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Observation of granulations in the basal body of ovarioles and follicular dilatations for the determination of physiological age of *Anopheles gambiae* s.s.

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ABSTRACT

Objective: To explore ovariole basal body granulations and follicular dilatations for determining physiological age in *Anopheles gambiae* s.s. (*An. gambiae* s.s.).
Methods: Mosquitoes were collected by using window trap catch and identified morphologically. For the first lot of mosquitoes, they were dissected, and ovary was left in distilled water for reading ovarian tracheoles and the second was cut and transferred to another blade in a physiological liquid for verification of ovariole basal body granulations. The same approach was followed with the second lot of mosquitoes where follicular dilatations were found after classic dilaceration of ovaries were transferred into physiological liquid. The other body parts of mosquitoes were used to identify the species of the *An. gambiae* s.s. complex by PCR.
Results: Among the 123 *An. gambiae* s.s. of the first lot, the method of Detinova determined the age of 89 mosquitoes versus 114 for the observation of granulations ($P > 0.05$). Among the 112 *An. gambiae* s.s. of the second lot, the method of Detinova determined the age of 84 mosquitoes versus 93 for the observation of follicular dilatations ($P > 0.05$). Unlike the method of Detinova, observation of follicular dilatations and basal body granulations of ovarioles were possible beyond the stage II Christophers.
Conclusions: Overall, the observation of follicular dilatations and ovariole basal body granulations are reliable for the determination of the physiological age in *An. gambiae* s.s. Furthermore, these two methods can be used beyond the stage II.

1. Introduction

The physiological age of malaria vectors is an important indicator in the evaluation of vector control means. The main objective of vector control means is to destroy vectors that are able to have sporozoites in their salivary gland[1]. The common method used to determine the physiological age of mosquitoes is the one described by Detinova, which is based on the aspect of ovarian tracheoles[2-4]. However, this method is not applicable in vectors of which the ovaries have evolved beyond stage II[2]. In this case, human landing catch is the appropriate type of mosquitoes sampling. Human landing catch allows capturing mosquitoes of stage I-II, and this favors ovarian tracheole method. Unfortunately, this kind of sampling causes ethical problems. Despite of all precautions to protect capturers, some are bitten by mosquitoes before catching them.

Window trap catch and the collection of residual mosquitoes in interior of houses have the advantage of providing starved mosquitoes, but they are fewer in quantity and some of them have already taken a partial blood meal. Consequently, among mosquitoes caught by using window trap or spray, there are females of beyond stage II-mean. It is, therefore, important to explore other determination methods of physiological age which can be used to examine the ovaries beyond stage II. Thus, this study was implemented to study other determination methods of physiological age of anopheles. If the observation of follicular dilatations and granulations of basal bodies have been used, they are not well known for the determination of physiological age of malaria vectors[5-7].

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2. Materials and methods

2.1. Study area

The study was conducted (between May and November 2013) in the region of Plateau in the south-east part of Benin and specifically at Itassoumba in the district of Ifangni (Figure 1). This region has an area of 3 264 km^2, with a total population of 407 116 inhabitants[8]. The number of malaria cases recorded there in 2011 and 2012 were 40 512 and 33 745 respectively[9-11]. The village of Itassoumba has rugged relief with the presence of some depressions. We found Guinean climate alternated during year between two dry and two rainy seasons: a long rainy season from March to July and a short dry season during the month of August, a short rainy season from September to November and a long dry season from December to February. Itassoumba recorded annual precipitation between 800 mm and 1 400 mm. The vegetation is composed of sacred forests, plantations of oil palms, shrubs and tall grasses. Itassoumba has swamps scattered all over. In the dry season, the breeding sites of *Anopheles gambiae* s.l. (*An. gambiae* s.l.) are scarce but permanent in Itassoumba due to the presence of fish ponds and marshes for market gardening. When animal feed reserved for fish gets onto the water's surface, it provides a source of food for the proliferation of malaria vectors, *Anopheles*.

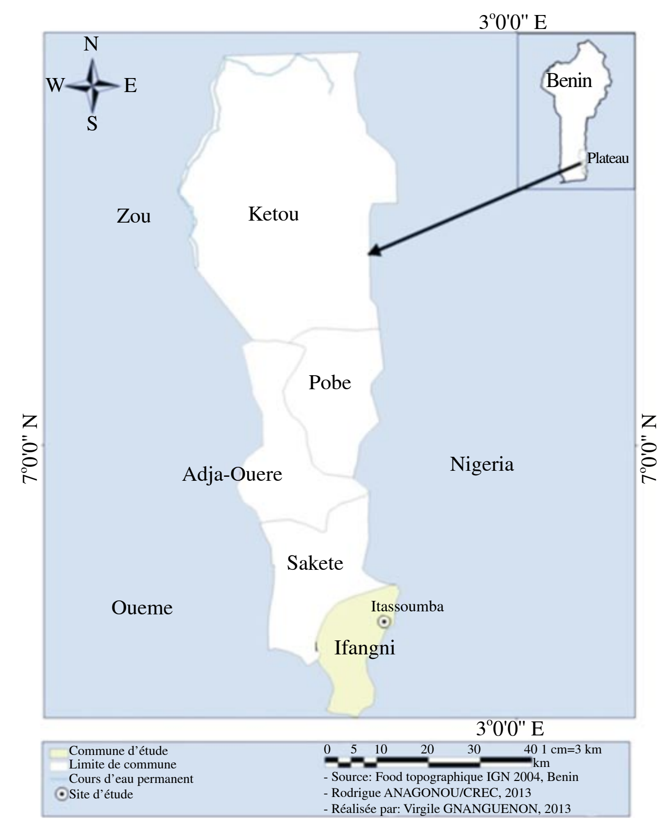

Figure 1. Map of the village of Itassoumba in the district of Ifangni, Benin.

2.2. Sampling of mosquitoes

Indoor biting and outdoor resting mosquitoes were collected by using window trap from 6 p.m. to 6 a.m.

2.3. Identification, dissection of ovaries and determination of physiological age of females An. gambiae s.l.

Anophelines collected were morphologically identified to species by using taxonomic keys of Aïkpon *et al.* and Ossè *et al.*[3,12]. The ovaries of the first lot of *An. gambiae* s.l. were dissected in distilled water with a microscope. Each mosquito ovary was left in distilled water and the second was cut and transferred to another blade in a physiological liquid (Natriumchlorid 0.9% + Neutral red 1/5000-1/3000). Similarly, the same approach was followed with the second lot of mosquitoes.

After drying, the ovarian tracheoles left in the distilled water were examined by the method of Detinova[2,3]. The presence of granulations was directly verified in the basal ovariole body transferred in the physiological liquid in the first lot of mosquitoes. The presence of granulations in the basal body of ovariole indicated that the mosquitoes were parous. Nulliparous females were without granulations[5]. In the second lot of mosquitoes, the presence of follicular dilatation was found after the classical dilaceration of ovaries transferred in the physiological liquid. Mosquitoes with at least a follicular dilatation were parous. Those without dilatation were nulliparous[2,6].

2.4. Characterization of An. gambiae species complex

Abdomen, wings and legs of dissected mosquitoes were used individually for *An. gambiae* species identification using PCR according to the protocol of Mala *et al.*[13,14].

2.5. Statistical analysis

To appreciate the reliability of methods based on the observation of follicular dilatations and the ovariole basal body granulations for the determination of physiological age, we compared the percentage of parous and nulliparous females between Detinova method and those of follicular dilatations and of granulations by calculating the *P*-values using Fisher's test. Also, pairwise comparison of unreadable lamellas following the ovarian development stages was done using pairwise test of comparison of multiple proportions with adjustment of *P*-value of Holm[2]. All analyses were done by using R.2.15.2[15,16].

3. Results

3.1. Structure of ovaries

Figures 2, 3 and 4 show the curled aspect of ovarian tracheoles, the basal body without granular ovarioles and the absence of follicular dilatations in nulliparous females respectively. However, Figures 5, 6 and 7 show the non-curled aspect of ovarian tracheoles, the presence of granulations in the basal body of ovarioles and the presence of a follicular dilatation in parous females respectively.

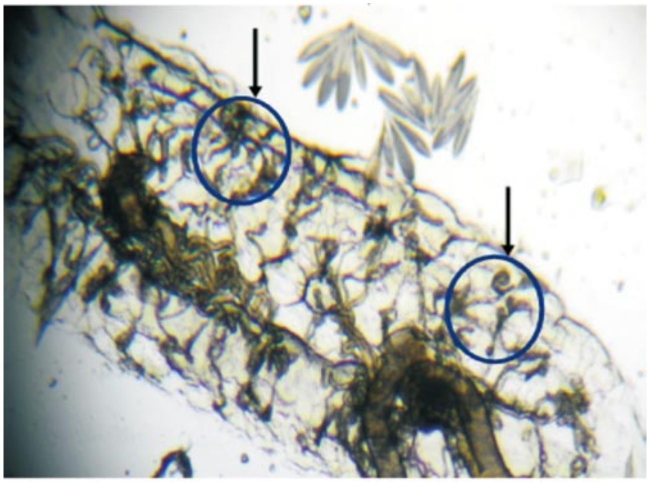

Figure 2. Nulliparous ovary (coiled tracheoles) according to Detinova (100 ×; CREC, 2013).

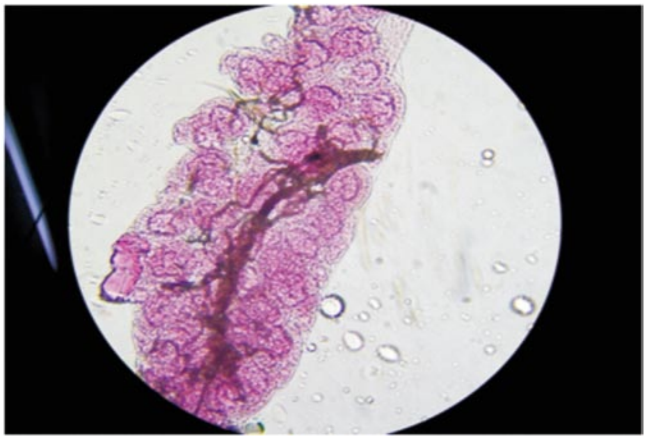

Figure 3. Nulliparous ovary (no granulations) according to Hoc (40 ×; CREC, 2013).

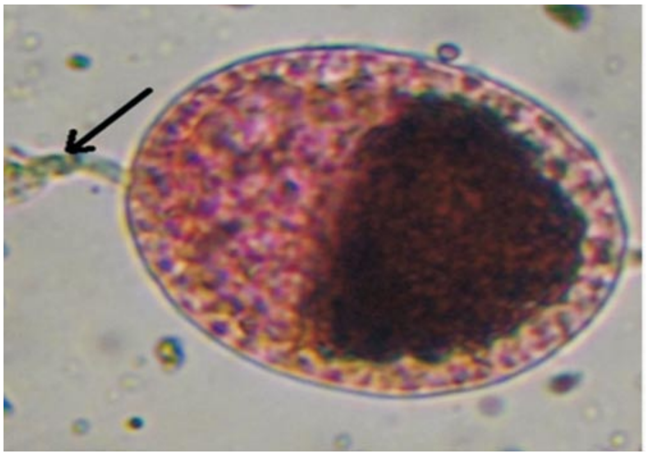

Figure 4. Nulliparous ovary (no dilatation) according to Lewis (40 ×; CREC, 2013).

3.2. Comparison of the reading of ovarian tracheoles and observation of granulations in the basal body of ovarioles

Among 123 females of *Anopheles gambiae* s.s. (*An. gambiae* s.s.) examined, 33.34% (n = 41) were identified nulliparous by the reading of ovarian tracheoles and 30.08% (n = 37) were identified nulliparous by the observation of granulations in the basal body of ovarioles (Table 1). No significant difference was observed between these two rates (P = 0.651). In the same lot of mosquitoes, 39.02% (n = 48) were identified parous from the reading of ovarian tracheoles and 62.60% (n = 77) were identified parous by the observation of granulations of ovarioles. The determination of physiological age based on the observation of granulation seemed to be more effective than the method based on the reading of ovarian tracheoles (P = 0.009) (Table 1). The use of granulations present in the basal body of ovarioles was a reliable method for the determination of physiological age in *An. gambiae* s.s.

Table 1

Reliability of the observation of granulations in the basal body of ovarioles for the determination of the physiological age in *An. gambiae* s.s.

Age	Tracheoles	Granulations	P-value
Nulliparous	41 (33.34%)	37 (30.08%)	0.651
Parous	48 (39.02%)	77 (62.60%)	0.009
Unreadable	34 (27.64%)	9 (7.32%)	-
Total	123 (100.00%)	123 (100.00%)	-

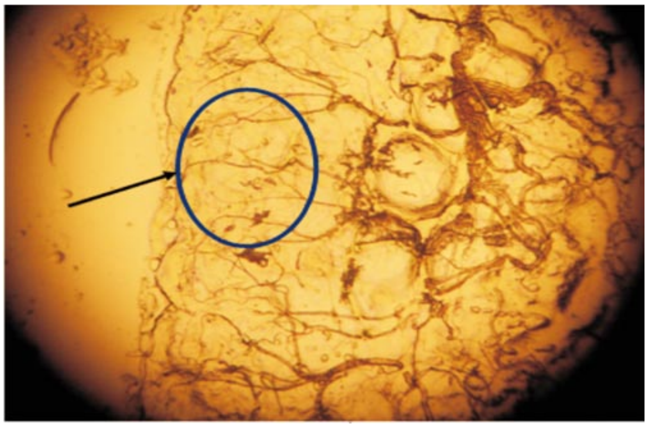

Figure 5. Parous ovary (unwound tracheoles) according to Detinova (100 × ; CREC, 2013).

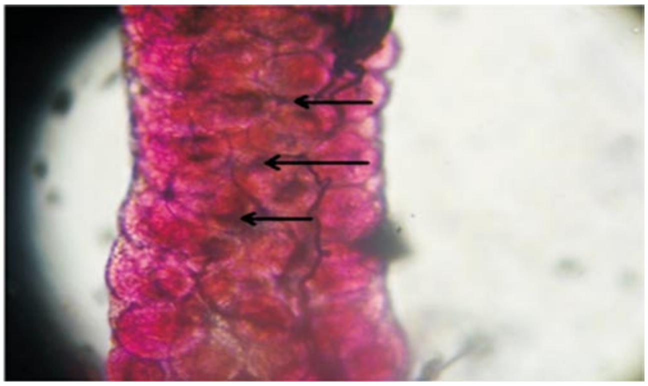

Figure 6. Parous ovary according to Hoc (40 ×; CREC, 2013).

3.3. Comparison of the reading of ovarian tracheoles and the observation of follicular dilatations

Among 112 *An. gambiae* s.s. examined, 17.86% (n = 20) nulliparous females were identified based on the reading of ovarian tracheoles and 14.29% (n = 16) were identified by the observation of follicular dilatations. No significant difference was observed between these rates (P = 0.505). Also, 57.14% (n = 64) of females were parous from ovarian tracheoles against 68.75% (n = 77) with the follicular dilatations. No significant difference was observed between these two rates (P = 0.314) (Table 2). The use of follicular dilatations also appeared to be reliable for the determination of the physiological age in *An. gambiae* s.s.

Table 2

Reliability of follicular dilatations for the determination of the physiological age in *An. gambiae* s.s.

Age	Tracheoles	Dilatations	*P*-value
Nulliparous	20 (17.86%)	16 (14.29%)	0.505
Parous	64 (57.14%)	77 (68.75%)	0.314
Unreadable	28 (25.00%)	19 (16.96%)	-
Total	112 (100.00%)	112 (100.00%)	-

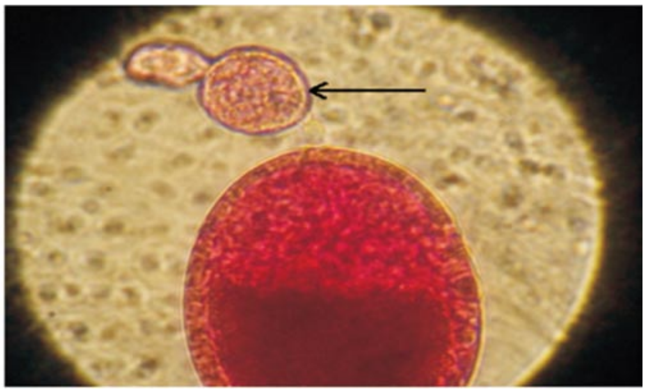

Figure 7. Parous ovariole (with a dilatation) according to Lewis (40 ×; CREC, 2013).

3.4. Influence of development stage of ovaries on ovarian tracheole reading, granulations in the basal body and follicular dilatation observation for the determination of anopheles physiological age

Among the 235 ovaries examined by Detinova method, 26.38% (*n* = 62) were unreadable. The illegibility rate increased significantly with the development of ovaries from stage II ($P < 0.001$) (Table 3). Concerning the method based on the observation of granulations in the basal body of ovarioles, only 7.32% (*n* = 9) of ovaries were not read out of a total of 123 and the fail rate showed the same tendency following the stages of ovarian development ($P > 0.05$) (Table 3). In the same way, regarding the use of follicular dilatations, the repartition of 16.96% (*n* = 19) of failures recorded among the 112 mosquitoes examined was the same ($P > 0.05$) following the ovarian development stages (Table 3). Nevertheless, the majority of failures (16/19) were observed between the stages I and II mean with the method of follicular dilatations (Table 3).

Table 3

Variation of indetermination rate of the physiological age following the ovarian development stages by three methods.

Stages	Tracheoles			Granulations			Dilatations		
	Total mstq	*N* (fails)	% (fails)	Total mstq	*N* (fails)	% (fails)	Total mstq	*N* (fails)	% (fails)
I	40	0	0.00^{b}	28	1	3.57^{a}	12	7	58.33^{a}
IId	91	6	6.59^{b}	41	2	4.88^{a}	50	5	10.00^{b}
IIm	39	6	15.38^{b}	20	2	10.00^{a}	19	4	21.05^{ab}
IIf	33	22	66.67^{a}	19	2	10.53^{a}	14	0	0.00^{b}
III	23	19	82.61^{a}	12	2	16.67^{a}	11	2	18.18^{ab}
IV	9	9	100.00^{a}	3	0	0.00^{a}	6	1	16.67^{ab}
Total	235	62	26.38	123	9	7.32	112	19	16.96

Percentages which carry the same letters in exposant were not significantly different ($P > 0.05$). d: Beginning; m: Mean; f: Aged; mstq: Mosquito. ^{a,b}: Standard letters used to differentiate the values that are statistically different.

4. Discussion

The follicular dilatations and the granulations of basal body of ovarioles are very useful for the determination of the physiological age in *An. gambiae* s.s. The method based on the use of follicular dilatations is reliable since Detinova method is usually used for the determination of the physiological age of mosquitoes. The same results have been observed with the method based on the observation of granulations in the basal body of ovarioles.

The reliability of granulations in the basal body of ovarioles for the determination of the physiological age of anopheles has been reported[5]. According to Darskaya *et al*., the density of granulations could increase after successive egg-laying, facilitating the reading of ovaries[7]. According to Anagonou *et al*., the observation of granulations in the basal body of ovarioles is possible beyond the stage II[6]. Yet, the three ovaries of stage IV dissected in our samples have been difficult to read. In stage IV of the ovaries' development, the eggs are almost formed and occupy all envelopes of ovarioles so that the basal body of ovarioles can not be observed directly. Despite this advantage, the method of granulations is expensive and requires good microscopy for its application. The observation of granulations is not possible when the installation of liquid is not well prepared (more concentrated or at least concentrated in neutral red) or late after preparation. These reasons explain the illegibility of 7.32% (*n* = 9) of ovaries in our sample.

The reliability of follicular dilatations for the determination of the physiological age of anopheles confirms the results of Anagonou *et al*.[2,6]. The observation of follicular dilatations is possible beyond the stage II, but it is not in stage IV when the eggs are almost formed and occupy the entire ovariole[5,6]. The isolation of ovarioles to allow the observation of dilatations requires more manual dexterity in mosquitoes in stage I-II mean. At the beginning of ovarian development, the ovarioles are joined to each other at the point where their isolation requires more application. This explains the majority of indetermination of age (*n* = 16, 84.21%) recorded in stage I-II mean with a total of 19. Thus, in a sample where the number of mosquitoes with the beginning ovarian development is important, the isolation of ovarioles becomes constraining and consequently leads to an enormous loss of mosquitoes of which the physiological age is not determined. A combination of the observation of follicular dilatations with the method of Detinova based on the aspect of the ovarian tracheoles could be ideal for the determination of the physiological age of mosquitoes. In addition, the pedicle of ovarioles lengthens with the successive egg-laying in parous mosquitoes[6]. This phenomenon produces an extreme fragility of pedicle susceptible to break during isolation by using needles[5,6]. Nevertheless, the classical dilaceration of ovaries has allowed us to observe a follicular dilation in parous females.

Detinova method allows examining 50 to 60 specimens each hour, and so does the observation methods of follicular dilatations and of granulations in the basal body of ovarioles. These two processes allow examining 60 to 80 mosquitoes per hour. Converse to the reading of ovarian tracheoles which was done after drying the ovaries, the observation methods of dilatations and of the granulations present the advantages that they can be directly applied after extraction of ovaries. Inefficiency of Detinova method from stage II development of ovaries has been reported in several articles[2,17]. About 19.35% (n = 12) of ovaries are unreadable in stage I-II, due to platoons compressed between the ovarioles when they arise profile. Indeed, from stage II age, the vitellus reserves become enormous and completely cover the tracheolar network making the ovaries illegible after drying[2]. This is not the case for mosquitoes at stage I- II whose reading of tracheoles is very easy.

In conclusion, the method based on the observation of follicular dilatations and of granulations in the basal body of ovarioles are applicable for the determination of the physiological age in *An. gambiae* s.s. Furthermore, the combination of the Detinova method and follicular dilatations would be very effective. Contrary to the usual method of Detinova based on the aspect of ovarian tracheoles that is not usable from the stage II, the observation of follicular dilatations and granulations in the basal body of the ovarioles is possible beyond stage II. The reading of tracheoles is the best method for determining the physiological age of anopheles from night captures. It is easy, fast, cheap and useful for a debutant technician. However, methods based on the follicular dilatations and granulations of basal body are indicated for mosquitoes captured in the houses. Both methods are relatively expensive and require technical expertise.

Conflict of interest statement

We declare that we have no conflict of interest.

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