for oviposition could be subject to intense interspecific competitions which could affect the fitness and the development of species.

Fitness studies indicated that the larval development time for *Cx quinquefasciatus* was shorter in trail containing high NPK concentrations. Similar observations were recorded for *Culex pipiens pipiens* exposed to organics fertilizers (urea) [43,44]. *An. coluzzii* larvae on the other side were found to display similar larval development time when reared in trail with different NPK concentrations. The following suggest increase tolerance of *An. coluzzii* population to organic pollutants and is in line with studies conducted in the city of Yaoundé suggesting high tolerance of this mosquito to organic pollutants [45]. Yet further studies are still needed to assess how this adaptation capacity is affecting mosquito fitness.

The present study provided additional information on the bionomic of *An. coluzzii* and *Cx quinquefasciatus* populations. These information could be key for improving control strategies against these vector species in Cameroon.

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**Author contribution:** Djiappi-Tchamen B: Data Curation, Formal Analysis, Investigation, Methodology, Visualization, Writing – Original Draft Preparation; Nchoutpouen E: Data Curation, Investigation, Writing – Review & Editing; Talipouo A: Writing – Review & Editing; NKahe D: Investigation, Writing – Review & Editing; Roland B: Investigation, Writing – Review & Editing; Formal Analysis, Software; Kopya E: Investigation, Methodology; Awono-Ambene P: Writing – Review & Editing; Tchuinkam T: Administration, Writing – Review & Editing; Antonio-Nkondjio C: Conceptualization, Funding Acquisition, Project Administration, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

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