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Using ovarian development stages in the determination of the physiological age in the females of *Anopheles gambiae s.l,* south-eastern Benin

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Abstract
Background: The physiological age of vectors is an indicator often used in the evaluation of the efficacy of malaria vector means. The purpose is to study the ovarian development stage for the determination of the physiological age of mosquitoes.
Methods: Mosquitoes were collected using human landing catch at night in the district of Itassoumba and Abomey-takplikpo, south-eastern Benin. Females *An. gambiae s.l* were identified morphologically using the determination keys. The ovaries were dissected in distilled water. Additionally, one ovary is cut and transferred into Natrichlorid physiological liquid for the reading of ovarian development stage. Mosquitoes, which are between the stages I and II-young are nulliparous and those which are between II-mean and III are parous. After drying, the tracheoles of ovary left in distilled water were examined with the usual Detinova method.
Results: Among 271 *An. gambiae s.l* examined at Itassoumba, 72 were nulliparous with the reading of ovarian tracheoles against 66 with the reading of the ovarian development stages (P=0.61). With the 199 remaining identified mosquitoes as parous with the examination of tracheoles, the reading of the ovarian development stages has recognized 173 as parous (P=0.18). At Abomey-takplikpo, on 197 specimens examined, 52 and 49 were nulliparous respectively with the reading of tracheoles and the examination of the ovarian development stages (P=0.71). Among the 145 remaining mosquitoes identified as parous with the tracheoles method, 119 were parous with the examination of the ovarian development stages (P=0.12).
Conclusion: The development stages of ovaries are usable for the determination of the physiological age of mosquitoes. Nulliparous mosquitoes are between the stages I and II-young. However, parous are between the stages II-mean and III.

Keywords: *Anopheles gambiae s.l*, ovaries, physiological age, stage, tracheoles

1. Introduction
In sub-saharan Africa, *Anopheles gambiae s.l.* is the major malaria vector [1]. In 2013, the number of malaria cases was estimated at 198 million in the world with 82% in Africa region and causing 584000 deaths [2]. In the same period, the number of deaths due to malaria was estimated at 2288 in Benin [2, 3]. Vector control is the main strategy for malaria prevention. The aim of vector control is to reduce the number of infectious vectors. Vectors longevity is one of the most used indicators for assessing the effectiveness of vector control programs [4]. Usually, longevity is expressed by the parturity rate of the vectors. Besides, blood meal ensures the maturation of ovaries which follows several stages described by Christophers [5]. Others authors [6-9] have shown that after egg-laying, the parous females are often beyond stage II-young of the ovarian development contrary to nulliparous females. We can think that it exists a link between the development stage of ovaries and the parturity of mosquitoes. Scientifically, we miss reliable data on this biological aspect of mosquitoes. The purpose of this study is to establish the correlation between the parturity and the ovarian development stage of vectors.

2. Methods
2.1. Study area
This study was carried out at Adjarra and Ifangni districts in Benin (Figure 1).

2.1.1. Adjarra

In Adjarra district, mosquitoes sampling was carried out in the village of Abomey-takplikpo (Figure 1). Adjarra district is located at 06°27'00' N and 01°56'00' E, in the department of Oueme with a population of 60,112 inhabitants and an area of 112 km^2 [10]. It is bordered in the north by Avrankou district, in the south by Seme-Podji district, in the west by Porto-Novo district and in the east by Nigeria. The climate is sub-equatorial with two dry seasons and two rainy seasons. The average rainfall is 1200 mm per year. The hydrographic network of this district includes the lagoon of Porto-Novo in the south and Aguidi River in the northeast. The vegetation is sparse; it is composed of shrubs, grass and by sacred forests relics.

2.1.2. Ifangni

Entomological surveys were conducted at Itassoumba (Figure 1) in the district of Ifangni (Province of Plateau) located at 06°38'56'N and 02°43'14'E with a population of 71,606 inhabitants and an area of 242 km^2 [10]. The climate is Guinean with two dry seasons and two rainy seasons. The annual precipitation is between 800 mm and 1400 mm. The vegetation includes sacred forest relics, plantations of oil palms, shrubs and tall grass. Itassoumba is crossed by swamps. In the dry season, the breeding sites of *An. gambiae s.l.* are scarce. However, breeding sites are particularly permanent in Itassoumba due to the presence of fish ponds and vegetable farming. Once animal feed reserved for small fry and fish are present on the surface of the water, they constitute a source of food favoring the proliferation of malaria vectors.

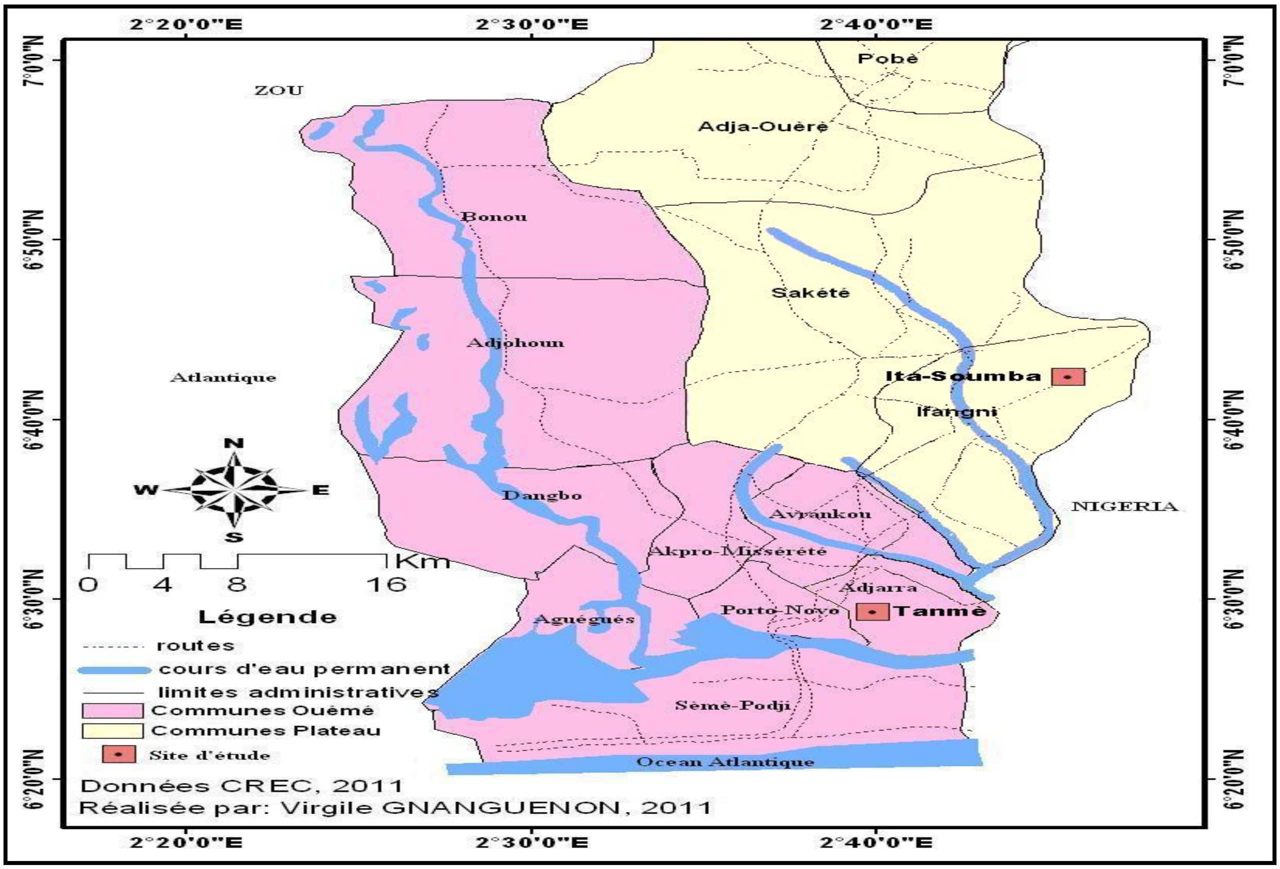

Fig 1: The villages of Itassoumba (district of Ifangni) and Abomey-takplikpo (district of Adjarra) in south-eastern Benin

2.2. Sampling of mosquitoes and identification of females *An. gambiae s.l.*

Mosquitoes were caught on man from 9:00 p.m. to 5:00 am. Collections were carried out inside and outside houses using hemolytic tubes and torch. After each collection, *An. gambiae s.l.* females were identified morphologically [11, 12].

2.3. Dissection of ovaries and determination of ovarian development stages and physiological age of females of *An. gambiae s.l.*

The ovaries of mosquitoes were dissected in distilled water using binocular microscope. One ovary of each mosquito is put on lamella in distilled water and the second on other lamella in physiological liquid (Natrichlorid 0.9% + Neutral red 1/5000-1/3000). After drying of ovary transferred in distilled and ovarian tracheoles were examined with a microscope (10X-40X) using Detinova method [4]. The tracheoles are wound (platoons) in nulliparous mosquitoes but are unwound in parous females. Moreover, the ovarian development stage was determined with ovaries transferred in physiological liquid. Mosquitoes found between the stages I and II-young are considered as nulliparous females. However, those found between the stages II-mean and III are parous females.

2.4. Statistical analysis

The comparison of nulliparous females' numbers and parous obtained with the reading of ovarian tracheoles and the observation of the ovarian development stage have been performed using khi 2 test. The analyses were performed with software R-2.15.2 [13].

2.5. Ethical consideration
Ethical approval for this study was received from the Ministry of Health [N°007/2010]. 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.

3. Results
3.1. Structure of ovaries showing physiological age in *An. gambiae s.l.*
Figures 2 and 3 showed respectively after microscopic observations, the curled aspect of ovarian tracheoles in nulliparous females and the unwound aspect of tracheoles in parous.

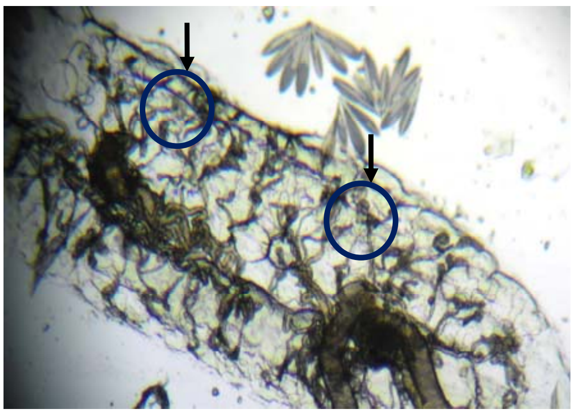

Fig 2: Nulliparous ovary (tracheoles coiled) according to Detinova (CREC, 2013)

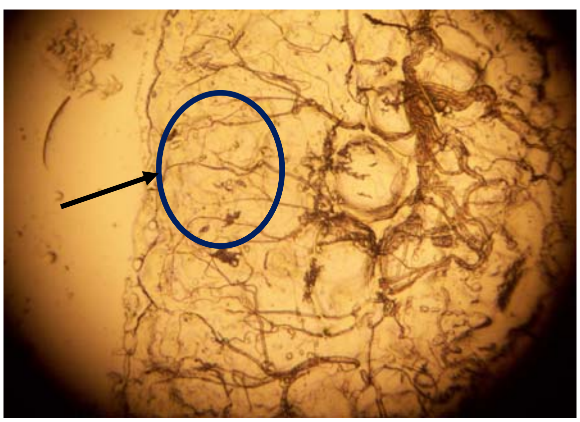

Fig 3: Parous ovary (tracheoles unwound) according to Detinova (CREC, 2013)

3.2. Ovarian development stages in *An. gambiae s.l.*
During dissection sections of *Anopheles* collected from Itassoumba and Abomey-takpliko, the ovarian follicles have been observed at different development stages in mosquitoes. The ovarian development stages in mosquitoes. The development stages are present as follow:

Stage I: Follicle is rounded, it is no vitellus grain. The oocyte nucleus do not differentiate from the one of nourishing cellular’s (Figure 4).

Stage II: It has been subdivided in three.

Stage II-young: Small vitellus granules appear around the oocyte nucleus (Figure 5).

Stage II-mean: The vitellus granules are visible with the objective (Figure 6).

Stage II-end: The vitellus occupies the half of follicle (Figure 7).

Stage III: The follicle is very oval and the vitellus occupies more than the half of follicle (Figure 8).

Stage IV: The follicle is lengthened and completely filled of vitellus (Figure 9).

Stage V: The egg is completely constituted with the floats (Figure 10).

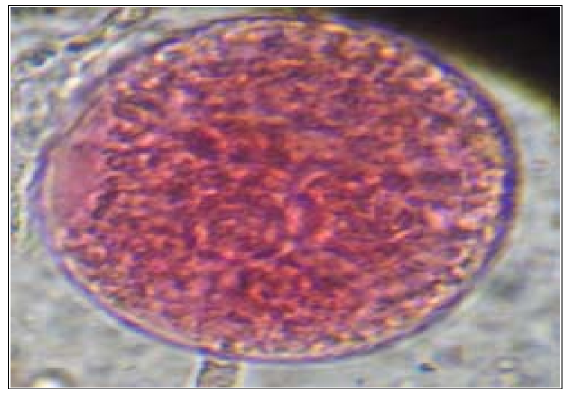

Fig 4: Follicle at stage I (40X) (CREC, 2013)

Fig 5: Follicle at stage II-young (40X) (CREC, 2013)

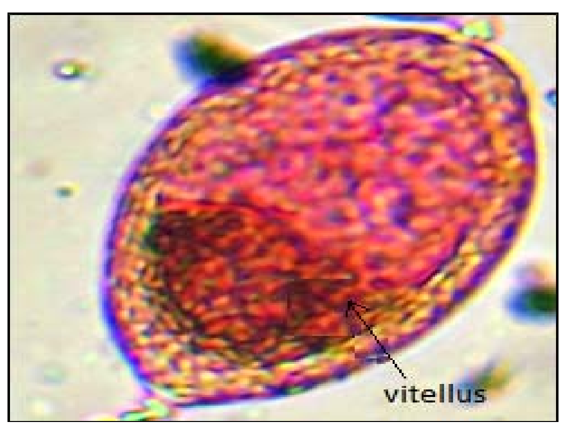

Fig 6: Follicle at stage II-mean (10X) (CREC, 2013)

Fig 7: Follicle at stage II-end (10X) (CREC, 2013)

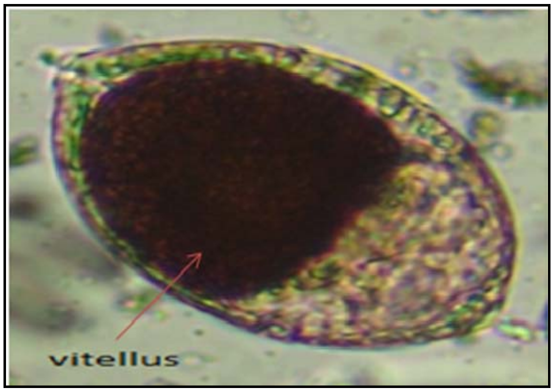

Fig 8: Follicle at stage III (10X) (CREC, 2013)

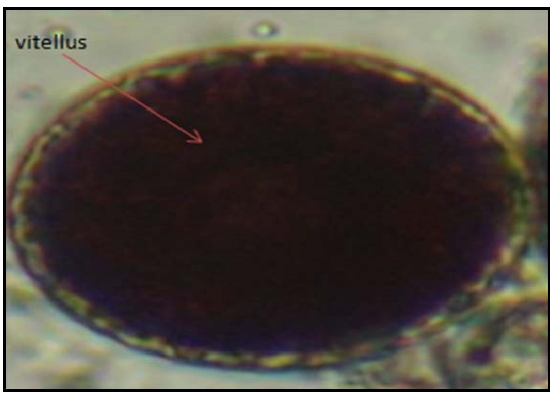

Fig 9: Follicle at stage IV (10X) (CREC, 2013)

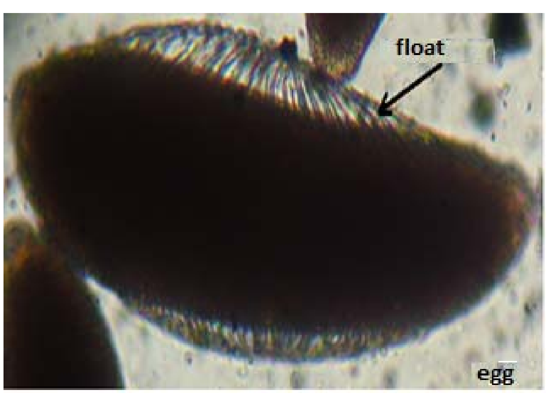

Fig 10: Follicle at stage V (10X) (CREC, 2013)

3.3. Relationship between the parturity and the development stages of ovaries in *An. gambiae s.l.*

At Itassoumba, among 271 females of *An. gambiae s.l.* examined by the reading of the ovarian tracheoles, 72 and 199 mosquitoes were respectively identified as nulliparous and parous females. Among the 72 nulliparous mosquitoes, 66 were found between the stages I and II-young. No significant difference has been observed between these two rates (p=0.61) (Table 1). Concerning the 199 parous mosquitoes, 173 are between the stages II-mean and III. No significant difference has also been observed between these two rates (p=0.18) (Table 1).

Besides, among the 197 mosquitoes examined by the reading of the ovarian tracheoles from Abomey-takplikpo, 52 have been identified as nulliparous against 145 parous. Among the 52 nulliparous females, 49 are found between the stages I and II-young. No significant difference has been observed between these two rates (p=0.77) (Table 1). The same tendency has been observed with the 145 parous females of which 119 were found between the stages II-mean and III (p=0.12) (Table 1).

As results, in Itassoumba and Abomey-takplikpo, the development stage of ovaries explains the parturity of mosquitoes. The nulliparous mosquitoes are between the stages I and II-young. However, the parous are between the stages II-mean and III.

Table 1: Comparison between the reading of ovarian tracheoles and the observation of the ovarian development stages in the determination of the physiological age in *An. gambiae s.l.*

	Age	Meth trach	Meth stage	p-value
Site 1	Nulliparous	72	66	0.61
	Parous	199	173	0.18
Site 2	Nulliparous	52	49	0.77
	Parous	145	119	0.12

Site 1: Itassoumba, site 2: Abomey-takplikpo, Meth: method, trach: tracheoles

4. Discussion

As we microscopically observed, the maturation of ovaries follows several steps. The similar observations were reported by several authors [4, 7, 8, 14]. Globally, the most of nulliparous females (more than 80%) are found at stage I of Christophers with a rounded follicle and the absence of vitellus grains [15]. However, according to the results obtained from study sites, 20 (16.12%) nulliparous specimens show the ovarian follicles of stage II-young and II-end. The slight ovarian development observed here could be linked to two reasons. First, these mosquitoes can be pre-gravid females. On the other hand, during human landing catch, some females succeed to take some blood meal before be caught. Thus, during the time between mosquito catch and dissection, the digestion of this blood meal has allowed to advance the development of ovarioles in these mosquitoes. Actually, human landing catch at night is uneasy to monitor. Spite of the precautions, some mosquito collectors under the tire effect, have been bitten by mosquitoes before capturing them.

Concerning the parous females obtained in both districts, it meets very few of specimens (n=16, 4.65%) presenting the ovaries at stage I of Christophers. The high frequencies of parous females are found at the stages II-mean and III. Likely, the residual of first blood meals have released a slight development of ovaries which has translated by the evolution of some ovarioles from stage I to stages II and III. Even if we do not neglect the blood meal took by some females before catching them, the residual effect of the previous blood meals is a determinant factor. From these observations, it bring out that in nulliparous and parous female, it exists two level of ovarian development. In parous females, the gonotrophic cycle is fast because it starts from more advanced stage (II-mean, III), while in nulliparous, it starts from the stage I, which justifies in pre-gravid females, the need to additional blood meal to complete the cycle. These results confirm the duration of gonotrophic cycle which is about 4 to 5 days in nulliparous mosquitoes and 2.5 days in parous [7, 15].

Conversely to our expectation, some gravid females at stage IV and V were caught on human seeking for blood meal. This phenomenon correspond likely to a reaction of defence against the lost of water by evaporation. The studies conducted by Hamon [16] have shown that this phenomenon is frequent in *An. superpictus* et *Phlebotomes sp* during a year hot and dry period in central Asia.

5. Conclusion

After egg-laying, the parous *An. gambiae s.l.* are between the stages II-mean and III of ovarian development. However, the nulliparous are in stages I and II-young. Thus, the ovarian development stages can be used in the determination of the physiological age of *An. gambiae s.l*, malaria vector.

6. Conflict of interests

The authors have not declared any conflict of interests

7. Authors' contributions

Rodrigue Anagonou and Martin Akogbéto conceived the study. Rodrigue Anagonou, Gil Germain Padonou and Martin Akogbéto have participated in the design of the study. Rodrigue Anagonou, Gil Germain Padonou and Martin Akogbéto carried out the field activities and the laboratory analyses. Virgile Gnanguenon has contributed to the mapping. Bruno Akinro did statistical analyzes. Rodrigue Anagonou, Fiacre Agossa, Gil Germain Padonou and Martin Akogbéto drafted the manuscript. Rodrigue Anagonou, Gil Germain Padonou and Martin Akogbéto critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

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