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The effects of oviposition site deprivation up to 40 days on reproductive performance, eggs development, and ovipositional behaviour in *Anopheles gambiae* (Diptera, Nematocera, Culicidae)

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The African malaria mosquito, *Anopheles gambiae*, depends on availability of suitable surface water for oviposition. The scarcity of breeding sites that characterizes droughts force gravid mosquitoes to delay oviposition and retain eggs in their ovaries. In laboratory conditions, we explored the possible consequences of preset duration of oviposition delay on reproductive capacity, egg viability, emergence and ovipositional behavior in gravid females of *A. gambiae* waiting for eggs laying in a context of oviposition delay. Overall, the mean anopheles egg batch size was not affected by the duration of the oviposition site deprivation. The embryo rates, hatchability and emergence rates decreased significantly gradually as the retention time is extended. However, the oviposition site deprivation has not been identified as a factor that can change the behavior of Anopheles in their choice of oviposition site.

Key words: *Anopheles gambiae*, oviposition delay, egg, ovaries, gravid females.

INTRODUCTION

Changes in climate and ecology are likely to affect the dynamics of vector populations and the distribution of vector-borne diseases (Tanser et al., 2003; McMichael and Githeko, 2001). For the past few decades, rise in temperature and precipitation has greatly modified the incidence of diseases transmitted by insects, ticks and rodents (McMichael et al., 1996). In Africa, despite rainfall scarcity and dreadful droughts in hot-dry savannahs, *Anopheles gambiae*, the main malaria vector, is able to survive, maintain and transmit the disease. Some studies showed that eggs of mosquitoes do not survive beyond 15 days in arid soil (Koenraadt et al., 2003), but can be retained during the whole dry period in aestivating female ovaries (Omer and Clousley-Thompson, 1970). Moreover, with the long-term absence of breeding sites (4-8 months) characterizing the unfavorable meteoro-logical conditions in arid ecosystems, it is no longer possible for gravid females of *A. gambiae* to lay eggs at the end of the gonotrophic cycle duration (Holstein, 1954; Warburg and Toure, 2010). These females undergo a very profound physiological reorganization to ensure survival (Omer and Clousley-Thompson, 1970). They also continue blood-feeding on humans but considerably slow down the rate of ovarian development (Yaro et al., 2012). The mechanisms that allow Anopheles' adaptation, particularly to difficult climatic conditions, have always been debatable (Lehmann et al., 2010).

In the current context of climate change marked by

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prolonged droughts events, it is essential to anticipate the behavior of malaria vectors, in order to sustain effective control in climatic conditions where aridity will intensify with time. This anticipation is based particularly on a deeper knowledge of the reproductive behavior of *A. gambiae* during harsh survival conditions, the fate of the eggs laid after long retention times in ovaries, the mechanisms involved, and the vector trophic behavior during the aestivation period. In order to evaluate the impact of forced egg-retention in *A. gambiae* due to the absence of breeding sites, we investigated egg laying ability, hatchability and emergence of larvae from eggs of Anopheles females that were blood-fed, and kept in cages for up to 40 days without oviposition opportunity.

MATERIALS AND METHODS

Biological material and mosquitoes rearing conditions

The study was performed with Kisumu strain of *A. gambiae s.s*. originating from Kisumu in Kenya. This particular standard strain has been reared at Centre de Recherche Entomologique de Cotonou for many decades. Mosquitoes were maintained in an insectary at 29 ± 2°C during the day and 24 ± 2°C during the night, at relative humidity (RH) ranging from 57 ± 4% (day) to 72 ± 5% (night), with a daily photoperiod of 12:12 h light:dark, using established procedures (Telang and Wells, 2004). These conditions mimic natural conditions prevailing in Cotonou, Benin. Larvae were fed daily on finely grinded fish food (TetraMin Tropical Flakes-Spectrum Brands, Inc) (Araùjo, 2012). Adult mosquitoes were kept in standard 30 × 30 × 30 cm cages in the adult insectary at 27 ± 2°C, 65 to 70% RH with 12:12 h (L–D) photoperiod, and were fed daily with 10% glucose solution.

Egg batch size in *Anopheles gambiae* deprived of oviposition box and blood meal

Five-day-old Kisumu female adults starved for 12 h were allowed to feed on rabbits for 10 min at 6:00 pm for each experimental trial. Fully blood-fed females were removed from the cage after their first blood meal and were fed once again 48 h later after complete digestion of the first blood meal. Following this, 9 batches (1 control batch and 8 tested batches) of Kisumu females that were fed twice, were submitted to single egg-laying events as follows:

Control: mosquitoes of this batch are used as control. Females are submitted to single laying immediately after the second blood meal.
Batch 1: females are submitted to single laying 5 days after the second blood meal.
Batch 2: females are submitted to single laying 10 days after the second blood meal.
Batch 3: females are submitted to single laying 15 days after the second blood meal.
Batch 4: females are submitted to single laying 20 days after the second blood meal.
Batch 5: females are submitted to single laying 25 days after the second blood meal.
Batch 6: females are submitted to single laying 30 days after the second blood meal.
Batch 7: females are submitted to single laying 35 days after the second blood meal.
Batch 8: females are submitted to single laying 40 days after the second blood meal.

Inside a given batch, one mosquito represents one replication. The oviposition material was composed of a white cup covered with a piece of white net. A piece of cotton wool moistened with water laid under a Whatman filter paper with a 5 cm radius, was placed at the bottom of the cup. On the netted cup, we placed a cotton pad moistened with 10% glucose solution to feed gravid females during the experiment. The feeding process was renewed and repeated every day.

In each batch (control and tested batches), the oviposition boxes were removed 3 days after gravid females had been isolated and set for individual spawning. The eggs laid by females from each batch were counted separately using a binocular microscope (PERFEX® Edu 3.0) and the number of embryonated eggs from each nest box was recorded. We dissected all mosquitoes that spawned to check if there were any eggs retained after spawning.

Viability of eggs laid after retention inside the ovaries of *Anopheles gambiae*

Each oviposition box was placed in a tray containing tap water used as artificial shelter for egg hatching. The tap water was boiled to neutralize any possible traces of chlorine and cooled prior to usage for mosquito rearing. We used 450 ml of water for the incubation of 100 eggs. After 24 h, the larvae emerging from the hatched eggs were counted using plastic pipettes and the hatching rate (HR) was recorded for each time of oviposition site deprivation. The nine retention types were recorded to assess the HR described as: (1) Control (no oviposition delay), (2) 5 days of oviposition site deprivation, (3) 10 days of oviposition site deprivation, (4) 15 days of oviposition site deprivation, (5) 20 days of oviposition site deprivation, (6) 25 days of oviposition site deprivation, (7) 30 days of oviposition site deprivation, (8) 35 days of oviposition site deprivation, (9) 40 days of oviposition site deprivation. The hatching rates were compared according to each modality of oviposition delay in order to evaluate the impact of the absence of oviposition box on the quality and viability of the eggs. The association between the rate of embryonated eggs and the hatching rate was determined in each case to assess the evolution of these two parameters (Hatching Rate and Embryo Rate).

Emergence rate evaluated in eggs laid after retention inside the ovaries of *Anopheles gambiae*

Larvae hatched after delayed oviposition were monitored daily until their emergence. The emergence rate was calculated and recorded according to the duration of oviposition delay (immediate egg-laying versus 10 to 40 days of eggs retention inside the ovaries of *A. gambiae* females).

Oviposition behavior in gravid females of *Anopheles gambiae* deprived of oviposition box and blood meal for 3 weeks

To access whether oviposition behavior and choice of breeding site are the same in both gravid females of *A. gambiae* forced to egg-retention and females submitted to egg laying immediately after blood meal, batches of gravid anopheles with 3-weeks oviposition delay were offered 3 kinds of oviposition cup. In our simulations, oviposition cups represent breeding sites in nature. Gravid females with no oviposition delay are used as control. An oviposition cup consisted of an open cylinder (10 cm diameter, 5 cm height) with a circular shaped Whatman paper placed on a hydrophilic cotton pad. We added water to a height of about 5 mm above the Whatman paper. The water samples used in the preparation of the 3 oviposition cups differed from each other. Three water samples were taken from three different breeding sites:

1. The first water sample was taken from a breeding site housing

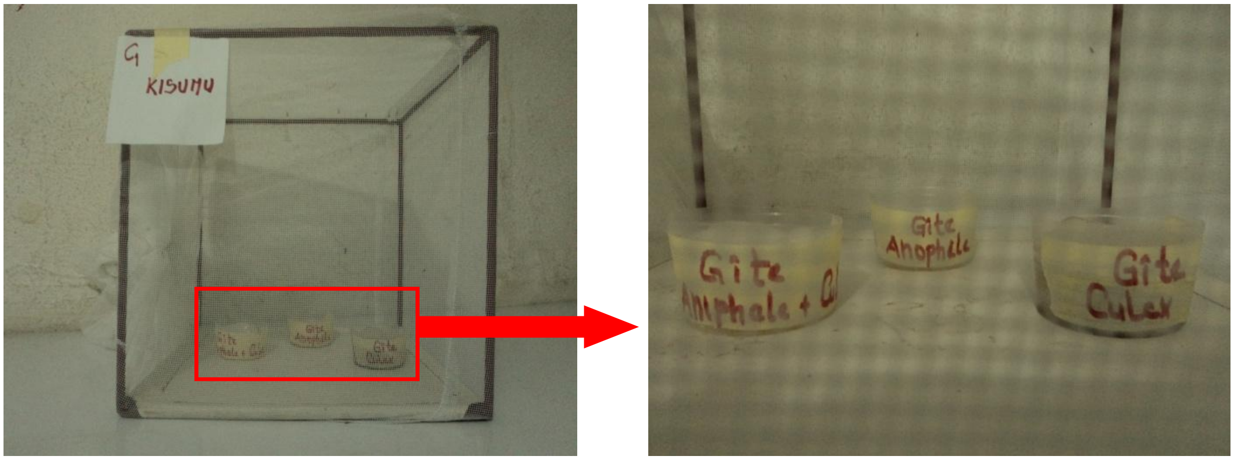

Figure 1. Experimental cage showing the 3 oviposition boxes used as breeding sites for gravid females of *Anopheles gambiae*.

exclusively larvae of *Anopheles spp* (oviposition cup 1).
2. The second water sample was taken from a breeding site housing exclusively *Culex* spp (oviposition cup 2)
3. The third sample was taken from a mixed breeding site of *Anopheles* spp. and *Culex* spp (oviposition cup 3).

The 3 prepared oviposition cups were placed inside 3 cages (30 × 30 × 30 cm) veiled with mosquito net in Figure 1. In each cage, we introduced 15 gravid specimens of *A. gambiae* that were deprived of oviposition cup and blood meal for 21 days. Three days later, the boxes were removed from the cages and the number of eggs on each Whatman paper was counted under binocular microscope (PERFEX® Edu 3.0). Furthermore, all mosquitoes' ovaries were dissected, to verify how many of them have effectively laid eggs. Each experiment was replicated three times. For the 3 replicates, nesting boxes were rotated to avoid side effect.

Data analysis

The influence of oviposition site deprivation on mosquito egg batch sizes was determined through the test of Kruskall Wallis. To access the impact of oviposition delay on the hatchability and the emergence, the binary logistic model was performed accompanied by the analysis of deviance. The choice in oviposition cup was assessed by calculating the rate ratio obtained with the unbiased estimate of the median (mid-p). The confidence interval was determined with a mid-p test and the pairwise comparison of the number of eggs laid per mosquito at each preset modality of oviposition delay was analyzed using the Poisson test. Odds ratios were calculated for the evolution of hatching eggs according to the difference.

RESULTS

Egg batch size in gravid nulliparous females of *A. gambiae* deprived of oviposition site and blood meal for up to 40 days

Overall, the egg batch size of *A. gambiae* in a context of egg retention was assessed from a total of 256 nulliparous females of Kisumu strain. The results showed very little variation in the average fecundity of gravid]mosquitoes depending on the length of oviposition delay. The number of eggs laid by different batches of Kisumu females ranges from 75.16 to 79.88 eggs/brood (ch^2 = 1.602, df = 8 and p = 0.991) (Figure 2). We observed similar results between the fecundity of the control batch (no oviposition delay) and the batches of gravid mosquitoes forced to retain eggs in the ovaries beyond the duration of the gonotrophic cycle. The oviposition site deprivation carried out in *A. gambiae* up to 40 days did not influence the average number of eggs laid at the end of the retention time Table 1.

Assessing the viability of eggs retained inside the ovaries of *A. gambiae*

A total of 19,716 eggs were monitored until hatching. Analysis of the results showed that the hatching rate decreases progressively as the retention time increases. The hatching rate decreased from 85.93% in the absence of any oviposition delay to 31.07% for eggs laid after a delay of 40 days (adjusted OR = 0.93; 95%-CI: [0.92 to 0.94]; p < 0.01) Table 2. The hatching rate in *A. gambiae* therefore appeared to be a decreasing function of the length of the oviposition delay (Figure 3).

Relationship between the hatchability and the embryonation

Both embryonation rates and hatching rates decreased progressively as the oviposition delay time increased but the embryonation rates remained above the hatching rate, regardless of the duration of egg-retention. Without any egg-retention, about 97% (3250/3346) of embryonated eggs have hatched while after 40 days of oviposition delay,

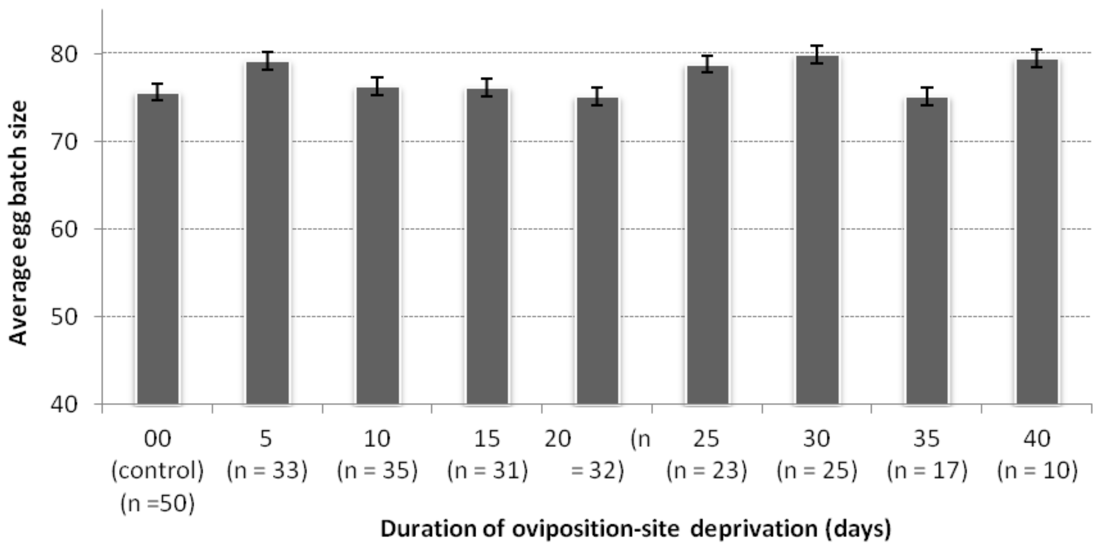

Figure 2. Egg batch size in gravid nulliparous females of *Anopheles gambiae* in preset types of oviposition site deprivation.

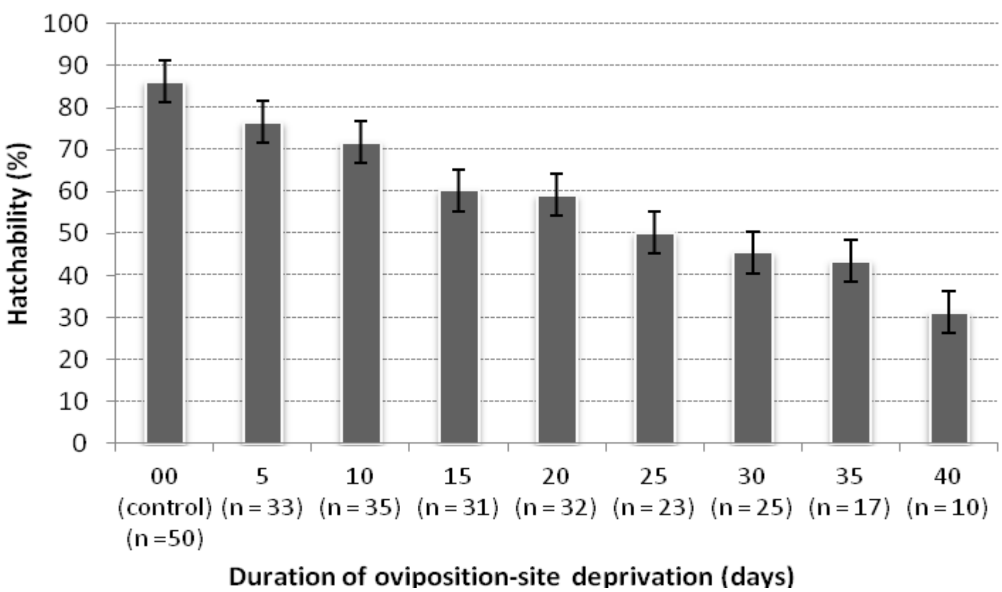

Figure 3. Hatchability in eggs laid by gravid nulliparous females of *Anopheles gambiae* in preset types of oviposition site deprivation.

only 79% (247/310) of embryonated eggs have hatched (Table 3). This implies that the embryonated status of an egg in *A. gambiae* at oviposition did not guarantee its hatchability.

Variation of the emergence rate in eggs laid after an oviposition delay of *Anopheles gambiae*

The results showed that the emergence rate of adult decreases as the duration of retention of eggs in the ovaries increased Table 4. This rate ranged from 77.60% in the absence of any oviposition delay to 24.40% after 40 days delay (adjusted OR = 0.941; 95%-CI: [0.932 to 0.949]; $p < 0.001$) (Figure 4).

Oviposition behavior in gravid females of *Anopheles gambiae* deprived of oviposition box and blood meal for 3 weeks

The Anopheles-exclusive egg laying box (oviposition cup 1) was the one that received most of the eggs laid by gravid females in the control batch (no delay). Oviposition

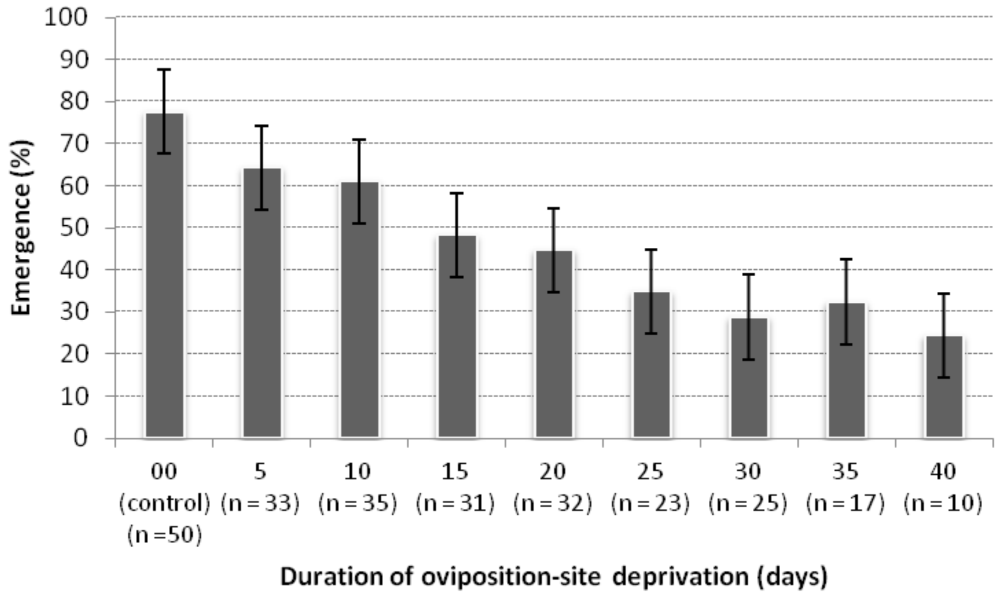

Figure 4. Emergence rate in eggs laid by gravid nulliparous females of *Anopheles gambiae* in preset types of oviposition site deprivation.

box 3 was also used for the egg laying by the gravid females in the control batch. However, gravid females waiting for oviposition since 21-days preferred oviposition cup 1 more (Table 5). Therefore, the phenomenon of oviposition site deprivation has not been identified as a factor that can change the behavior of Anopheles in choosing their breeding sites for oviposition.

DISCUSSION

Eco-climatic factors in ecosystems influence the dynamics of populations of Anopheles malaria vectors and their reproductive performance. Mosquitoes depend on availability of suitable surface water for oviposition. Short and long dry spells occur throughout the year in many parts of their range that limit their access to oviposition sites. The mosquito populations' dynamics are so affected (Dieter et al., 2012). The simulations in this study aimed at exploring the egg batch size, the eggs' development, and the preference in choice of breeding sites in gravid females of *A. gambiae* that were forced to hold eggs inside their ovaries for up to 40 days after the blood meal.

The gravid females of the reference strain Kisumu received two blood meals from rabbits. The second blood meal occurred 48 h after the first one in order to make sure that it was completely digested. The two blood meals are justified by the fact that in nulliparous females of Anopheles, there is a mandatory pre-gravid phase following the first blood meal, then ovarian maturation and oviposition can occur after a second meal of a "normal" volume (Carnevale et al., 1979). The data showed that the average number of eggs laid by the females that are not subject to an egg-retention does not vary significantly from the fecundity of females forced to keep their eggs beyond the duration of the gonotrophic cycle, respectively after 5, 10, 15, 20, 25, 30, 35 and 40 days of follow-up. The delay in oviposition, even after 40 days, because of a lack of breeding sites (egg laying box) has not therefore been identified as a factor influencing the fecundity in gravid females of *A. gambiae* at the end of the retention period. However, recent studies have shown that

Table 1. Hatchability and embryonation in eggs laid by gravid nulliparous females of *Anopheles gambiae* in preset types of oviposition site deprivation.

Duration of OSD[1] (day)	Status	Total number (N)	Total of eggs laid (E)	Embryonated and hatched rate (%) $\frac{N}{E} x\ 100)$	Odds ratio (OR)	IC-95% (OR)[2]	p.value
00 (Control)	Embryonated	3346	3782	88.47[a]	1.00	-	-
	Hatched	3250	3782	85.93[b]	1.26	[01.10-01.44]	0.001068
05	Embryonated	2173	2615	83.10[a]	1.00	-	-
	Hatched	1962	2615	75.03[b]	1.64	[01.43-01.87]	<0.00001
10	Embryonated	2171	2669	81.34[a]	1.00	-	-
	Hatched	1939	2669	72.65[b]	1.64	[01.44-01.87]	<0.00001
15	Embryonated	1607	2362	68.04[a]	1.00	-	-
	Hatched	1453	2362	61.52[b]	1.33	[01.18-01.50]	0.0000031
20	Embryonated	1374	2260	60.80[a]	1.00	-	-
	Hatched	1279	2260	56.59[b]	1.19	[01.07-01.34]	0.0045112
25	Embryonated	1170	1757	66.59[a]	1.00	-	-
	Hatched	836	1757	47.58[b]	2.20	[01.92-02.52]	<0.00001
30	Embryonated	1109	1997	55.53[a]	1.00	-	-
	Hatched	830	1997	41.56[b]	1.76	[01.55-01.99]	<0.00001
35	Embryonated	635	1278	49.69[a]	1.00	-	-
	Hatched	527	1278	41.24[b]	1.41	[01.20-01.65]	0.000021
40	Embryonated	310	0795	38.99[a]	1.00	-	-
	Hatched	247	0795	31.07[b]	1.42	[01.15-01.74]	0.001100

[1]Oviposition site deprivation and [2]Confidence Interval of the odds ratio.

Table 2. Ovipositional behavior in gravid nulliparous females of *Anopheles gambiae* with 3 weeks oviposition delay.

Batches of mosquitoes	Type of oviposition cup (OC)	No. of mosquitoes tested	Number of eggs laid	Rate ratio (RR)	IC-95% (RR)[1]	p. value
Control batch (females with no oviposition site deprivation)	OC 1	42	1687[a]	1.00	-	-
	OC 2		530[b]	0.31	[00.28-00.35]	<0.000001
	OC 3		1129[c]	0.67	[00.62-00.72]	<0.000001
Gravid females with 21 days oviposition delay	OC 1	37	1219[a]	1.00	-	-
	OC 2		0763[b]	0.63	[00.57-00.69]	<0.000001
	OC 3		0818[b]	0.67	[00.61-00.73]	<0.000001

[1]Confidence Interval of the rate ratio.

Table 3. Egg batch size in gravid nulliparous females of *Anopheles gambiae* in preset types of oviposition site deprivation.

Duration of OSD[1] (day)	Total number of mosquitoes (M)	Total number of eggs laid (E)	Average egg batch size (E/M)	Rate ratio (RR)	IC-95% (RR) [2]	p-value
00 (Control)	50	3782	75.64[a]	1.00	-	-
05	33	2615	79.24[a]	1.05	[01.00-01.10]	0.0676
10	35	2669	76.26[a]	1.01	[00.96-01.06]	0.7474
15	31	2362	76.19[a]	1.01	[00.96-01.06]	0.7803
20	32	2405	75.16[a]	0.99	[00.94-01.05]	0.8064
25	23	1813	78.83[a]	1.04	[00.99-01.10]	0.1492
30	25	1997	79.88[a]	1.06	[01.00-01.11]	0.0591
35	17	1278	75.18[a]	0.99	[00.93-01.06]	0.8513
40	10	0795	79.50[a]	1.05	[00.97-01.13]	0.2031

[1]Oviposition site deprivation and [2]Confidence interval of the rate ratio.

Table 4. Hatchability in eggs laid by gravid nulliparous females of *Anopheles gambiae* in preset types of oviposition site deprivation.

Duration of OSD[1] (day)	Total number of eggs laid (E)	Total number of eggs hatched (H)	Hatching rate (%) $(HR = \frac{H}{E} \times 100)$	Odds ratio (OR)	IC-95% (OR) [2]	p-value
00 (Control)	3782	3250	85.93[a]	01.00	-	-
05	2615	1962	75.03[b]	02.03	[01.79-02.31]	<0.00001
10	2669	1939	72.65[b]	02.30	[02.03-02.61]	<0.00001
15	2362	1453	61.52[c]	03.82	[03.38-04.32]	<0.00001
20	2405	1330	55.30[d]	04.94	[04.37-05.58]	<0.00001
25	1813	0836	46.11[e]	06.73	[05.90-07.67]	<0.00001
30	1997	0830	41.56[f]	08.59	[07.56-09.76]	<0.00001
35	1278	0527	41.24[f]	08.71	[07.54-10.06]	<0.00001
40	0795	0247	31.07[g]	13.55	[11.37-16.16]	<0.00001

[1]Oviposition site deprivation and [2]Confidence interval of the odds ratio.

several factors may be involved in the number of eggs laid by mosquitoes. This include, for instance, the quantity and quality of protein reserves accumulated by the mosquito during the larval stages (Klowden et al., 1988; Amalraj et al., 2005), the larval rearing temperature (Carvalho et al., 2002; Alto and Juliano, 2001) the size of the mosquito, the diet and physiological age of the mosquito (Gary and Foster, 2001).

In our study, the feeding of the reference strain *A. gambiae* Kisumu larvae was carried out using 10 g of tetramin fish food (TetraMin Tropical Flakes-SpectrumvBrands, Inc) for 100 larvae. Meanwhile, adults were fed with a cotton pad moistened with 10% glucose solution which was

Table 5. Emergence rate in eggs laid by gravid nulliparous females of *Anopheles gambiae* in preset types of oviposition site deprivation.

Duration of OSD[1] (day)	Total of emergence (E')	Total of eggs laid (E)	Emergence rate (%) $(\frac{E'}{E} \times 100)$	Odds ratio (OR)	IC-95% (OR)[2]	p-value
00(Control)	2935	3782	77.60[a]	1.00	-	-
05	1681	2615	64.28[b]	1.92	[01.72-02.15]	<0.0001
10	1629	2669	61.03[c]	2.21	[01.98-02.47]	<0.0001
15	1140	2362	48.26[d]	3.71	[03.32-04.15]	<0.0001
20	1076	2405	44.74[d]	4.28	[03.83-04.78]	<0.0001
25	0632	1813	34.86[f]	6.48	[05.72-07.32]	<0.0001
30	0575	1997	28.79[g]	8.57	[07.57-09.69]	<0.0001
35	0414	1278	32.39[f]	7.23	[06.29-08.32]	<0.0001
40	0194	0795	24.40[h]	10.73	[08.98-12.83]	<0.0001

[1]Oviposition site deprivation and [2]confidence interval of the odds ratio.

daily renewed.

The hatchability of eggs laid by different batches of females significantly decreased as the retention period was extended. Our results confirm the study by Deieter et al. (2012) in G3 laboratory colony of *A. gambiae* adults where a drastic decrease of hatching rate (0 to 2% within 7 days) has been reported (Dieter et al., 2005). This is due to the fact that the oviposition delay is detrimental to the survival of embryos because the number of non-embryonated eggs increases gradually along with the long oviposition site deprivation. Several factors are known to influence hatchability in mosquito eggs: these include the temperature drop and water quality (Holstein, 1954; Yaro et al., 2006). In natural conditions, hatching occurs in response to a decrease in oxygen tension of water under the action of microorganisms present in stagnant water deposits (Foster, 2001).

In our study, the preference in the choice of oviposition site was also investigated in gravid females of *A. gambiae* with 3 weeks oviposition delay. The three oviposition cups that we fashioned for oviposition represented breeding sites. This aimed at exploring how 'egg-retention females' behaved compared to a control cohort of gravid females directly submitted to laying eggs. The results do not show a change in the oviposition behavior after being forced to retain eggs in their ovaries beyond 21 days. According to Subra (1971) and Adebote et al. (2008), the choice of oviposition site in mosquitoes is mainly determined by chemicals contained in the breeding environment. Moreover, Ikeshoji and Mulla (1970) and Sattler et al. (2005) reported that there is, in each breeding site, a specific factor attractive for the species housed in. Those raised surveys could explain the choice of oviposition cups in the context of our study. But it should be better to include in these simulations the measure of physicochemical parameters of each water sample for a full understanding in the choice of oviposition site by gravid mosquitoes. However, since the primary outcome of interest in this study was not the physiochemical determinants controlling oviposition, this limitation should not greatly affect interpretation of our results.

The data recorded in this study are very encouraging. However, further investigations need to be conducted under natural conditions to have a better understanding of the mechanisms allowing females *A. gambiae* to survive long absence of breeding sites.

Conclusion

This study has helped to record data measuring the possible consequences of a prolonged gonotrophic cycle on the reproductive capacity of females *A. gambiae*. It has been shown that the absence of breeding sites does not affect the egg batch size and the ovipositional behavior of *A. gambiae*, but leads to a decrease in the hatching rate of the eggs in proportion to an increase in oviposition delay time. This experiment was based on simulations carried out under laboratory conditions. We believe that further studies would be necessary to repeat this experiment under natural conditions in order to have a better understanding of the various conditions allowing females *A. gambiae* to survive during the long absence of breeding sites.

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