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Development of new combined method based on reading of ovarian tracheoles and the observation of follicular dilatations for determining the physiological age of *Anopheles gambiae* s.s.

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The conventional dilaceration of ovaries is binding at the beginning of ovary development. However, reading ovarian tracheoles was very easy in stage I-II mean. The combination of both methods can really reduce the number of age indeterminations recorded with the two methods separately. The present study aimed to identify a method capable of determining physiological age of mosquitoes regardless of their ovarian development stage. In the present study, mosquitoes were caught in houses using window traps. After identification of *Anopheles gambiae* s.l species, their ovaries were dissected in distilled water. An ovary was left in distilled water for tracheoles reading and the other in a physiological liquid to search for follicular dilatation after dilaceration of ovary. The other body parts of mosquitoes were used to identify the species of the *A. gambiae* complex by polymerase chain reaction. The ovarian tracheoles reading method was unable to determine physiological age of 25% (n=28) of 112 *A. gambiae* s.s. analyzed. With follicular dilations observation method, the physiological age of 16.96% (n=19) mosquitoes was not determined; but, the age indetermination rates were reduced to 0.89% by combining the two methods. The combination of ovarian tracheoles reading method and follicular dilatation observation method significantly reduced (almost null) the number of physiological age indeterminations recorded using the two methods separately.

Key words: *Anopheles gambiae* s.s, ovary, physiological age, ovarian tracheoles, classical dilacerations, follicular dilatation.

INTRODUCTION

In 2013, the number of malaria cases was estimated at 198 million in the world with 82% in Africa region and causing 584000 deaths (WHO, 2014). In the same period, the number of deaths due to malaria was

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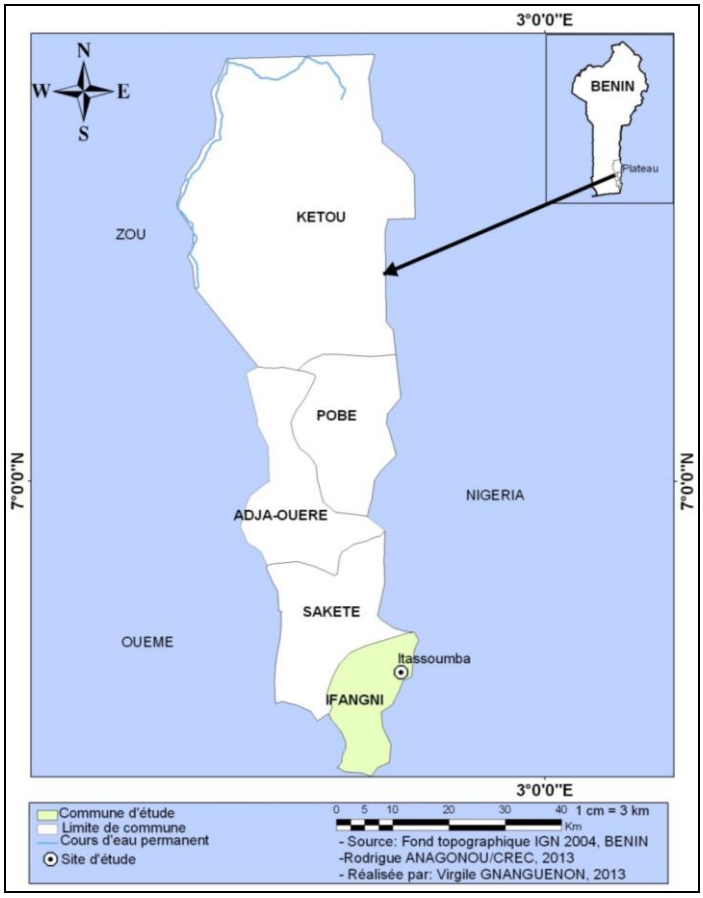

Figure 1. Map showing the village of Itassoumba in the district of Ifangni, Benin.

estimated at 2288 in Benin (OMS, 2014; MS, 2014). Vector control is the main strategy for malaria prevention. The aim of vector control is to reduce the number of infectious vectors (Hamon et al., 1961). Vector longevity is one of the most used indicators for assessing the effectiveness of vector control programs. According to Polovodova method, the number of dilatations observed on ovarioles should be equal to the number of egg-laying (physiological age) in females of mosquitoes (Hugo et al., 2008). Difficulties observed when applying Polovodova method justify the use of Lewis method (Mondet, 1996) to facilitate the identification of nulliparous females which have not laid and those which have laid at least once during their live cycle. With Lewis method, ovarioles are separated in a physiological serum and the absence (nulliparous females) or presence (parous females) of dilatations on ovarioles (follicles) is verified with a microscope (Hamon et al., 1961). At the beginning of ovaries' development, ovarioles are more joined one to others and their isolation is not easy during classical dilaceration of ovaries. In a sample where the number of mosquitoes at beginning of their ovarian development is important, Lewis method becomes difficult and therefore leads to an impossibility to determine physiological age of mosquitoes dissected. However, the usual method of Detinova based on ovarian tracheoles aspects is easily with mosquitoes in stage I-II mean of their ovarian development (Hugo et al., 2008). In a representative sample where about 1/5 of the specimens are not examined, the representativeness of the sample size is weaken. The combination of ovarian tracheoles reading method and follicular dilatations observation method can really reduce the number of mosquitoes whose physiological age cannot be determined when both methods are used separately. The aim of this study was to explore a method capable of determining physiological age of all mosquitoes submitted for examination regardless of their ovarian development stage.

METHODOLOGY

Study area

Entomological surveys were carried out between May and November 2013 at Itassoumba (Figure 1) in Ifangni district (Province of plateau) located 06°38'56''N and 02°43'14''E in South-east of Benin with 71606 inhabitants (INSAE, 2002). Itassoumba

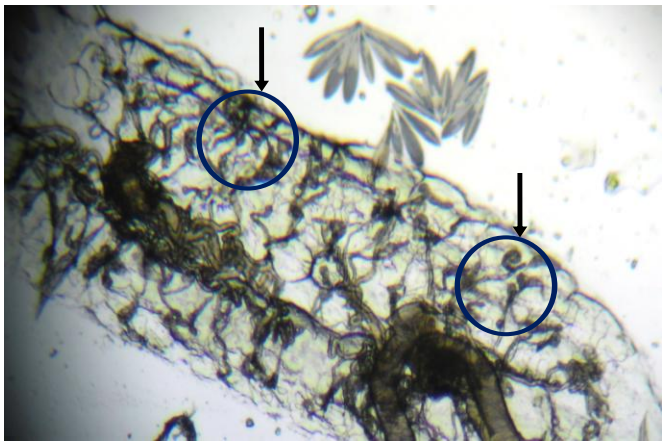

Figure 2. Nulliparous ovary (tracheoles coiled) according to Detinova (CREC, 2013).

has a rugged relief with the presence of some depressions. The climate is Guinean with two dry and rainy seasons: a great rainy season from March to July, a small dry season in August, a small rainy season from September to November and a great dry season from December to February. Itassoumba recorded an annual rainfall between 800 and 1400 mm water. The vegetation includes relics of sacred forests, plantations of oil palms, shrubs and tall grasses. Itassoumba is crossed by swamps. In the dry season, the breeding sites of *Anopheles gambiae* s.l are scarce. They are particularly permanent in Itassoumba due to the presence of fish ponds and marshes for vegetable farming.

Sampling of mosquitoes

Mosquitoes were caught using window traps from 6 to 18 h. Indoor biting and outdoor resting mosquitoes were collected and kept separately in Eppendorf tube until taken to the laboratory.

Identification, dissection of ovaries and determination of physiological age of females of *A. gambiae* s.l

Mosquitoes species collected were morphologically identified (Gillies and Coetzee, 1987; Gillies and De Meillon, 1968). 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, tracheoles of ovaries were examined with a microscope (4-10x) using Detinova method (Hugo et al., 2008). The tracheoles are wound (platoons) in nulliparous mosquitoes but are unwound in parous females. Moreover, the presence of follicular dilatation was verified after classical dilaceration of ovaries transferred in physiological liquid. According to Lewis method (Mondet, 1996), parous mosquitoes have at least one dilatation on their ovarioles but the nulliparous females do not have one.

Characterization of *A. gambiae* species complex

Abdomen, wings and legs of dissected mosquitoes were used for *A. gambiae* species identification using PCR (Scott et al., 1993).

Statistical analysis

To assess the reliability of method based on the observation of follicular dilatations for the determination of physiological age, we have compared the percentage of parous and nulliparous females between Detinova and Lewis methods by estimating the p-values using Fisher test.

Pairwise comparison of unreadable lamellas following the ovarian development stages was done using pairwise comparison test of multiple proportions (Robert, 1998) with Holm adjustment of p-value (Benjamini and Yekutieli, 2001). The same approach was used for the comparison of indetermination rate obtained by combining Lewis and Detinova methods to that obtained using these methods separately. All statistical analyses were done using R.2.15.2 (Development Core Team, 2011).

RESULTS

Structure of ovaries and ovarioles showing physiological age in *A. gambiae* s.s.

Figures 2 and 3 shows respectively after microscopic observations, the curled aspect of ovarian tracheoles and the absence of follicular dilatation in nulliparous females. However, Figures 4 and 5 show respectively the absence of curled aspect of tracheoles and the presence of a follicular dilatation in parous females.

Comparison of ovarian tracheoles reading method and follicular dilatations observation method for determination of physiological age in *A. gambiae* s.s.

On 112 females of A. gambiae s.s. examined, 20 (17.86%) and 16 (14.28%) nulliparous females were identified respectively by ovarian tracheoles aspect and the observation of follicular dilatations. No significant difference was observed between these two rates (p=0.505) (Table 1). 64 (57.14%) females were parous according to ovarian tracheoles method against 77 (68.75%) for follicular dilatations observation method. No

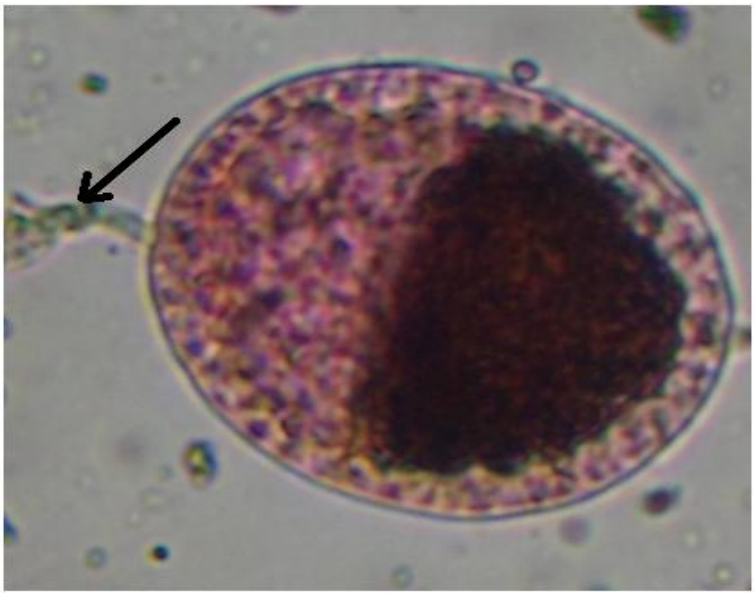

Figure 3. Nulliparous ovary (no dilatation) according to Lewis (CREC, 2013).

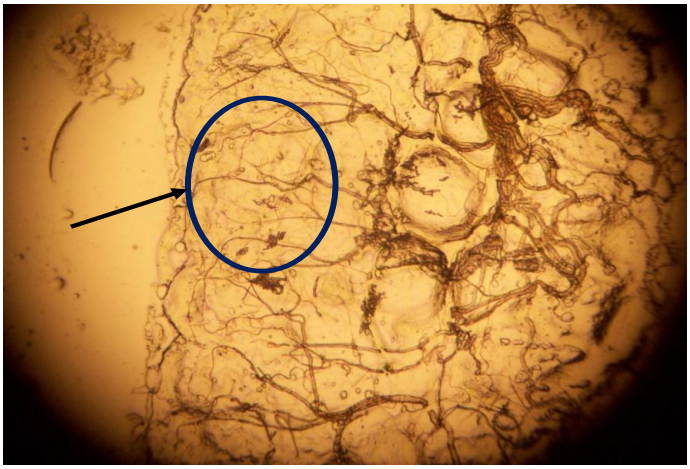

Figure 4. Parous ovary (tracheoles unwound) according to Detinova (CREC, 2013).

significant difference was observed between these two rates (p=0.314) (Table 1). These results confirm that the use of follicular dilatations is a reliable method for A. gambiae s.s physiological age determination.

Influence of ovarian development stage on ovarian tracheoles reading method and the follicular dilatations observation method for determination of *A. gambiae* s.s. physiological age

With 112 mosquitoes examined by ovarian tracheoles reading method, 25% (n=28) were unreadable and this rate increased significantly with ovaries development ($p<0.05$) (Table 2). The unreadable ovaries were 4.94% at stage I-II mean against 85.71% at stage II aged-IV of ovaries development (Table 2). According to the follicular dilatations observation method, the distribution of 16.96% (n =19) of mosquitoes whose age was not determined was homogenous following the ovarian development (Table 2). The majority of indeterminations (84.21%) was recorded in stage I-II mean with the observation of follicular dilatations (Table 2). Overall, the indeterminations of physiological age recorded with ovarian tracheoles reading method were associated with ovaries development. However, with follicular dilatations observa-

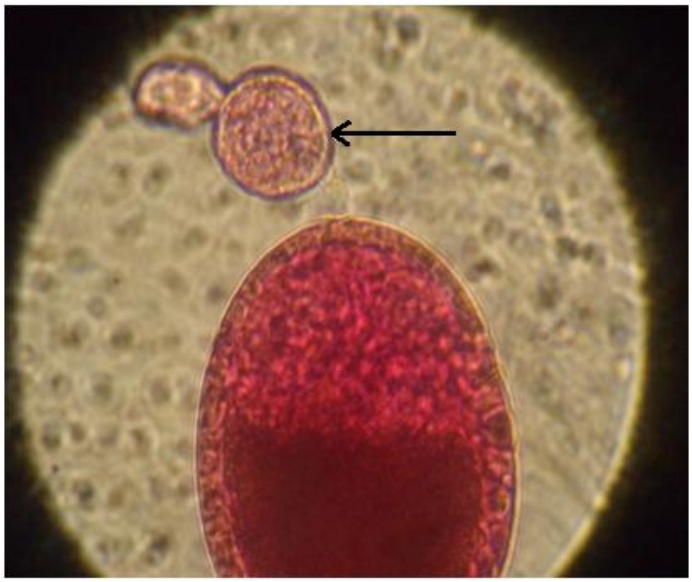

Figure 5. Parous ovariole (having a dilatation) according to Lewis (CREC, 2013).

Table 1. Reliability of the observation of follicular dilatations for the determination of physiological age of *A. gambiae s.s*.

Parity	Tracheoles	Dilatation	p-value
Nulliparous	20 (17.86%)	16 (14.28%)	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%)	-

tion method, no association was observed between the indeterminations of physiological age and the development stage of ovaries.

Efficacy of combination ovarian tracheoles reading method and follicular dilatations observation method for the determination of physiological age of *A. gambiae* s.s.

The two methods based on the reading of ovarian tracheoles and the observation of follicular dilatations were applied simultaneously on 112 females *A. gambiae* s.s (Table 3). The ovarian tracheoles reading method was not able to determine the physiological age of 25% (n=28) *A. gambiae* s.s. while follicular dilatations observation method was not able to determine 16.96% (n=19). The indetermination rate of the physiological age was almost null (00.89%) with the combination of the two methods on the same sample (Table 3). A significant difference was observed between the indeterminations obtained with the two methods separately and those obtained with their combination ($p<0.001$).

Overall, the combination ovarian tracheoles reading method and follicular dilatations observation method significantly reduced (almost null) the number of mosquito whose physiological age could not be determined using these methods separately.

DISCUSSION

The results of this study confirm the inefficacy of Detinova method (1962) observed from stage II age of ovarian development. Several authors (Germain et al., 1974; Cornet et al., 1978) showed that from stage II age, the reserves of vitellus become enormous and recover completely tracheoles network making ovaries unreadable after drying. Reading of ovarian tracheoles is the basic method used for the determination of physiological age of mosquitoes. This method is rapid, cheaper and easy to apply once the ovaries are extracted (Beklemishev et al., 1959). In addition, mosquitoes collected using human landing catch are more indicated for the application of Detinova method based on the ovarian tracheoles aspect. Human landing catch provides overall starve and pre-gravid mosquitoes which are in

Table 2. Variation of indetermination rate of physiological age following the development of ovarian stages after the reading of ovarian tracheoles and the observation of follicular dilatations.

Stage	Total Mstq	Tracheoles		Dilatation	
		N (i)	% (i)	N (i)	% (i)
I	12	0	0.00[b]	7	58.33[a]
IId	50	2	4.00[b]	5	10.00[b]
IIm	19	2	10.53[b]	4	21.05[ab]
IIf	14	9	64.29[a]	0	0.00[b]
III	11	9	81.82[a]	2	18.18[ab]
IV	6	6	100.00[a]	1	16.67[ab]
Total	112	28	25.00	19	16.96

d: Beginning, m: mean, f: aged, N: number, Mstq: mosquito, i: indetermination. Percentages which carry same letters in exposant were not significantly different (p>0.05)

Table 3. Results of the combination of the reading of ovarian tracheoles and the observation of follicular dilatations for the determination of the physiological age of *A. gambiae s.s.*

Methods age	N mstq	N (i)	% (i)
Dilatations	112	19	16.96a
Tracheoles	112	28	25a
Trac & dil	112	1	0.89b

Trac: Tracheole, dil: dilatation, N: number, mstq: mosquito, i: indetermination. Percentages which carry same letters in exposant were not significantly different (p>0.05).

stage I-II mean of their ovarian development. However, window trap catch and indoor residual morning spray catch have the advantage to provide not only, starve and pre-gravid mosquitoes, but also blood fed mosquitoes whose ovaries are over stage II mean.

Classical dilaceration of ovaries requests technical delicacy for physiological age determination of vectors (Mondet, 1996). Ovarioles isolation is not easy at stage I and II, beginning of ovaries development. At the beginning of ovaries' development, the ovarioles are joined one to another and their separation requests manual dexterity; this is not the case for mosquitoes at stage II aged-IV. This phenomenon explains the high number of indetermination age (16/19) recorded in mosquitoes at stage I-II mean of their ovarian development.

The fragility of pedicle of ovarioles was demonstrated (Mondet, 1993; Hoc and Wilkes, 1995b). In nulliparous mosquitoes, the pedicle of ovarioles is very short; this facilitates maintaining ovarioles intact during their isolation. However, in parous females, the pedicle of ovarioles elongates with successive egg-laying (Mondet, 1996). This phenomenon generates and extreme fragility of pedicle susceptible to breakage on the smallest needle moves during isolation of ovarioles (Mondet, 1993; Giglioli, 1965b). After ovarioles isolation, we observed at least a follicular dilatation in parous females during dissections.

This study shows that, the combination of ovarian tracheoles reading method and the follicular dilatations observation was very efficient for determination of *A. gambiae* s.s. physiological age. On 112 mosquitoes examined with the combination of the two methods, the physiological age of only one mosquito (0.89%) was not determined. These results show that the combination of Detinova and Lewis methods is able to determine the physiological age of all mosquitoes submitted for examination. This efficiency confirms the reciprocal complementarily of efficacy that exists between ovarian tracheoles reading method and follicular dilatations observation method. In addition, the combined application of the two methods is constraining when it is not implemented in team. Even for an experimented researcher, the combination of both methods on a

representative sample requests more time. For a systematic use in vector control evaluation, an easy and very rapid physiological age estimation method must be encouraged (Hamon et al., 1961).

Conclusion

The combination of ovarian tracheoles reading method and follicular dilatations observation method is very efficient for *A. gambiae* s.s. physiological age determination. It can be used over stage II age without physiological age indetermination. However, the combination of both methods requires technical expertise and delicacy. Also, the application seems difficult when it is not realized in team.

Conflict of interests

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

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