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Biting behavior of multiparous female of *Anopheles gambiae s. s.* and transmission of *Plasmodium falciparum* in South-Eastern of Benin

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Abstract

Background: The *Plasmodium falciparum* transmission occurs mainly at night. *Anopheles* mosquitoes that transmit *P. falciparum* are those that have taken blood feed and laid eggs several times. Unfortunately, the biting behavior of these mosquitoes is little known. This study aims to assess the impact of the biting behavior of multiparous vectors on malaria transmission.
Methods: Mosquitoes were collected by hour from 21. 00 hours to 05. 00 hours in two villages, Abomey-Takplikpo and Itassoumba, south-easten Benin. *Anopheles gambiae s.l.* was morphologically identified and dissected using the method of Polovodova and oil injection technique. The head-thoraces were assessed using enzyme-linked immunosorbent assay (ELISA CSP) and the others body parts were used for molecular identification by Polymerase Chain Reaction (PCR).
Results: Among the parous *An. gambiae s.l.* collected at Abomey-Takplikpo and Itassoumba, old *An. gambiae s.l.* that laid eggs several times bite more frequently in the second half of the night ($p<0.05$) than young *An. gambiae s.l.* that were more likely to bite earlier in the houses. On the 1008 and 868 head-thoraces of *An. gambiae s.s.* confirmed by PCR and analyzed by the ELISA-CSP respectively at Itassoumba and Abomey- Takplikpo, 2.98% (n = 30) and 2.53% (n = 22) were tested positive for the circumsporozoitic antigen of *P. falciparum*. Overnight, the infectivity distribution is homogeneous in both localities ($P> 0.05$). However, in the second half of the night, infectious mosquitoes were more frequent.
At Itassoumba and Abomey-Takplikpo, the biting behavior of multiparous females explains respectively 89.51% ($R^2 = 0.8951$) and 90.17% ($R^2 = 0.9017$) of the variability of mosquitoes' infectivity.
Conclusion: Young *Anopheles gambiae s.s.* bite earlier in houses than old *An. gambiae s.s.* that bite more in the second half of the night. Infectious *An. gambiae s.s.* bite at any time of the night but more frequent after midnight. In addition, the biting behavior of multiparous *An. gambiae s.s.* was significantly associated with *P. falciparum* transmission.

Keywords: *Anopheles gambiae s.s*, multiparous, biting behavior, infectivity, *Plasmodium falciparum* transmission, Benin

Introduction

Many insects are vectors of diseases. Among them, mosquitoes are more dangerous due to their abundance and the diseases they transmit. They are vectors of malaria, lymphatic filariasis and arboviral [1]. Among the major endemic tropical diseases, malaria has most impact in children mortality and countries' economy [2]. According to the World Health Organization (WHO), this disease continues to significantly impact on the health and the economic development in Sub-Saharan Africa [2]. In 2013 the number of cases of malaria was estimated to 198 million with 82% in the African region [2]. In the same year, the number of deaths due to malaria was estimated to 2,288 in Benin [2, 3].
In Benin, *An. gambiae s.s.* is the major vector of malaria [6]. The main parasite responsible for the disease is *Plasmodium falciparum*, the most virulent *Plasmodium* species causing the deadly forms of the malaria [7].
Vector control is the main preventive strategy against malaria transmission. Currently, indoor

residual spraying of insecticide and distribution of Long Lasting insecticidal nets (LLINs) are the two major methods of vector control in Benin [8]. One of the objectives of vector control is to avoid man contact with malaria vectors [9]. If the infectivity of mosquitoes depends on the availability of men carrying gametocytes, the longevity of the vectors is a determining factor [10]. Several methods are used to assess the physiological age (longevity) of vectors. But the computing of follicular dilatations is the only method that estimates the number of egg-laying in the mosquito [11]. A recent study showed that infectious mosquitoes, epidemiologically dangerous, are the females that have laid eggs at least twice [11]. In addition, the transmission of malaria essentially occurs at night, it is then useful to study the biting activity of multiparous *Anopheles gambiae s.s.* as well as the periods favorable to their infectivity in *P. falciparum* during the night. In Africa, very few studies documented this important information and the existing data need to be update.
The present paper describes the biting activity of multiparous *An. gambiae s.s.* and its connection with the transmission of *P. falciparum*.

Material and Methods

Study area

This study was conducted in two villages namely Abomey-Takplikpo and Itassoumba between January 2012 and February 2015. These villages are covered with LLINs.

Abomey-Takplikpo

Abomey-Takplikpo is located in the district of Adjarra, south-eastern Benin (Figure 1). The district of Adjarra is located in the department of Oueme with a population of 60112 inhabitants [12] and an area of 112 km^2. Adjarra is bordered in north by the district of Avrankou, in the south by the district of Seme-Podji, in the west by the district of Porto-Novo and in the east by Nigeria. The district of Adjarra has a humid tropical climate characterized by two dry seasons and two rainy seasons. The average rainfall is 1200 mm. The relief of this district is homogenous and very little rough. The vegetation is sparse and dominated by the oil palm (*Eleasis guineensis*).

Itassoumba

Itassoumba village is located in the district of Ifangni in the south-eastern of Benin in the department of Plateau with a population of 71606 inhabitants [12] and an area of 242 km^2 (Figure 1). The climate is guinean with two dry seasons and two rainy seasons. The district of Ifangni is located on Pobe-Sakété plateau with an average altitude of 100 m. It has a little uneven relief with small and medium depressions. The district of Ifangni is crossed by the wetlands used for the production of against-season crops, market gardening and the installation of plant nurseries of various species that promote the creation of pockets of water serving as breeding sites to mosquitoes. The vegetation consists of forests relics, oil palm, high grass and shrubs.

Mosquito collection and identification of female *An. gambiae s.l.*

Mosquitoes were collected in two houses in each village using Human Landing Catches (HLCs) method from 21. 00 hours to 05. 00 hours, twice per month. Two mosquito collectors, one indoor and one outdoor, with a flashlight and haemolysis tubes, collected all landing mosquitoes on their feet at each house. Due to the possible risk of malaria transmission, collectors received an antimalarial prophylaxis to prevent malaria. A monitoring of malaria symptoms was also conducted and treatment was given when necessary. The females of *An. gambiae s.l.* were identified using morphological identification keys [13, 14].

Dissection of the ovaries and determination of the physiological age of female *An. gambiae s.l.*

Dissection of the ovaries and the determination of the physiological age of the vectors were made by time slot with the help of Polovodova method based on the oil injection technique [11].

Conservation of the biological material

The head-thorax and the carcass of each mosquito dissected are cut and stored in two Eppendorf tubes containing silica gel at -20 °C. Eppendorf tubes were labeled according to the collection site, date, time and the mosquito identification number.

Identification of *An. gambiae.* complex species and positivity of females *An. gambiae s.s.* to circumsporozoite protein of *P. falciparum*

The head-thoraces were used for identification of the circumsporozoitic antigen of *P. falciparum* by the method of Enzyme Linked Immunosorbent Assay (ELISA-CSP) described by Wirtz *et al.* [15]. Furthermore, the carcasses were used for the identification of species of the complex *An. gambiae* by Polymerase Chain Reaction (PCR) with the method of Scott *et al.* [16].

Statistical analysis

The average number of bites received per man per hour (b/m/h) was calculated by dividing the number of vectors collected by hour by the number of collection nights. The comparison of b/m/h indoor and outdoor was performed using the test of Poisson. By age group, the proportion of mosquitoes collected by hour was divided by the total number of vectors collected in the time period. The infectivity rates to *P. falciparum* by time period were estimated dividing the number of infected vectors by the number of vectors tested. The pairwise comparison of parturity rates (proportions) by age group and infectivity rates (proportions) by time period was performed using the Khi2 test of multiple proportions comparison [17], and the method of adjustment of p-value of Holm [18]. The estimation of the correlation coefficient allowed us to estimate the level of variability of vector's infectivity explained by of the biting behavior of the multiparous females. All analyzes were performed using R. 2.15.2. [19].

Results

Species of *An. gambiae* complex at Itassoumba and Abomey-Takplikpo

On 2538 and 988 *An. gambiae s.l.* tested by PCR respectively at Itassoumba and Abomey-Takplikpo, *An. gambiae s.s.* was the only species of the complex *An. gambiae* found in both villages.

Biting behavior of female *An. gambiae s.s.* indoor and outdoor at Itassoumba

At Itassoumba, on 2538 specimens of *An. gambiae s.s*, 49.05% (n = 1245) were collected indoor versus 50.95% (n =

1293) outdoor. No significant difference was observed between the two biting rates (p = 0.187).

At Itassoumba, the biting activity of female *An. gambiae s.s.* is as follows:

From 21. 00 hours, the low biting rate of 0.5 bites/man/hour (b/m/h) indoor and 0.88 b/m/h outdoor increased progressively to reach respectively 5.91 and 6.41 b/m/h at 02. 00 hours (Figure 2). Between 02. 00 hours and 05. 00 hours, the maximum biting rate of 7b/m/h, was constant outdoor (Figure 2). Indoor, the biting activity increased to 7.66 b/m/h between 03. 00 hours and 04. 00 hours then dropped to 5.78 b/m/h at 05. 00 hours (Figure 2).

Indoor and outdoor, female *An. gambiae s.s.* showed a high biting activity during the second half of the night. Biting activity was more pronounced outdoor towards the end. Overall, high biting activity (7.45 b/m/h) of *An. gambiae s.s.* was observed between 03. 00 hours and 04. 00 hours at Itassoumba (Figure 2).

Biting behavior of female *An. gambiae s.s.* indoor and outdoor at Abomey-Takplikpo

At Abomey-Takplikpo, on 988 *An. gambiae s.s*, 55.36% (n=547) and 44.64% (n=441) were caught respectively indoor and outdoor. There was a significant difference between the number of *An. gambiae s.s.* collected indoor and outdoor (p<0.001). At Abomey-Takplikpo the biting activity of female *An. gambiae s.s*. is as follows:

From 21. 00 hours, the low biting rate of 0.14 b/m/h indoor and 0.21 b/m/h on outdoor increased gradually to reach respectively 3.11 and 3.29 b/m/h at 01. 00 hour (Figure 3). Indoor, between 01. 00 hour and 03. 00 hours, the maximum biting rate of 3 b/m/h remained almost steady before gradually decreasing to 1.61 b/m/h at 05. 00 hours (Figure 3). Outdoor, the biting activity increased and reached 4.25 b/m/h between 01. 00 hour and 02. 00 hours and then decreased to 2.5 b/m/h at 05. 00 hours (Figure 3).

Indoor and outdoor, female *An. gambiae s.s.* showed higher biting activity during the second half of the night. Overall, the biting activity showed a peak (3.57 b/m/h) between 01. 00 hour and 03. 00 hours at Abomey-Takplikpo (Figure 3).

Biting activity of female *An. gambiae s.s.* according to their physiological age at Itassoumba and Abomey-Takplikpo

At Itassoumba, 1008 female *An. gambiae s.s.* were dissected. In this group of mosquitoes, 28.77% (n=290) were nulliparous (have never laid eggs); 28% (n=383) were uniparous (laid eggs once); 25.89% (n=261) were biparous (laid eggs twice); 5.56% (n=56) were triparous (laid eggs three times) and 1.79% (n=18) were quadriparous (laid eggs four times). Of the 868 females dissected in Abomey-Takplikpo, 29.15% (n = 253) were nulliparous females; 37.90% (n = 329) uniparous; 26.15% (n = 227) biparous; 5.18% (n = 45) triparous and 1.61% (n = 14) of quadriparous.

During all the collection hours (from 21. 00 hours to 05. 00 hours), no significant difference in the proportion of nulliparous females collected at Itassoumba (Figure 4) and Abomey-Takplikpo (Figure 5) was observed. However, in both villages, an unequal distribution of uniparous mosquitoes according to the collection hours was observed (Figures 4 and 5). The same trend was observed with multiparous females (Figures 4 and 5). Indeed, high proportions of uniparous mosquitoes were collected from 22. 00 hours to 05. 00 hours and from 23. 00 hours to 05. 00 hours for biparous females (Figures 4 and 5).

At Itassoumba, high proportions of triparous (66.07%; n=37) and quadriparous females (66.67%; n=12) were collected later in the night between 01. 00 hour and 04. 00 hours (Figure 4). The same trend was observed at Abomey-Takplikpo were high proportions of triparous (84.44%; n=38) and quadriparous (85.71%; n=12) females were collected between midnight and 04. 00 hours (Figure 5).

Overall, in the two study villages, nulliparous mosquitoes have a permanent biting activity during the night. However, parous females were more likely to enter houses lately during the night for blood feeding. In this parous females group, young females (uniparous and biparous) bite earlier indoor than old females (triparous and biparous).

Hourly distribution of *An. gambiae s.s.* infected with *P. falciparum* at Abomey-Takplikpo and Itassoumba

Among the 1008 and 868 head-thorax of *An. gambiae s.s.* tested respectively at Itassoumba and Abomey-Takplikpo for identification of circumsporozoitic (CS) antigen of *P. falciparum*, 2.98% (n= 30) were positive at Itassoumba versus 2.53% (n= 22) at Abomey-Takplikpo (Table 1). In the two villages, the distribution of infected mosquitoes was homogeneous during the collection hours from 21. 00 hours to 05. 00 hours (P>0.05) (Table 1). However, at Itassoumba, *P. falciparum* were most identified in female *An. gambiae s.s* collected (80%; n = 19) between midnight and 04. 00 hours (Table 1). The same trend was observed at Abomey-Takplikpo where 68.18% (n=15) of CS antigen of *P. falciparum* were identified in mosquitoes collected between 01. 00 hour and 04. 00 hours (Table 1). In summary, infectious mosquitoes bite at any time during the night but their biting frequency was high in the second half of the night.

Correlation between biting behavior of multiparous female *An. gambiae s.s* and transmission of *P. falciparum* at Itassoumba and Abomey-Takplikpo

Figures 6 and 7 show the evolution of infectivity according to the biting activity of multiparous females *An. gambiae s.s.* at Itassoumba and Abomey-Takplikpo. The analysis of correlation in both villages showed that there was a significant association between the biting activities (p<0.02). At Itassoumba, the biting activity of multiparous females explains 89.51% (R^2=0.8951) of the variability of mosquitoes' infectivity with *P. falciparum* (Figure 6). Similarly, 90.17% (R^2=0.9017) of the variability of mosquitoes' infectivity are explained by the biting activity of multiparous females in Abomey-Takplikpo (Figure 7).

Discussion

The only vector *An. gambiae s.s.* present at Abomey-Takplikpo and Itassoumba should facilitate the control of malaria transmission. Indeed, when two species of the complex coexist, malaria control becomes more difficult due to the difference in biting behavior between species [20-22]. At Abomey-Takplikpo, outdoor biting of *Anopheles gambiae s.s.* is higher than indoor during the night. This behavior is different from what is known on the classic endophagy of mosquitoes in rural Africa with more attraction to human indoor. This change in feeding behavior is probably related to the excito-repellent effect of LLINs that are in use in the houses. These LLINs were freely distributed in 2011 by the National Malaria Control Program (NMCP) in Benin [23]. The same reasons explain the similar biting indoor and outdoor at

Itassoumba. The trend in exophagy is associated with a deliberate exophily characterized by low densities of blood fed, semi-gravid and gravid females and a short rest post-meal [24]. The exophily of *An. gambiae s.s.* may be a resistance behavior to pyrethroids [25-27]. Indeed, several studies have shown that resistance to the lethal effect of insecticides is associated with resistance to their repellent effect by changing behavior of resistant individuals [24, 27] that leave the houses before having contact with the insecticide used for impregnation of LLINs. According to other authors [28, 29], this change in feeding behavior of the vectors were similar to the optional exophagy in urban areas.

The biting behavior of females *An. gambiae s.s.*, characterized by a peak at midnight, was also reported in West African savanna areas [30, 31]. This behavior demonstrates that mosquitoes were adapted to the behavior of its human host. Particularly at Itassoumba, during the last hour of our collections (between 04. 00 hours and 05. 00 hours), the overall biting activity of *An. gambiae s.s* (6.63 b/m/h) represents 88.99% of biting activity of mosquitoes (7.45 b/m/h), suggesting that the biting activity of vectors continues after 05. 00 hours. This observation suggests the implementation of a specific study on the biting activity of *Anopheles* mosquitoes at Itassoumba. Furthermore, in parous females group characterized by a biting activity during the second part of night [30], the young females *Anopheles gambiae s.s* enter houses s earlier than old female. Fernandes & Briegel [32] showed that females of *An. gambiae* fed daily with blood and sugar juice have a maximum longevity of 54 days and 20 days when the females are only fed with sugar juice. This shows that the blood provides a lot of energetic elements allowing mosquitoes to live longer. In addition, the number of gonotrophic cycles depends on the number of blood meals taken. These two observations suggest that the number of gonotrophic increase the energetic reserves in the mosquitoes. The study also demonstrated that young *An. gambiae s.s.* express their need to feed on human before old ones that are likely to have more energetic reserves. According to Detinova [33], the decline of mobility due to the age should not be neglected in old individuals group in a natural population.

In our two study areas, the biting behavior of multiparous females explained about 90% of the variability of mosquitoes' infectivity to *P. falciparum*. These results show that there is a good correlation between the biting behavior of multiparous females and transmission of *Plasmodium falciparum*. High frequencies of mosquitoes carrying gametocytes was also observed at Itassoumba [34] and Abomey-Takplikpo [3]. This shows the endemic nature of malaria in both villages. Indeed, the availability of individuals carrying gametocytes increases the likelihood of infection of epidemiologically dangerous mosquitoes (multiparous). So, it is normal that the biting behavior of multiparous females explains the variability of malaria transmission. Furthermore, the closed link observed between biting activity of multiparous females and malaria transmission justifies once again the main objective of vector control that aims to kill vectors before they reach the age of hosting sporozoites in their salivary glands in malaria endemic areas [9]. That explains why, vector longevity remains one of the most important indicators when evaluating the effectiveness of vector control tools. Usually, the longevity of mosquitoes is expressed by the parturity rate which is the ratio of the number of mosquitoes that have laid eggs at least once (parous) on the total number of mosquitoes dissected [35]. In Africa, studies reported that the increase of the infectivity rates depends on the parous rate in *Anopheles* vectors [6, 10]. However, conflicting results was obtained by other authors [36].

At Itassoumba and Abomey-Takplipko, quadriparous and triparous females *An. gambiae s.l.* were more active during the second half of the night between midnight and 04. 00 hours. Although mosquitoes that have laid eggs twice are epidemiologically dangerous, the likelihood that they are infected is low compared to females that have laid eggs three and four times [11]. Between midnight and 04. 00 hours where malaria transmission is higher, the sleeping human population constitutes an easy prey for mosquitoes [30]. So, the use of LLINs is an effective preventive method against malaria [37]. According to a recent study conducted in southern Kenya, a drastic decline of over 90% of the entomological inoculation rate (EIR) of *An. gambiae* was observed after 13 years of use of LLINs with a coverage rate of over 86% and an average of 1 LLIN for 2.5 persons [38]. Akogbeto *et al.* [6] also showed 72% of reduction of the aggressive density of *Anopheles* and 83.3% of EIR reduction at Dangbo, a district located in the department of Oueme (southern Benin) after massive use of LLINs. So, it is important to use vector control tools to prevent malaria.

Table 1: Time distribution of infectivity rate of *An. gambiae s.s.*with *P. falciparum*

Hours	Itassoumba			Abomey-Takplikpo		
	Nb tested mosq	Nb CS+	% CS+	Nb tested mosq	Nb CS+	% CS+
21h-22h	48	1	2,08[a]	33	0	0,00[a]
22h-23h	86	1	1,16[a]	74	1	1,35[a]
23h-00h	140	2	1,43[a]	121	2	1,65[a]
00h-01h	145	5	3,45[a]	128	2	1,56[a]
01h-02h	168	8	4,76[a]	150	5	3,33[a]
02h-03h	154	6	3,90[a]	137	5	3,65[a]
03h-04h	157	5	3,18[a]	133	5	3,76[a]
04h-05h	110	2	1,82[a]	92	2	2,17[a]
Total	1008	30	2,98	868	22	2,53

h: hours, mosq: mosquito, Nb: number, CS+: positivity in circumsporozoitique protein of *P. falciparum*

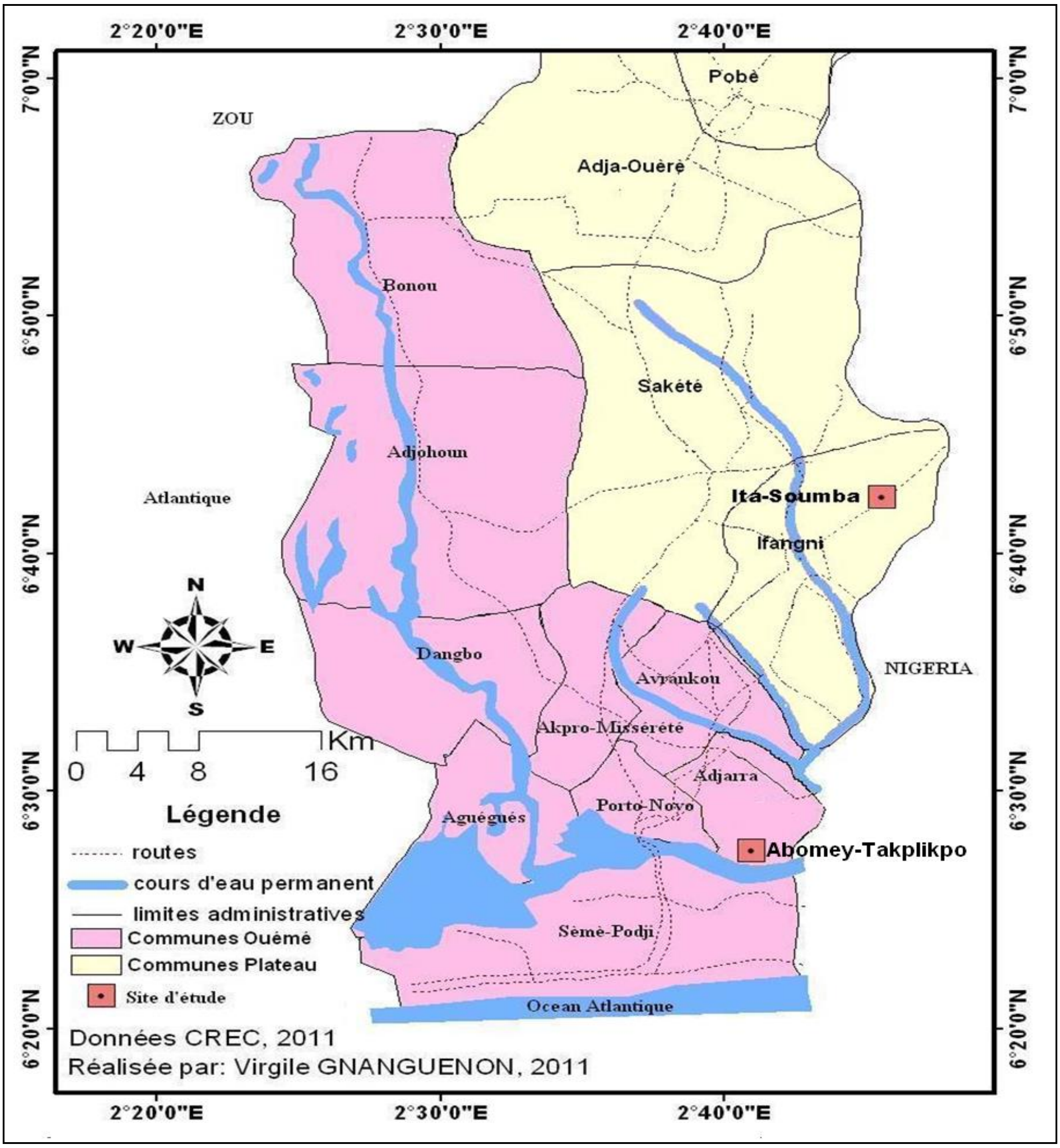

Fig 1: The villages of Itassoumba (district of Ifangni) and Abomey-Takplikpo (district of Adjarra) in south-eastern Benin [11]

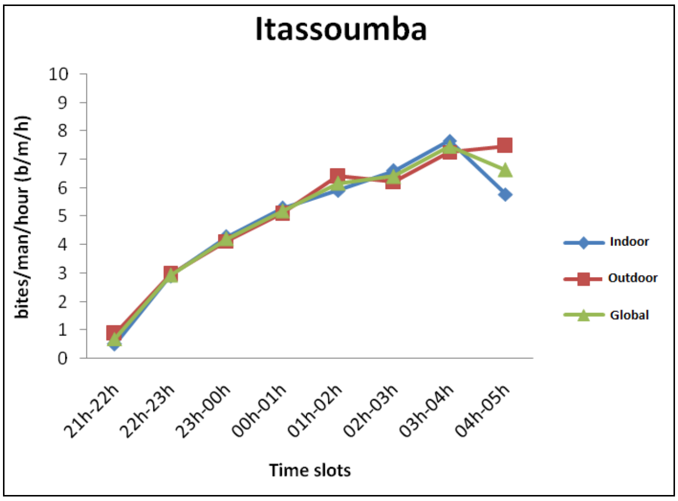

Fig 2: Cycle of aggressiveness of females *An. gambiae s.s.* caught on man inside and outside of dwellings at Itassoumba

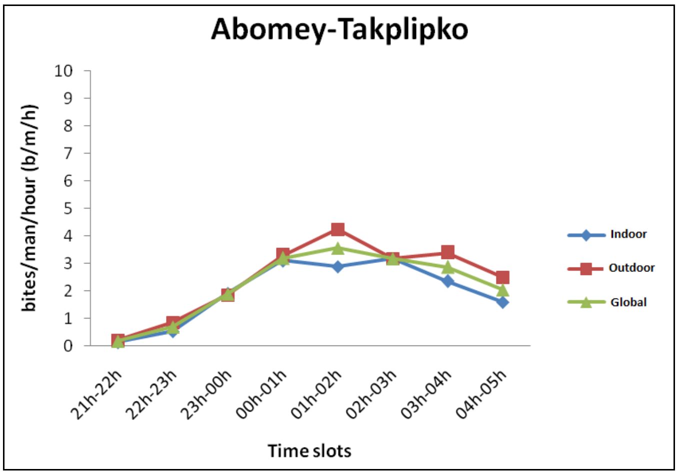

Fig 3: Cycle of aggressiveness of females *An. gambiae s.s.* caught on man inside and outside of dwellings at Abomey-Takplikpo

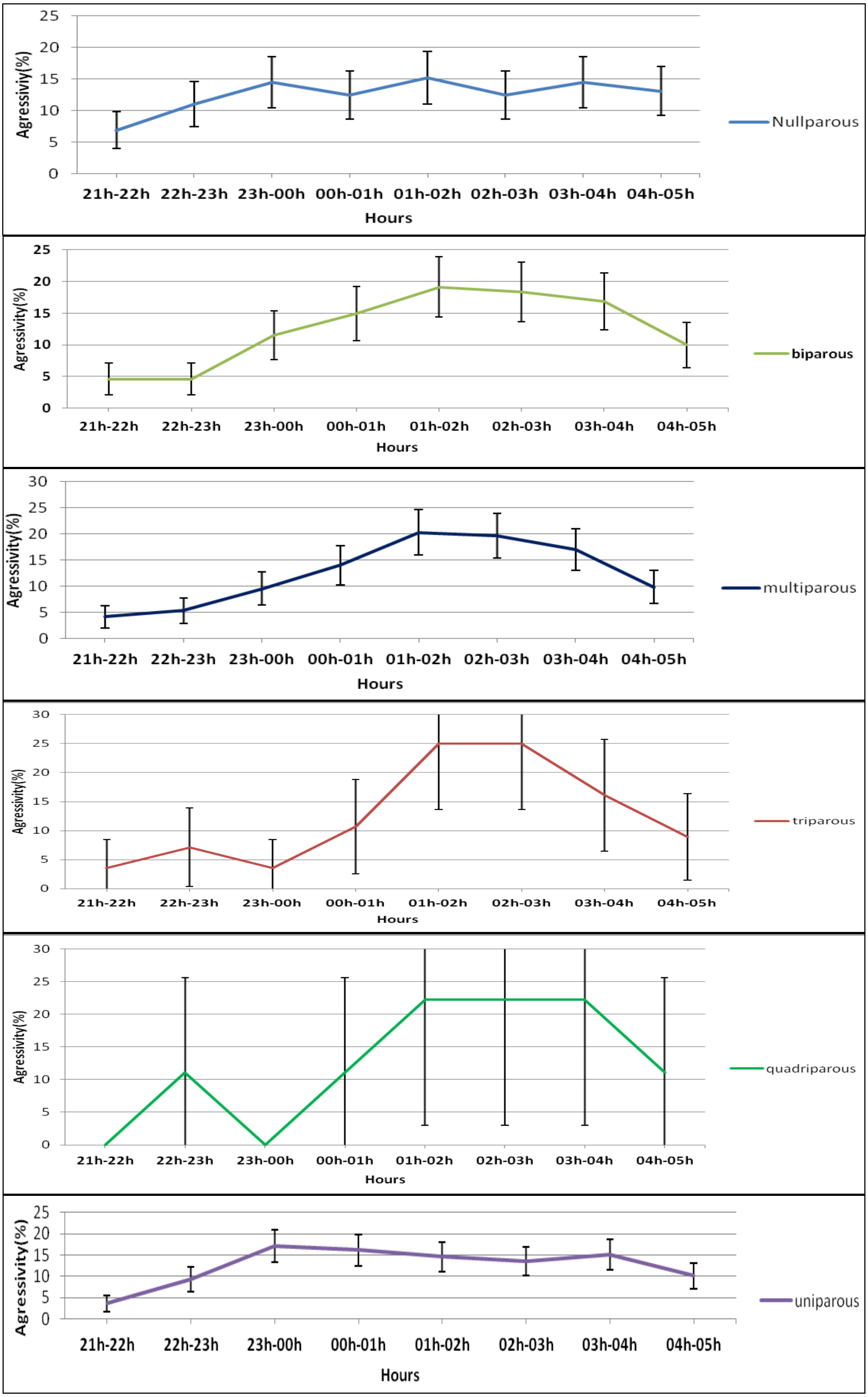

Fig 4: Rhythm of activity according to the physiological age of females *An. gambiae s.s.* at Itassoumba

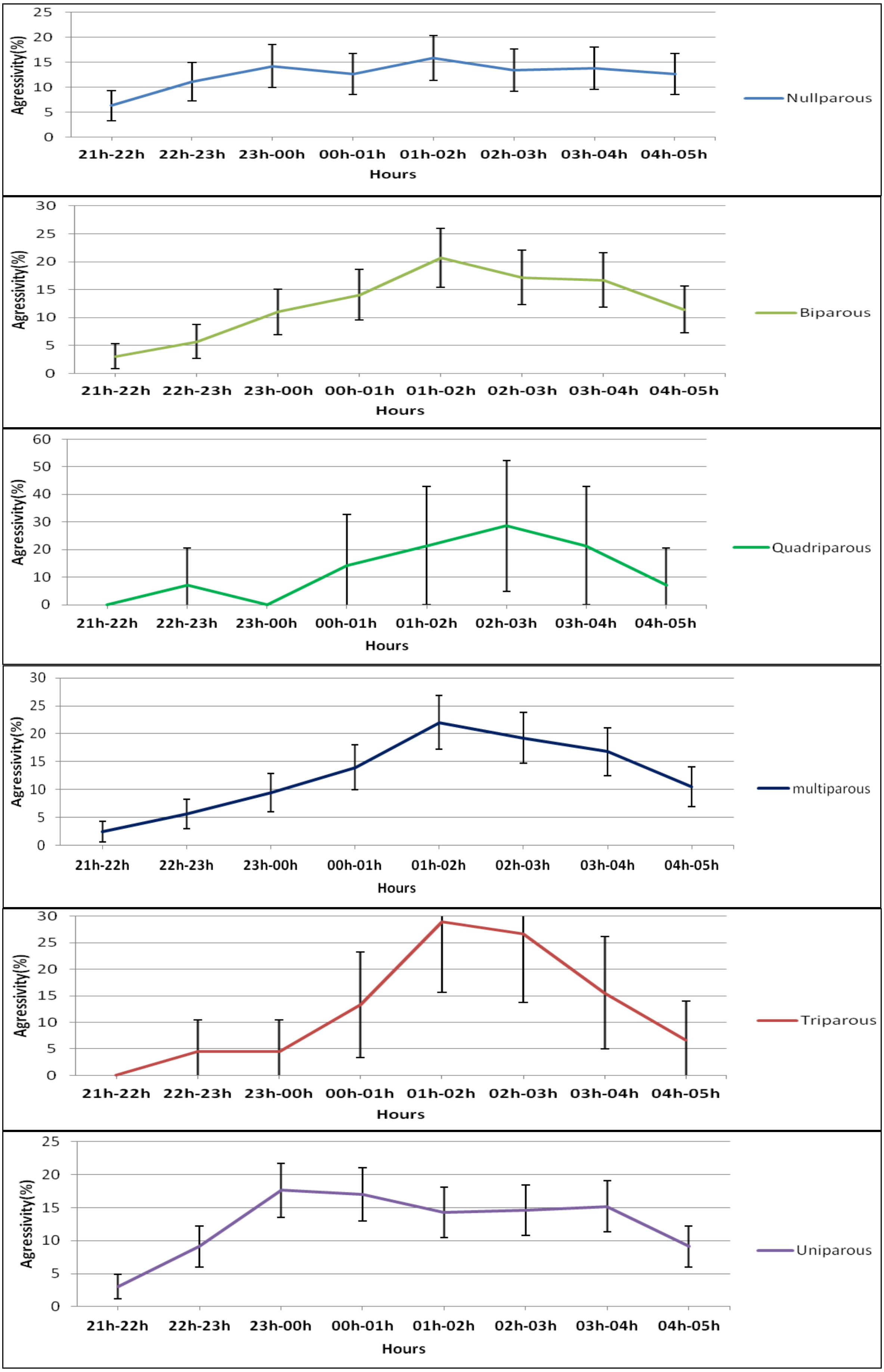

Fig 5: Rhythm of activity according to the physiological age of females *An. gambiae s.s.* at Abomey-Takplikpo

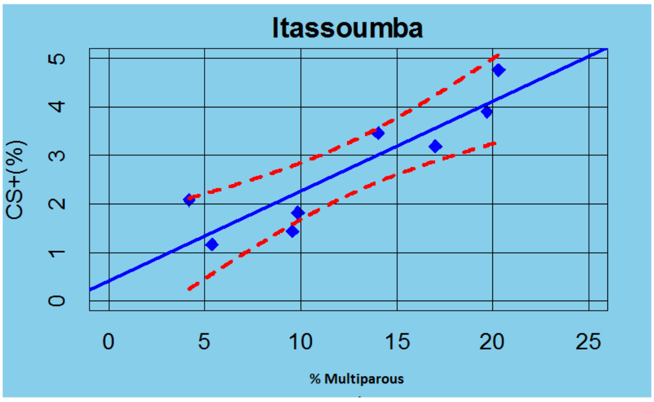

Fig 6: Evolution of the infectivity depending on the aggressiveness of multiparous females of *An. gambiae s.s.* at Itassoumba

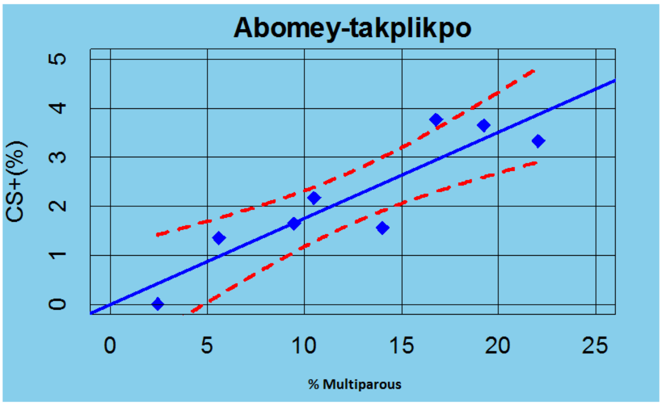

Fig 7: Evolution of the infectivity depending on the aggressiveness of multiparous females of *An. gambiae s.s.* at Abomey-Takplikpo

Conclusion
An. gambiae s.s. biting activity was permanent throughout the night with high frequencies in the second half of night at Itassoumba and Abomey-Takplikpo. In the parous females *An. gambiae s.s* group, young females bite earlier indoor than old females. Furthermore, the biting behavior of multiparous females explains the variability of *P. falciparum* transmission. While the infective bites were low during the first part of night, in the second half, period during which the multiparous females are regular, the infective bite were higher and more frequent. An appropriate use of LLINs should be effective to prevent malaria in these areas. Moreover, when people are awake at night, the use of repellents products should be an alternative against infective mosquito bites.

Abbreviations
WHO: World Health Organization; b/m/h: bites per man per hour; CSP: Circumsporozoitic antigen of *P. falciparum*; ELISA: Enzyme Linked Immunosorbent Assay; PCR: Polymerase Chain Reaction; LLIN: Long Lasting Insecticidal Nest; EIR: Entomological Inoculation Rate; NMCP: National Malaria Control Program; CREC: Entomological Research Center of Cotonou; PMI: President Malaria Initiative; USAID: United States Agency for International Development.

Ethics approval and consent to participate
Approval [N°007/2010] was obtained from the ethic committees of the Ministry of Health of Benin. Mosquito collectors gave prior informed consent and they were vaccinated against yellow fever. They were also subjected to regular medical check-ups with preventive malaria treatments.

Consent for publication
All authors have read and agreed the content of manuscript as well as its publication, and that any experimental research that is reported in the manuscript has been performed with the approval of appropriate ethics committee. The corresponding author is Rodrigue Anagonou

Availability of supporting data and materials
The data and materials are under the custodianship of Entomological Research Center of Cotonou (CREC) and are accessible upon request addressed to CREC**.**

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Author's Contributions
RA and MAK conceived the study. RA, GP, MAK, VG, ID, ASA and MA have participated in the design of the study. RA, AS, ASA and FA have carried out the field activities and the laboratory analyses. RA, ASA, FA, AS and VG have participated in laboratory studies. VG has contributed to the mapping. BA did statistical analyzes. RA, ID, ASA and MAK drafted the manuscript. RA, ID, IA, AH, RAI, RAZ, ASA, RG, IT, MI, UN and MAK critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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