



RESEARCH ARTICLE

REVISED **Water source most suitable for rearing a sensitive malaria vector, *Anopheles funestus* in the laboratory [version 2; referees: 2 approved]**

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Abstract

Background: The insecticide susceptibility status of *Anopheles funestus*, one of the main malaria vectors in the Afrotropical regions, remains under-studied due to the difficulty of working with this mosquito species. Collecting their larvae in natural breeding sites, rearing and maintaining them in normal laboratory conditions have been a difficult task. Forced-egg laying technique has been a very good tool to generate eggs from adult mosquitoes collected from the wild but rearing these eggs to obtain satisfying portion as adults has always been the problem. In this study, we optimized the development of mosquito species larvae under standard laboratory conditions for desired production of adult mosquitoes that can be useful for insecticide susceptibility tests.

Methods: A forced-egg laying technique was used to obtain eggs from gravid female *Anopheles funestus* collected from Kpome locality in Benin. Eggs were reared in three different water samples (water from the borehole, and two mineral water namely FIFA and Possotômè) and larvae were fed with TetraMin baby fish food. The physico-chemical parameters of the waters were investigated prior to use for egg incubation (introduction of eggs' batches into water).

Results: In contrast to mineral water that had no contamination, the borehole water source was contaminated with lead (2.5mg/L) and nitrate (118.8mg/L). Egg hatching rates ranged as $91.9 \pm 4.4\%$, $89.1 \pm 2.5\%$ and $87.9 \pm 2.6\%$ in FIFA, Possotômè and borehole water respectively. High emergence of larvae to adult mosquitoes was recorded as in FIFA (74.3%) and Possotômè (79.5%) water. No adult mosquito was obtained from larvae reared in borehole water.

Conclusions: This study gave insight on the water sources that could be good for rearing to mass produce *An. funestus* in the laboratory. More analysis with other local mineral water sources in our environments could be considered in the future, hopefully giving better outputs.

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2

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report



report

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REVISED Amendments from Version 1**Introduction**

1. We have made some minor changes: "at" was replaced by "from".
2. Some sentences were reformulated.
 - From "Insecticide susceptibility the laboratory".
 - From "Unfortunately malaria vector".
 - From "Sometimes high sensitivity".
 - The end of the introduction has been re-written for a better understanding.
 - The word "insecticide" has been introduced in appropriate place in the sentence.

Material and methods

- The small cups used in this study were described;
- Details have been given in the revised manuscript to explain what the word Calavi is.
- We have specified that the total number of eggs reared was different between the 3 types of water and that larvae rearing duration is the time from L1 to pupae.
- The paragraphs "Data analysis" and "Ethical statement" were also corrected.

Results

1. Some sentences have been corrected:
 - "in" was replaced by "at" in the sentence "nitrate... borehole water".
 - "recorded" by "obtained" in the sentence "No adult mosquitoes... borehole water".
 - "adult stage" by "pupae stage" in the sentence "Rearing of *An. funestus*... 15 days".
1. More details have been given to explain what is meant by "egg hatching rate of all incubation days" and "eggs incubation".
2. Statistics were added to show if larval mortality and emerged adults were statistically different between the two mineral waters.

Discussion

1. The word "was" was removed from the sentence "These pH...laboratory conditions".
2. The following sentences have been reformulated:
 - "This research....egg shells".
 - "This nitrate concentration...the larvae development".
- 1 Also, "larvae mortality" has been replaced by "larval mortality" throughout the revised manuscript.

Figure 1

The quality of pictures of [Figure 1](#) has been improved.

See referee reports

Introduction

Anopheles funestus remains a main malaria vector, and is thereby also responsible for malaria morbidity and mortality in Sub-Saharan Africa¹. Breeding of this mosquito species like other mosquitoes also requires an aquatic environment where larvae emerge to adult mosquitoes. Water is an important component of the ecosystem of this insect, and the quality of the water is an important determinant in egg laying, for adequate growth

and development from larval stages until adults²⁻⁵. *An. funestus*, unlike the other known malaria vector, *An. gambiae*, in Sub-Saharan Africa breeds in natural/artificial permanent and semi-permanent water bodies with floating or emerging vegetation like edges of swamps, in weedy and grassy parts of rivers, streams, furrows, ditches and ponds with low salinity and little richness in organic matter⁶. There are reports that *Anopheles* mosquitoes breed in clear waters with temperatures between 22°C and 32°C⁷. However, decreased oxygen levels caused by water flow and flooding are always responsible for physical damage of mosquito larvae⁸. In addition, breeding water with a pH range of 6.08 to 7.02 is good for weakening the egg shell, so that first instar larvae can emerge⁹. Generally, these chemical properties of larval habitat, including ammonia, nitrate and sulphate concentration, influence larval development and their aquatic survival³. Most experiments that study the biology of *Anopheles* mosquitoes, such as assessments of insecticide susceptibility, use laboratory reared colonies¹⁰. It is therefore crucial to better understand suitable conditions for rearing of field collected mosquitoes. *An. funestus* is a difficult mosquito species to handle: not only because its larvae are rarely found during field survey, but also because of their inability to survive in normal laboratory conditions¹¹. Since *Anopheles funestus* represents an important malaria vector across Sub-Saharan Africa¹²⁻¹⁵, it is therefore necessary to find all means to study this malaria vector. Forced-egg laying technique has been a very helpful tool to generate a first filial generation of *An. funestus* mosquitoes from adults collected on the field¹⁶. As much as this field tool has been of help, obtaining the desired quantity of *An. funestus* when rearing its larvae under laboratory conditions for insecticide susceptibility testing has been a big challenge. Sometimes, we recorded high mortality rate even when *An. funestus* mosquitoes are kept under recommended laboratory conditions. This observation prompted us to rear *An. funestus* larvae generated from forced-egg laying technique with different water sources but under the same laboratory conditions. Therefore, the aim of this study was to determine the most suitable water source which will obtain the best quantity of F₁ *An. funestus* for laboratory experiments.

Materials and methods**Mosquito collection**

Blood fed *An. funestus* resting indoors were collected in selected rooms at Kpome, a village (6°55'N, 2°19'E) located in the South of Benin. Collection was carried out between 06:00 and 10:00 am using electric aspirators. The collection period corresponds to the dry season in southern Benin when *An. funestus* densities are likely to be higher. Mosquitoes collected were morphologically identified as *An. funestus* group using the key of Gillies and Meillon. (1968)⁶, kept in small cups and transferred to the laboratory (Insectariums of the International Institute of Tropical Agriculture of Benin) for rearing of the F₀ to produce the F₁ generation.

Mosquito rearing in the laboratory

In the insectary (T=25°C, RH=70–80% and L/D=12:12), blood fed, semi-gravid and gravid females were kept in the small paper cups (Diameter: 7.3 cm; height: 7.8 cm; capacity: 20cl) for 5 to

6 days after collection until fully gravid stage. Gravid females were then introduced individually and gently into 1.5ml Eppendorf tubes containing cotton soaked in water and surmounted by a filter paper (Wattman 3 mm/1 cm diameter) (Figure 1)¹⁶. Each Eppendorf tube was checked daily to identify females of *An. funestus* that have laid eggs, mosquitoes were gently removed from the tubes and transferred to a new Eppendorf tubes containing cotton and silica gel and stored at -20°C for subsequent experiments. Twenty-four hours post-oviposition, eggs from a single mosquito were divided into 3 batches and were allowed to hatch in small cups for larvae emergence, which were later transferred into rearing bowls containing 3 different types of water (Borehole water collected in Calavi, a southern Benin locality, and local mineral waters named FIFA and Possotômè) (Figure 1). Water of each larvae bowl was changed every two days to reduce mortality and larvae were fed daily with Tetramin™ baby fish food. The larvae were monitored daily during the four larval stages of the mosquito up to the adult stage (F1 *An. funestus*). Each experiment was repeated at least 8 times with each type of water. The growth and development yield was evaluated based on:

- (i) eggs hatching duration,
- (ii) larvae rearing duration corresponding to duration from L1 stage till the first pupae stage,

(iii) larval mortality rate

(iv) adult emergence rate.

Sheets were established to collect data manually on these parameters, such as the number of eggs incubated in each type of water, the number of hatched eggs, larval and pupae mortality and number of daily emerged adult mosquitoes.

Physico-chemical parameters of breeding water

Each water sample was analyzed for physico-chemical parameters in the laboratory of water and food quality of Agriculture Environment and Health (AgroEcoHealth) platform of IITA-Benin. Temperature and pH of each water sample were determined using the pH meter WAG-WE30200 (Wagtech Projects, Berkshire, UK). Conductivity and Total dissolved solid (TDS) were also determined using the conductivity/TDS meter WAG-WE30210 (Wagtech Projects, Berkshire, UK). Before analysis, electrodes of these materials were sensitized, calibrated and rinsed with deionized water. Water samples were then analyzed, and all parameters were read and recorded.

The quantity of nitrate, nitrite and chloride was determined using the Photometer 7100: WAG-WE10441 (Wagtech Projects, Berkshire, UK). Three replicates of each water sample were

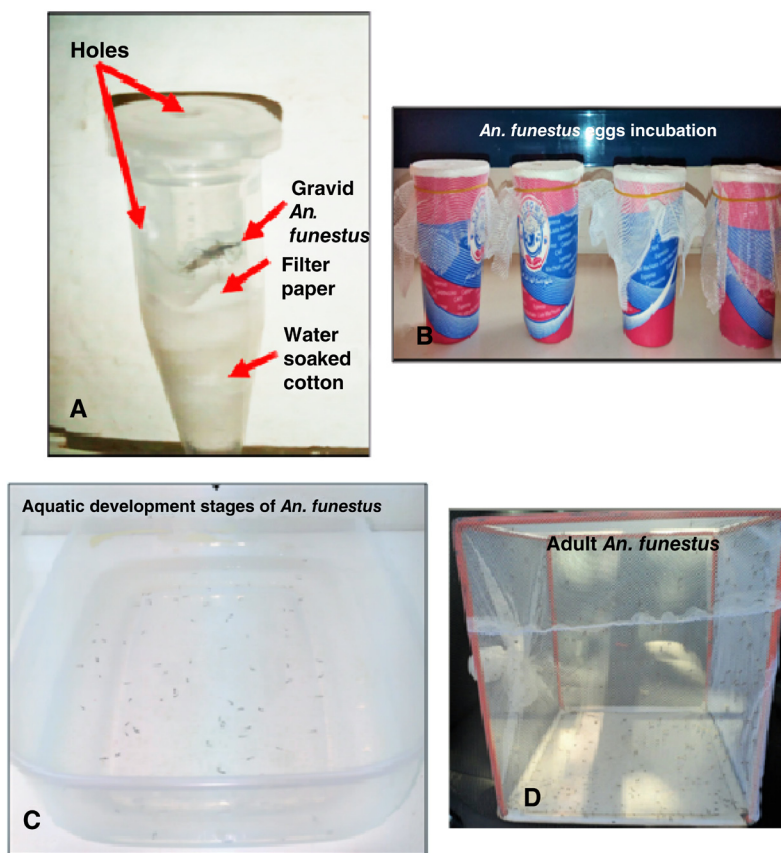


Figure 1. Developmental cycle of wild population of *An. funestus* in forced-eggs laying conditions: Oviposition of *An. funestus* (A), Incubation and hatching of *An. funestus* eggs (B), rearing of aquatic stage of *An. funestus* (C), Adults emergence (D).

introduced into beakers and the reagents were added as recommended by the manufacturer (Wagtech Projects, Berkshire, UK). The mixture was incubated to stand for 10 min (Nitrate and nitrite) and for 2 min (chlorine) to allow the appearance of color. Each beaker was then inserted into the photometer and concentrations were directly displayed and recorded.

Calcium and fluorine were quantified using the W-22XD.23XD HORIBA multi-probe (HORIBA, Ltd Japan). The electrodes were also calibrated and rinsed with sterile deionized water and then with the water samples to be analyzed. The quantities of calcium and fluorine were recorded after homogenization of the samples.

Heavy metals (Cadmium, Lead and Copper) were quantified using METALYSER HM 3000 (TRACE20, Berkshire, UK) by the reverse voltammeter method. The electrodes were placed and conditioned according to the desired metal as recommended by the manufacturer. Heavy metal was quantified in 70 ml of water with appropriate reagents (buffer and standard) according to the desired metal. After 5 mins of incubation, the concentration of the metal and the corresponding graphs (in the form of a peak) were displayed on a tablet connected to the machine.

PCR-based species identification

All females used for individual oviposition were identified using PCR as belonging to the *An. funestus* group. DNA was extracted from a total of 94 mosquitoes using the Livak protocol¹⁷, followed by PCR species identification using the protocol described by Koekemoer *et al.* (2002)¹⁸.

Data analysis

Data were inserted on excel sheets and analyzed using SPSS 17.0. Chi square test was used to analyze the difference between hatching rate in different water samples. The easy to use online

software (Fischer exact test) was used to assess the difference in larval mortality rate and adult emergence rate according to the water samples. The significance level was set at 5%.

Ethical statement

The request for ethical approval was not applicable for this study, according to the International Institute of Tropical Agriculture (IITA) Ethical Committee (IITA, 08 P.O. Box 0932, Tri-Postal, Cotonou, Benin). However, there was a focus group discussion with the community and household heads where verbal and written consent was obtained for mosquito collections in the community after the study aims and objectives were explained. Since mosquitoes were collected using electrical aspirators, no insecticide spray or human bait methods were used for mosquito collections during this study.

Results

Species identification

Molecular identification of 94 females used for forced-eggs technique revealed that they all belonged to *An. funestus s.s.*

Physico-chemical parameters of water samples

The physico-chemical parameters of the different water types used for *An. funestus* rearing are summarized in Table 1. These results showed that Possotômè mineral water had a pH of 7.9 whereas the pH obtained with FIFA mineral water and borehole water were 5.9 and 6.2, respectively. The total dissolved solids in Possotômè water (386 mg/l) was high compared to borehole water (131mg/l) and FIFA water (29.9mg/l). The same trend was observed with the total conductivity characterized by a high quantity of minerals in Possotômè water (775 μ S/cm), which was significantly different to FIFA water (60 μ S/cm) and borehole water (265 μ S/cm) ($p < 0.05$). Indeed, calcium and chloride concentration were higher in Possotômè mineral water (54 mg/l calcium, 110 mg/l chloride) than in FIFA

Table 1. Physico-chemical parameters of mineral/bottle waters (FIFA, Possotômè) and Borehole water.

Physico-chemical parameters	Borehole water	FIFA mineral water	Possotômè mineral water
pH	6.2	5.9	7.9
Total dissolved solids (mg/l)	131	29.9	386
Conductivity (μ S/cm)	265	60	775
Calcium (mg/l)	0.00031	3.1	54
Chloride (mg/l)	12.2	10	110
Nitrate (mg/l)	118.8	4.04	0
Nitrite (mg/l)	0.0759	0	0.0462
Lead (mg/l)	2.58	0	0
Copper (mg/l)	0.003399	0.00679	0.042
Fluoride (mg/l)	0.38	0	0.3
Cadmium (mg/l)	0	0	0
Temperature ($^{\circ}$ C)	25.5	25	25.2

mineral water (3.1 mg/l calcium, 10 mg/l chloride) and borehole water (0.00031 mg/l calcium, 12.2 mg/l chloride). Nitrate was almost absent in both mineral water (Possotome and FIFA) but was present at high concentration of 118.8mg/l in borehole water (Table I). Lead was completely absent in mineral water while, it was detected in borehole water at a concentration of 2.5mg/l. Copper was also detected in all water samples (FIFA water: 0.00679 mg/l, Possotômè water: 0.042 mg/ml and Borehole water: 0.003399 mg/l) but at nontoxic-doses. No trace of cadmium was detected in all of water samples used for rearing. Temperatures recorded from pH meter were 25°C, 25.2°C and 25.5°C in FIFA, Possotômè and borehole water, respectively.

Rearing yield of wild populations of *An. funestus*

A total of 355, 1124 and 830 *An. funestus* eggs were bred in borehole, FIFA and Possotômè mineral waters, respectively. There was no significant difference in the egg hatching duration between the borehole water (5 days) and mineral waters (4 days) ($P = 0.0722$). The eggs hatching rate of all repetitions (experiments) of each water sample are summarized in Table 2. These hatching rates varied between incubation days (the day

the eggs were introduced in the water sample) and ranged from $12.61 \pm 0.11\%$ to $41.21 \pm 6.11\%$ for borehole, 16% to $55.57 \pm 6.46\%$ for FIFA and from $19.6 \pm 2.38\%$ and $43.77 \pm 5.01\%$ for Possotômè. No significant difference was found in the daily hatching rates for borehole ($p = 0.0637$), FIFA ($p = 0.1450$) and Possotômè waters ($p = 0.080$). Overall, no significant difference in eggs hatching rate was observed between the three waters (FIFA $91.9 \pm 4.4\%$, Possotômè $89.1 \pm 2.5\%$ and borehole $87.9 \pm 2, 6\%$) ($P < 0.05$). Larval mortality rates obtained were respectively, 97.36% , 17.5% and 14.06% in borehole, Possotômè and FIFA waters (Table 3). There was a significant difference in larval mortality with borehole water and mineral waters ($p < 0.05$). No significant difference was observed in larval mortality between FIFA and Possotômè mineral waters ($P = 0.3573$). The percentage of adult mosquitoes that emerged from FIFA and Possotômè mineral waters were respectively 74.36% and 79.50% . No adult mosquitoes were obtained from borehole water (Table 3). Another observation was that the rate of emerged adults in Possotômè was slightly higher than in FIFA mineral water but not significant ($P = 0.1823$). Rearing of *An. funestus* larvae to adults with both mineral waters took about 10 days,

Table 2. Monitoring of emerged larvae of *Anopheles funestus* during the hatching period indifferent water samples.

Water samples	Number of replicates	Mean number of eggs per replicate	Eggshatchingdays	Meanhatching rate/day (%)	p value
Borehole water	8	44	1	0	0.0637
			2	41.21 ± 6.11	
			3	22.01 ± 4.82	
			4	12.07 ± 1.9	
			5	12.61 ± 0.11	
			Total	87.9 ± 2.60	
FIFA mineral water	15	75	1	0	0.1450
			2	55.57 ± 6.46	
			3	20.33 ± 11.54	
			4	16	
			Total	91.9 ± 4.45	
			Possotômè mineral water	17	
2	43.77 ± 5.01				
3	25.76 ± 2.63				
4	19.6 ± 2.38				
Total	89.13 ± 2.51				

Table 3. Adult productivity rate of *Anopheles funestus* rearing in different water samples.

Water samples	Borehole water	FIFA mineral water	Possotômè mineral water
Number of replicates	8	15	17
Mean number of larvae/replicate	38	64	40
Mortality rates (%)	97.36 (± 5.08)	14.06 (± 8.52)	17.5 (± 11.78)
Emerged adult rates (%)	0	74.36 (± 9.8)	79.5 (± 11.3)
larvae development duration (Days)	15	10	10

while for borehole water, rearing of larvae to pupae stage took as long as 15 days.

Discussion

Water quality is an important factor for oviposition of the female mosquito, and also influences the emergence of adult mosquitoes from larvae stages⁴. However, physico-chemical parameters such as temperature, pH, dissolved oxygen, nitrate and sulphate concentrations are likely to affect the development and survival of mosquito larvae³. The different values of physico-chemical parameters obtained in this study could give a better understanding on the environmental requirements needed to produce good yield of *F₁ An. funestus* mosquito in the laboratory from the ones collected in the wild. The pH ranges (5.9 to 7.9) recorded in the different water sources could be considered suitable for mosquito breeding. These pH were similar to that found in breeding water samples, with pH values ranging from 4.0 to 7.8 considered favorable for normal development of *An. gambiae* under laboratory conditions^{9,19}. pH values recorded in all sampling waters might not have an effect on the eventual yield of *An. funestus* in this study. A previous study also revealed that mosquito larvae can thrive well in water with neutral or slightly alkaline pH^{1,20}. The temperature of water samples (25°C) used in this study is known to be suitable for *An. funestus* mosquito breeding⁷.

This research also showed similar high hatching rates of eggs with FIFA, Possotômè and borehole waters, indicating that physico-chemical compositions of the different water samples do not influence the weakening of *An. funestus* egg shells. However, a high larval mortality rate (97%) was observed with borehole water compared to mineral water samples that produced good emergence rates. High larval mortality rate recorded could be attributed to the high nitrate dose in borehole water (118.8mg/l). This nitrate concentration in this water sample is higher than the maximum limit of 50mg/l nitrate dose authorized for human consumption²¹, and could also be toxic for mosquito larvae. The main toxic action of nitrate on aquatic animals is the conversion of oxygen-bearing pigments (hemoglobin and hemocyanin) into

the inhibited forms (methaemoglobin) which are not able to fix and carry oxygen²²⁻²⁴. Therefore, the lack of oxygen can cause the death of the mosquito larvae. The high nitrate content in the borehole water could be justified by the phreatic origin of this water, because ground water has always been described to contain toxic nitrate concentrations exceeding the standard^{9,21}. Water sources that should be used for the breeding of aquatic stages of *An. funestus* should not have similar physico-parameters as ground water. Nitrate and phosphate residues derived from chemical fertilizers used in agriculture are other potential pollutants that could be found in aquatic waters²⁵. Ndenga *et al.*, (2012)²⁶ was able to establish a non-significant positive correlation between the presence of nitrate at 1.8 to 3.6 mg/l and the development of *Anopheles* larvae. It has also been demonstrated that the toxicity of nitrate decreased with increasing salinity of water²⁷⁻²⁹. This could further explain the high salinity of Possotômè mineral water, which had a high conductivity and high amount of total dissolved solids. Some studies have shown that at around 15.85 g/l of sodium chloride (NaCl) in water, salinity becomes a discriminate dose that kills *An. gambiae* s.s, *An. coluzzii* and *An. merus* larvae³⁰. It would always be good to assess any trace of phosphate and nitrate in rearing water before use for breeding *An. funestus* in the laboratory. However, the definite effect of nitrate on larvae development should be further investigated.

Despite a relatively high salinity of 110 mg/l in Possotômè water, more than 70% of adults were able to emerge. This correlates with the observation of Koekemoer *et al.*, (2014)³¹, where they reported that salinity does not affect the emergence of *An. funestus* adults. Similar to egg hatching rates, rates of emergence of adult *An. funestus* in FIFA and Possotômè mineral waters were statistically similar, although the larval mortality rate was relatively low in FIFA water compared to Possotômè. No adult mosquitoes emerged from larvae reared with borehole water in this study. This could be explained by the fact that some larvae that reached pupal stage did not have enough energy to get out from their cuticle and become adults. This may be due to the lack of oxygen certainly related to high concentration of nitrate in borehole water²²⁻²⁴.

Conclusions

This study highlighted the impact of some physico-chemical factors of breeding waters on *An. funestus* development under laboratory conditions. It showed that *An. funestus* could develop well in FIFA and Possotômè mineral waters, which both have similar physico-chemical characteristics. However, further studies should be performed to measure other physico-chemical parameters, such as phosphate, dissolved oxygen, alkaline content. This information will be of immense help to improve *An. funestus* rearing in order to obtain desired F₁ progenies for more analysis.

Data availability

The raw data underlying the findings reported in this study can be found at: <http://doi.org/10.17605/OSF.IO/AES4P32>.

Competing interests

No competing interests were disclosed.

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References

- WHO: **World Malaria Report 2015**. Geneva, Switzerland; 2015. [Reference Source](#)
- Kearney M, Porter W: **Mechanistic niche modelling: combining physiological and spatial data to predict species' ranges**. *Ecol Lett*. 2009; **12**(4): 334–50. [PubMed Abstract](#) | [Publisher Full Text](#)
- Mutero CM, Ng'ang'a PN, Wekoyela P, et al.: **Ammonium sulphate fertiliser increases larval populations of *Anopheles arabiensis* and culicine mosquitoes in rice fields**. *Acta Trop*. 2004; **89**(2): 187–92. [PubMed Abstract](#) | [Publisher Full Text](#)
- Piyaratne MK, Amerasinghe FP, Amerasinghe PH, et al.: **Physico-chemical characteristics of *Anopheles culicifacies* and *Anopheles varuna* breeding water in a dry zone stream in Sri Lanka**. *J Vector Borne Dis*. 2005; **42**(2): 61–7. [PubMed Abstract](#)
- Varun T, Sharma A, Yadav R, et al.: **Characteristics of the larval breeding sites of *Anopheles culicifacies* sibling species in Madhya Pradesh, India**. *J Mal Res and Rev*. 2013; **1**(5): 47–53. [Reference Source](#)
- Gillies M, De Meillon B: **The Anophelinae of Africa south of the Sahara (Ethiopian zoogeographical region)**. 1968; 343. [Reference Source](#)
- Lyons CL, Coetzee M, Chown SL: **Stable and fluctuating temperature effects on the development rate and survival of two malaria vectors, *Anopheles arabiensis* and *Anopheles funestus***. *Parasit Vectors*. 2013; **6**: 104. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Okogun GR, Anosike JC, Okere AN, et al.: **Ecology of mosquitoes of Midwestern Nigeria**. *J Vector Borne Dis*. 2005; **42**(1): 1–8. [PubMed Abstract](#)
- Mehdaoui A, Venant AM, Fekhaoui M: **Contamination par les pesticides organochlorés et les nitrates de la lagune de Moulay Bouselham, Maroc**. *Cahiers d'études et de recherches francophones / Santé*. 2001; **10**(6): 381–8. [Reference Source](#)
- WHO: **World Malaria Report 2016** [Internet]. Geneva, Switzerland; 2016. [Reference Source](#)
- Dia I, Guelbeogo MW, Ayala D: **Advances and Perspectives in the Study of the Malaria Mosquito *Anopheles funestus***. *Intech Open Sciences*. 2013. [Publisher Full Text](#)
- Dabire KR, Baldet T, Diabate A, et al.: ***Anopheles funestus* (Diptera: Culicidae) in a humid savannah area of western Burkina Faso: bionomics, insecticide resistance status, and role in malaria transmission**. *J Med Entomol*. 2007; **44**(6): 990–997. [PubMed Abstract](#) | [Publisher Full Text](#)
- Mulamba C, Irving H, Riveron JM, et al.: **Contrasting *Plasmodium* infection rates and insecticide susceptibility profiles between the sympatric sibling species *Anopheles parensis* and *Anopheles funestus* s.s.: a potential challenge for malaria vector control in Uganda** [Internet]. *Parasit Vectors*. 2014; **7**: 71. Available from: Parasites & Vectors. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Djouaka RJ, Atoyebi SM, Tchigossou GM, et al.: **Evidence of a multiple insecticide resistance in the malaria vector *Anopheles funestus* in South West Nigeria**. *Malar J*. 2016; **15**(1): 565. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Djouaka R, Riveron JM, Yessoufou A, et al.: **Multiple insecticide resistance in an infected population of the malaria vector *Anopheles funestus* in Benin**. *Parasit Vectors*. 2016; **9**: 453. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Morgan JC, Irving H, Okedi LM, et al.: **Pyrethroid resistance in an *Anopheles funestus* population from Uganda**. *PLoS One*. 2010; **5**(7): e11872. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Livak KJ: **Organization and mapping of a sequence on the *Drosophila melanogaster* X and Y chromosomes that is transcribed during spermatogenesis**. *Genetics*. 1984; **107**(4): 611–34. [PubMed Abstract](#) | [Free Full Text](#)
- Koekemoer LL, Kamau L, Hunt RH, et al.: **A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (Diptera: Culicidae) group**. *Am J Trop Med Hyg*. 2002; **66**(6): 804–11. [PubMed Abstract](#) | [Publisher Full Text](#)
- Patz JA, Graczyk TK, Geller N, et al.: **Effects of environmental change on emerging parasitic diseases**. *Int J Parasitol*. 2000; **30**(12–13): 1395–1405. [PubMed Abstract](#) | [Publisher Full Text](#)
- Pelizza SA, López Lastra CC, Becnel JJ, et al.: **Effects of temperature, pH and salinity on the infection of *Leptolegnia chapmani* Seymour (Peronosporomycetes) in mosquito larvae**. *J Invertebr Pathol*. 2007; **96**(2): 133–137. [PubMed Abstract](#) | [Publisher Full Text](#)
- Halwani J, Baroudi BO, Wartel M: **[Nitrate contamination of the groundwater of the Akkar Plain in northern Lebanon]**. *Sante*. 1999; **9**(4): 219–23. [PubMed Abstract](#)
- Grabda E, Einszporn-Orecka T, Felinska C, et al.: **Experimental methemoglobinemia in rainbow trout**. *Acta Ichthyol Piscat*. 1974; **4**(2): 43–71. [Publisher Full Text](#)
- Scott G, Crunkilton RL: **Acute and chronic toxicity of nitrate to fathead minnows (*Pimephales promelas*), *Ceriodaphnia dubia*, and *Daphnia magna***. *Environ Toxicol Chem*. 2000; **19**(12): 2918–2922. [Publisher Full Text](#)
- Cheng SY, Chen JC: **Study on the oxyhemocyanin, deoxyhemocyanin, oxygen affinity and acid-base balance of *Marsupenaeus japonicus* following exposure to combined elevated nitrite and nitrate**. *Aquatic Toxicol*. 2002; **61**(3–4): 181–193. [PubMed Abstract](#) | [Publisher Full Text](#)
- Petrus R, Warchol JK: **Heavy metal removal by clinoptilolite. An equilibrium study in multi-component system**. *Water Res*. 2005; **39**(5): 819–830. [PubMed Abstract](#) | [Publisher Full Text](#)
- Ndenga BA, Simbauni JA, Mbugi JP, et al.: **Physical, chemical and biological characteristics in habitats of high and low presence of anopheline larvae in western Kenya highlands**. *PLoS One*. 2012; **7**(10): e47975. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Camargo JA, Ward JV: **Short-term toxicity of sodium nitrate (NaNO₃) to non-target freshwater invertebrates**. *Chemosphere*. 1992; **24**(1): 23–28. [Publisher Full Text](#)
- Camargo JA, Ward JV: **Nitrate (NO₃-N) toxicity to aquatic life: a proposal of safe concentrations for two species of nearctic freshwater invertebrates**. *Chemosphere*. 1995; **31**(5): 3211–3216. [Publisher Full Text](#)
- Tsai SJ, Chen JC: **Acute toxicity of nitrate on *Penaes monodon* juveniles at**

- different salinity levels. *Aquaculture*. 2002; **213**(1–4): 163–170.
[Publisher Full Text](#)
30. Smith KE, VanEkeris LA, Okech BA, *et al.*: Larval anopheline mosquito recta exhibit a dramatic change in localization patterns of ion transport proteins in response to shifting salinity: a comparison between anopheline and culicine larvae. *J Exp Biol*. 2008; **211**(Pt 19): 3067–76.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
31. Koekemoer LL, Waniwa K, Brooke BD, *et al.*: Larval salinity tolerance of two members of the *Anopheles funestus* group. *Med Vet Entomol*. 2014; **28**(2): 187–192.
[PubMed Abstract](#) | [Publisher Full Text](#)
32. Yessoufou A: Water source most suitable for rearing a sensitive malaria vector, *Anopheles funestus* in the laboratory. 2017.
[Data Source](#)

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Version 1

Referee Report 07 December 2017

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Emmanuel Elanga Ndille

Research Institute for Development (IRD), MIVEGEC (Infectious Diseases and Vectors : Ecology, Genetics, Evolution and Control), Montpellier, France

This manuscript try to describe some qualities needed for water used for rearing *Anopheles funestus* mosquito species in the laboratory. The study deals with an important issue all medical entomologist faces to when trying to establish a lab stain of *Anopheles funestus* mosquito. Results obtained this manuscript of Tchigossou *et al* are very interesting and very informative. However even if the manuscript and data presented are interesting, there are some minor concerns I will like the authors to take into consideration so that their work will be more efficient.

Comments:

1) In the section Material and methods, it would certainly be interesting for authors to give an approximate size of the small cup they used to keep their mosquitoes after identification. This aspect could be important as the manuscript try to present some interesting condition for the rearing of *Anopheles funestus* mosquito in the laboratory

2) In the text (mosquito rearing in the laboratory) authors speak about "Calavi". I think they should give more details on what is Calavi. Is not easy for the reader with no information about this locality to know what Calavi is.

3- In the "mosquito rearing" section, it would have been interesting for authors to be precise that the total number of eggs reared was different or the same between the 3 types of water.

4- As the present manuscript is trying to describe an experimental design to improve the rearing of *Anopheles funestus* in the laboratory, I think authors should ameliorate the quality of the pictures in figure 1. This will be helpful for someone who will try to reproduce what was done in the present study. Also it could be interesting for the reader if authors could give more details on "eggs incubation"

5- The two last sentences of the results section (Rearing yield of wild population of *An. funestus*) seem to be contradictory. Indeed, on one side authors said "No adult mosquitoes were recorded from borehole water", on another side they said "... for borehole water, rearing of larvae to adult stage took as long as 15 days. It's a bit difficult for the reader to understand how authors did to estimate these 15 days to obtain adult from borehole water, when it's said by authors that with this water no adult mosquitoes were recorded? So maybe authors could clarify this last part of the "results" section.

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

Yes

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?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: Medical Entomology

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

Author Response 14 Jan 2018

Akadiri Yessoufou, University of Abomey, Calavi, Benin

Answers, point by point, to the Reviewer's comments

The Reviewer approved our manuscript. However, he raised some minor points which we have now addressed and rectified accordingly in this revised version of the MS. The answers to his comments are as follows:

Reviewer 1: Emmanuel Elanga Ndille, Research Institute for Development (IRD), MIVEGEC (Infectious Diseases and Vectors: Ecology, Genetics, Evolution and Control), Montpellier, France

1) We agree with the Reviewer's comments. The characteristics of small cup used in this study were added in the revised manuscript. Please see in the *Material and Methods* section.

2) We agree with the Reviewer's comment. More details have been given in the revised manuscript to explain what is Calavi. Please see in the *Material and Methods* section

3) We agree with the Reviewer's comment. This precision has been given in the revised manuscript. Please see in the *Material and Methods* section

4) As per Reviewer's suggestion, the quality of the pictures of figure 1 has been improved.

5) "eggs incubation" means introduction of the eggs batches into water. As per Reviewer's suggestion, this precision has been given in the revised manuscript.

6) We agree with the Reviewer's comment. Larvae reared in this water sample reached to pupae

stage but no emergence was recorded. So, for this water sample “adult stage” was replaced by “pupae stage” in the revised manuscript. Please, see in the *Results* section.

Competing Interests: The authors have nothing to declare as far as the conflict of interest is concerned

Referee Report 01 December 2017

doi:[10.21956/wellcomeopenres.14033.r27837](https://doi.org/10.21956/wellcomeopenres.14033.r27837)



Cyrille Ndo

Malaria Research Laboratory, Organization of Coordination for the Fight against Endemic Diseases in Central Africa (OCEAC) , Yaoundé, Cameroon

General comment

This is a very interesting paper, especially for all people working with *Anopheles funestus*. This species is difficult to colonize in insectary and this has for long time prevented research on *An. funestus*. By defining water conditions that could promote or impede larval development of this species, it will henceforth be easy to generate large F1 colonies from wild collected gravid females that could be used for various experiments.

Recommendation

I recommend the paper to be accepted with minor revisions. Especially, the language in the abstract need to be improved.

Reviewer's Comments and questions

Please check everywhere you have **bold** to see the change done

Introduction

1) ... for adequate growth and **development from larval stages** until adults

2) Insecticide susceptibility studies are important for checkmating the effectiveness of control tools used to control malaria vectors¹⁰. Achieving success in this field requires a good understanding of the bio-ecology of the vector. This will guide us to organize suitable conditions to maintain mosquito samples collected from fields in the laboratory.

Comment: For me this transition is not appropriate. I suggest the authors to say rather that "most of experiments to study the biology of Anopheles mosquitoes, such assessment of insecticide susceptibility, use laboratory reared colonies. Is this therefore crucial to better understand suitable conditions for rearing of field collected mosquitoes".

3) Unfortunately, there are reports that *An. funestus* is involved in malaria transmission across Africa^{12 15}. It is therefore necessary to find all means to study this malaria vector.

Comment: I suggest the authors to say rather "Since Anopheles funestus represents an important malaria vector across Sub-Saharan Africa, it is therefore necessary to find all means to study this malaria vector."

4) for **insecticide** susceptibility testing.....

5) Sometimes, we also have the problem of recording a high mortality and low hatching rate of field mosquitoes under suitable laboratory conditions because of their high sensitivity.

Question: This sentence is not clear. Can the author reformulate it?

6) **Comment:** I found the end of the introduction laborious from " Sometimes, we also have the problem..... " to the end

Material and methods

7) larvae rearing duration corresponding to duration from oviposition till the first pupae stage

Comment: Most of the time, larvae rearing duration is the time from L1 to Puape

8) **Comment:** Larval mortality, not larvae mortality

Data analysis

9) Chi square test was performed to analyze the difference in hatching rate between different water samples and hatching duration according to the water types.

Comment: I guess that hatching duration was expressed in days and that the authors rather compared means. I don't understand why the chi square test was used.

Ethical statement

10) Mosquitoes were collected in the morning using electrical aspirators. No insecticide spray or human bait methods were used for mosquito collections during this study.

Comment: I suggest the authors to say rather "Since mosquitoes were collected using electrical aspirators, no insecticide spray or human bait methods were used for mosquito collections during this study."

Results

11) Nitrate was almost absent in both mineral water (Possotome and FIFA) but was present in high concentration of 118.8mg/l in borehole water (Table I).

Comment: Nitrate was almost absent in both mineral water (Possotome and FIFA) but was present at high concentration of 118.8mg/l in borehole water (Table I).

12) **Comment and question:** I don't understand what the authors mean by egg hatching rate of all incubation days. Could they please explain or use a more clear expression?

13) There was a significant difference in larvae mortality with borehole water and mineral waters ($p < 0.05$).

Comment: The authors didn't mention if larval mortality was statistically different or not between the two mineral waters

14) No adult mosquitoes were recorded from borehole water (Table 3).

Comment: No adult mosquitoes were **obtained** from borehole water (Table 3).

15) Another observation was that the rate of emerged adults with Possotome was slightly higher when compared to FIFA mineral water.

Comment: Please add statistics

Discussion

16) These pH were similar to that **was** found in breeding water samples, with pH values ranging from 4.0 to 7.8 considered favorable for normal development of *An. gambiae* under laboratory conditions^{9,19}.

Comment: Remove "was"

17) This research also showed **similarly** high hatching rates of eggs with FIFA, Possotome and borehole waters, indicating that physico- chemical compositions of the different water samples do not influence the weakening of *An. funestus* egg shells

Comment: Say rather "similar high..." or "comparable high...."

18) **Question:** Can the authors use the term "larval mortality" rather than "larvae mortality"?

19) This nitrate concentration in this water sample is higher than the maximum limit of 50mg/l nitrate dose authorized for human consumption²¹, and could also be toxic for the larvae development.

Comment: Say rather "This nitrate concentration in this water sample is higher than the maximum limit of 50mg/l nitrate dose authorized for human consumption²¹, **and could also be toxic mosquito larvae.**"

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

Yes

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?

Partly

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

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

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

Author Response 14 Jan 2018

Akadiri Yessoufou, University of Abomey, Calavi, Benin

Answers, point by point, to the Reviewer's comments

The Reviewer approved our manuscript. However, he raised some minor points which we have

now addressed and rectified accordingly in this revised version of the MS. The answers to his comments are as follows

Reviewer 2: Cyrille Ndo, Malaria Research Laboratory, Organization of Coordination for the Fight against Endemic Diseases in Central Africa (OCEAC) , Yaoundé, Cameroon

Introduction

- 1) We agree with this correction and we have already incorporated the change in the revised manuscript. Please see in the Introduction section.
- 2) As per Reviewer's suggestion, we have now rectified the sentence accordingly. Please see in the *Introduction* section.
- 3) As per Reviewer's suggestion, we have now reformulated the sentence. Please, see in the Introduction section
- 4) As per Reviewer's suggestion, we have now introduced the word "insecticide" in appropriate place. Please see in the *Introduction* section.
- 5) As per Reviewer's suggestion, we have now reworded the sentence. Please see last paragraph of introduction section: "Sometimes, we recorded high mortality rate even when *An. funestus* mosquitoes are kept under recommended laboratory conditions."
- 6) As per Reviewer's suggestion, we have now reformulated the last sentence of the introduction: "Sometimes, we recorded high mortality rate even when *An. funestus* mosquitoes are kept under recommended laboratory conditions. This observation prompted us to rear *An. funestus* larvae generated from forced-egg laying technique with different water sources but under the same laboratory conditions. Therefore, the aim of this study was to determine the most suitable water source which will permit to obtain the best quantity of F₁ *An. funestus* for laboratory experiments."

Material and methods

- 7) We agree with the Reviewer's comment. "larvae rearing duration is the time from L1 to pupae". We have now corrected this sentence in the revised manuscript.
- 8) We agree with the Reviewer's comment. "larvae mortality" has been replaced by "larval mortality" throughout the revised manuscript.

Data analysis

- 9) We agree with the Reviewer's comment. Chi square test was used to analyze the difference in hatching rate (means) between different water samples only not to compare duration (days). This statement has been corrected in the revised manuscript.

Ethical statement

- 10) As per Reviewer's suggestion, we have now corrected the sentence. Please see in the *Material and Methods* section

Results

- 11) As per Reviewer's suggestion, we have now corrected the sentence. Please see in the *Results* section
- 12) The tests have been repeated several times (see table 2: number of replicate). We have now corrected the sentence to better explain the idea: "The eggs hatching rate of all repetitions (experiments) of each water sample are summarized in Table 2"
- 13) We agree with Reviewer's comment. Larval mortality is not statistically different between the two mineral waters ($P = 0.3573$). We have now mentioned this in the revised manuscript.
- 14) As per Reviewer's suggestion, we have now replaced "recorded" by "obtained" in the revised manuscript. Please see in the *Results* section

15) We agree with Reviewer's comment. We have now corrected the sentence and added statistics to this statement in the revised manuscript (P = 0.1823).

Discussion

16) As per Reviewer's suggestion, we have removed 'was' from the sentence in the revised manuscript. Please see first paragraph of the *Discussion* section

17) As per Reviewer's suggestion, we have now corrected the sentence in the revised manuscript. Please see in the *Discussion* section

18) We agree with the Reviewer's comment. "larvae mortality" has been replaced by "larval mortality" throughout the revised manuscript.

19) We agree with the Reviewer's comment. The comment has been addressed in the manuscript and the sentence has been modified as suggested.

Competing Interests: The authors have nothing to declare as far as the conflict of interest is concerned