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Mating of unfed, engorged, and partially to fully gravid *Aedes aegypti* (Diptera: Culicidae) female mosquitoes in producing viable eggs

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Abstract

Background Understanding the relationship between blood-feeding and mating is important in effectively managing the most well-adapted vector insect, *Aedes aegypti* (Linnaeus). Although extensive studies have investigated the behavioural aspects of *Aedes* such as blood-feeding, mating, and their relationship, several knowledge gaps still exist. Therefore, the present study was undertaken to determine the possibility of successful mating by unfed, engorged, and partially to fully gravid (up to 5 days after blood-feeding with fully developed eggs) female *Ae. aegypti* mosquitoes and production of viable eggs.

Methods Mating of sexually mature adult *Aedes aegypti* was allowed in three different ways. In control 1, the females were allowed to mate before taking blood meal, and in control 2, the females were not at all allowed to mate. In the experiment, the females were separated into six categories, viz. D-0 to D-5. In D-0, the females were allowed to mate immediately after the bloodmeal and, in D-1, the females were allowed to mate on the first day of blood feeding, likewise, the females of D-2, D-3, D-4 and D-5 were allowed to mate on 2nd, 3rd, 4th and 5th day of blood feeding. Ovitrap was uniformly kept on the 4th day of blood feeding for the cages D-0 to D-3 for 1 h and then removed and for the cages D-4, and D-5, the ovitrap was kept on 4th and 5th day of blood feeding for 1h immediately after mating. The total number of eggs and the total number of hatching were counted. In the subsequent days, the entire experiment was replicated two times with different cohorts of mosquitoes, and the mean value of three experiments was used to draw Excel bars with 5% error bars and also for the statistical analysis.

Results It was found that mating just before oviposition was sufficient to produce 1581 eggs (70% compared with control) and fertilize 1369 eggs (85% compared with total eggs laid), which is far higher than the 676 non-hatching (unfertilized) eggs (30%) laid by unmated females. Although mating is not essential for producing eggs, our study shows that even brief exposure to the semen and seminal fluids greatly enhances the oviposition and hatching efficiency, even if the mating occurs just before oviposition. However, those females mating before blood-feeding and those mating after blood-feeding produced 2266 and 2128 eggs, with hatching rates of 96.78% and 95.54%, respectively. Hence, the retention time of seminal fluid in the female seems to influence the number of eggs laid and the number of eggs hatched.

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Conclusions In general, mating is possible in *Ae. aegypti* even minutes before oviposition and is sufficient to produce a greater number of viable eggs.

Keywords *Aedes aegypti*, Mating, Unfed, Fully fed, Gravid, Engorged, Viable eggs

Background

The mosquitoes *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) are a major public health concern, responsible for the transmission of viruses causing dengue [1], Zika [2], chikungunya [3], and yellow fever [4]. Their development in tropical and subtropical regions is intrinsically linked to human dwellings and activities such as rapid urbanization, which offer adequate larval growing habitats and the blood meal hosts required for their reproduction [5]. Among the diseases transmitted by *Ae. aegypti* and *Ae. albopictus*, dengue alone threatens nearly half of the world's population across 129 countries, with approximately 96 million clinical cases and 40,000 deaths every year [6]. Despite vaccine availability since the 1930s, yellow fever remains a major threat to the people living in Africa and in South and Central America [7]. Two vaccine candidates (CYD-TDV [Dengvaxia, Sanofi Pasteur] and TAK-003 [Qdenga, Takeda Pharmaceuticals]) have been licensed for dengue, and a third candidate (Butantan-DV) has recently passed a phase 3 trial in Brazil [8], but none of them is currently available for public use in the affected countries. As there are no effective drugs available for dengue and similar *Aedes*-transmitted diseases such as yellow fever, the treatment relies solely on symptomatic supportive therapy [9]. Although vector control strategies using appropriate larvicides and adulticides play an important role in dengue control programs [10–12], dengue control efforts are complicated by the development of resistance in *Aedes* mosquito populations to the commonly used insecticides [13]. For the transmission of the dengue virus, or any other arboviruses, an infected host needs to be bitten by a female mosquito for a blood meal to develop her first batch of eggs, and subsequent bites for laying second or further cycles of eggs after a considerable period [14]. Hence, as mating and blood-feeding are connected with host-seeking and disease transmission, every aspect of the crosstalk between male and female *Aedes* mosquitoes needs to be investigated. Immediately after emergence, the female *Ae. aegypti* are unreceptive to mating [15], and males are also unable to mate until their abdominal terminalia rotate, which takes approximately 24 h. Once the female initiates flight, the sexually mature males will attempt to mate by engaging their genitalia [16].

Although many researchers have conducted extensive studies on the behavioural aspects of *Aedes*, such as blood-feeding, mating, and their relationship [17–22],

existing knowledge gaps have yet to be explored [23]. A report that blood-fed *Aedes* females were generally inactive and had lower chances for copulation [24, 25] contradicts reports that the rate of mating after blood-feeding was high [26, 27]. A very recent report by Ramirez-Sanchez [28] connects female *Ae. aegypti* age to blood-feeding, insemination, sperm storage, and fecundity. In female *Ae. aegypti*, mating results in decreased sexual receptivity [29, 30] and changes in gene expression in female reproductive tissues [31], including the long-term sperm storage sac [29], the spermatheca [32, 33], which produces products essential for optimal mosquito fertility [33, 34]. It is not known whether semen holding time or retention time in spermathecae is short when mating occurs just before oviposition. Irrespective of mating, a fully fed, engorged female has to spend 4 to 5 days digesting the blood meal and subsequent development of eggs. Hence, an unmated but blood-fed female has an enormous length of time, from taking a blood meal to oviposition, to achieve successful copulation. However, the success rate of laying viable eggs if the female encounters a male during the time from immediately after taking the blood meal to just before oviposition is not known. Although female *Ae. aegypti* require a blood meal to develop their eggs, the age at which they would have their first blood meal is unclear [35]. Reports are also not clear on whether females mate before or after a blood meal [15].

Closing this knowledge gap in mating versus blood-feeding is important, particularly for vectors such as *Aedes*, in which re-mating of females is rare [36–38]. As mating behaviour is one of the most understudied aspects of mosquito biology, the present study will provide some important pieces of information on the reproductive biology of *Aedes* mosquitoes to better understand the process. Therefore, this study was undertaken to determine the possibility of successful mating and production of a total number of eggs and total number of viable eggs by unfed, engorged, and partially to fully gravid (up to 5 days after blood-feeding with fully developed eggs) female *Ae. aegypti* mosquitoes.

Methods

Aedes aegypti collection and colony maintenance

Adult *Ae. aegypti* mosquitoes were collected from Madurai (latitude: 9°56′10.97″ N, longitude: 78°08′09.33″ E; elevation: 140 m) in Tamil Nadu State, India, by indoor

resting collection and stabilized in the mosquito colony of this centre (Indian Council of Medical Research-Vector Control Research Centre [ICMR-VCRC] Field Station, Madurai, Tamil Nadu, India) for three generations before being used in the experiment. The standard operating procedure as described by Zheng et al. [39] for mass rearing, egg storage, and hatching of *Ae. aegypti* was followed, with minor modifications. Briefly, the adult *Ae. aegypti* populations were maintained in 60 × 60 × 60 cm cages (BugDorm-6610, India) at room temperature (RT) of 27 ± 1 °C and relative humidity (RH) of 85 ± 5% with a photoperiod of 12:12 h (L:D). A cotton pad soaked in 10% glucose along with wet raisins was given in each cage as a sugar source for the adult mosquitoes. The glucose pad and wet raisins were changed at an interval of 2 days to avoid fungal growth. After 2 days of glucose feeding, the females were allowed to take a chicken blood meal and then were allowed to rest for 3 days before being placed for oviposition. A filter paper (Whatman) strip was kept half-soaked in each half-water-filled bowl for oviposition.

Developing adults for mating experiment

Approximately 1500 freshly laid *Ae. aegypti* eggs were kept for 3 days for stabilization (to complete embryo development) and then floated in 1 l of deionized water (non-chlorinated) taken in metallic enamel-coated trays [18, 40]. Powdered dog biscuits and baker's yeast pellets at a 3:2 ratio were given in adequate amounts for 2 days of larval food for the newly emerged larvae. The larval-rearing water was changed every 2 days for optimal larval growth and to reduce larval mortality. Trays were completely enclosed by wire mesh and maintained in optimal laboratory conditions as mentioned before, with routine monitoring.

A total of 1000 pupae were collected from the larval rearing tray, and each pupa was carefully placed in a 1.5-ml Eppendorf tube with 0.5 ml deionized water to avoid the chances of mating at the emergence of adults from the pupal stage [23]. Three tiny holes were made through the lid of each tube to facilitate the respiration of the pupa, and the tubes were maintained at 27 ± 1 °C and RH of 85 ± 5% with a photoperiod of 12:12 h (L:D) for 24 h. The sex of the emerging adults in the transparent Eppendorf tube was determined using a stereomicroscope (Nikon SMZ800, Japan). In contrast to the pilose (less hairy) antennae of females, the plumose (feather-like) antennae of males can be easily distinguished. By skipping the early pupae, we were able to ensure that we selected more female pupae than males.

Blood-feeding versus mating

As we required only 480 females [60 females in each of eight cages (six for experiment+two for controls)] and

420 males [60 males in each of seven cages (six for experiment+one for control)], the excess males and females were kept as substitutes. Only active and healthy males and females were selected by discarding the inactive and sluggish individuals. Sixty emerged females were kept separately in each 60 × 60 × 60 cm cage labelled from day 0 (D0) to day 5 (D5) and control 1 and 2 as shown in the schematic diagram in Fig. 1, and all the males were kept together in one cage. Both adults were kept for 48 h with a cotton pad soaked in 10% glucose along with wet raisins as a sugar source to attain sexual maturity.

For control 1, a set of 60 sexually mature males and females were allowed to mate individually by releasing one male and one female in each of the 60 flat-bottom glass tubes (length 10 cm; diameter 2.5 cm) with a cotton plug. Generally, mating occurred within 10–15 min, with a few exceeding 30 min. Unmated pairs were noted and replaced by an equal number of other pairs to bring the total mated pairs to 60. After mating, the female from each tube was released back into a 60 × 60 × 60 cm cage, and the male was removed from each tube and discarded. The females were allowed to take a chicken blood meal for 2 h, after which they were examined for distended abdomen through the transparent cage for the status of individual feeding. Then, using a mechanical aspirator, the unfed and half-fed individuals were removed, keeping 50 fully fed females in the cage. A cotton pad soaked in 10% glucose along with wet raisins was given in each cage as a sugar source. On the fourth day of blood-feeding, an ovitrap was provided for oviposition. Since it has been established that for more than 90% of females, oviposition occurs within the first 30 min, the ovitraps were kept for 1 h to achieve maximum egg-laying. For control 2, 60 females were released into a 60 × 60 × 60 cm cage and allowed to take a chicken blood meal. Then the unfed and partially fed females were removed, leaving 50 fully fed females which were maintained as described for control 1.

For the experiment, six 60 × 60 × 60 cm cages were taken and marked as D0 to D5. Sixty sexually mature females were released in each cage and were allowed to take a chicken blood meal for 2 h. On the mating day, the engorged and partially/fully gravid females from cages D0 to D5 were taken separately from the cage and allowed to mate with individually released males as described for control 1 (Fig. 1). Utmost care was taken when collecting the fully fed females to avoid rupturing the distended abdomen. The mating was observed, and the glass tube with the mated female was marked and released back into the respective cage and maintained under the conditions noted earlier for colony maintenance. All the mated males were euthanized. The ovitrap was kept on the fourth day of blood-feeding for the cages marked D0 to D3

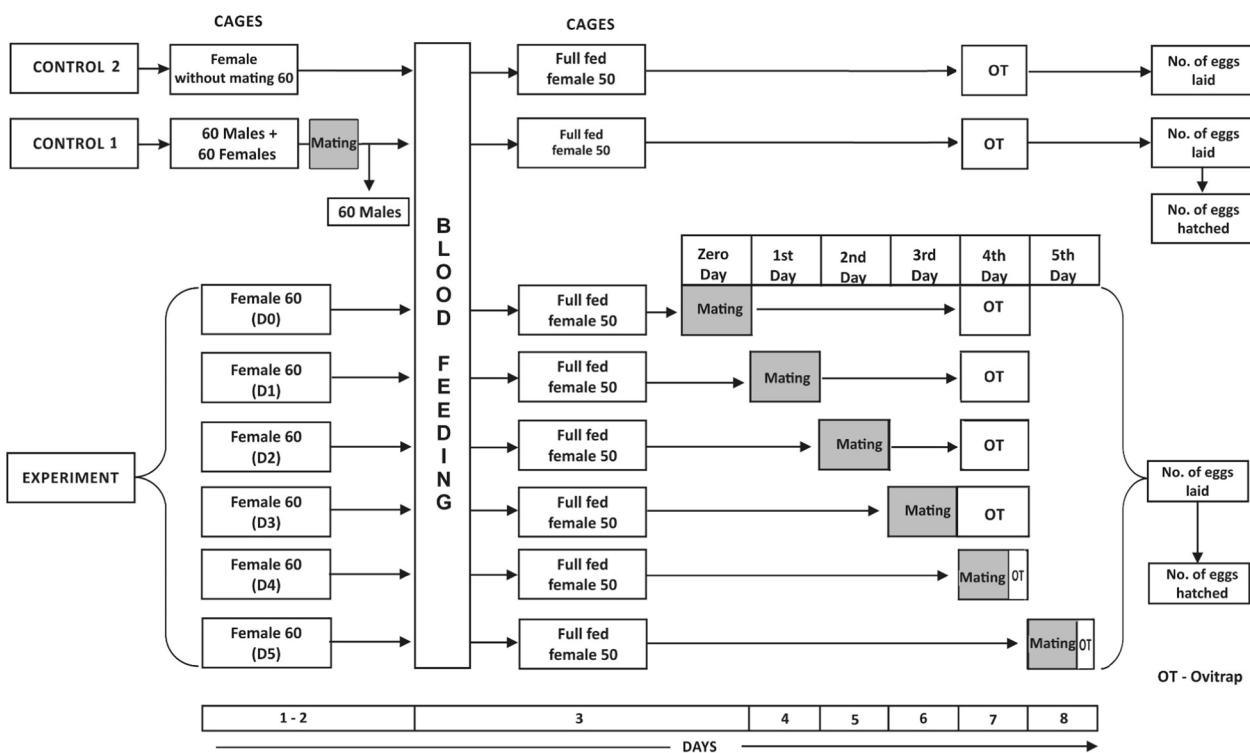


Fig. 1 Schematic representation of the methodology followed

and collected after 1 h. For the cages marked D4 and D5, the ovitrap was kept immediately after mating on the same day, fourth, and fifth day, respectively, and collected after 1 h.

Plastic bowls (height 6 cm, upper diameter 7 cm, lower diameter 4.5 cm), half-filled with deionized water (200 ml), were used as ovitraps to collect the eggs. A filter paper (Whatman) strip was circularly placed around the inner wall of each bowl and kept half immersed in the water. The egg-laid papers were air-dried, and the number of eggs was counted under a stereomicroscope and recorded. The air-dried and 3-day-stabilized eggs in the Whatman filter papers were separately floated in deionized water and kept in enamel-coated metallic trays for hatching, as described above. The number of larvae hatched from the eggs in each cage were counted and recorded. In the subsequent days, the entire experiment was replicated twice with different cohorts of mosquitoes, and the mean value of the three experiments was used for the analysis.

Statistical analysis

For statistical analysis, we first tested the normality of all data using the D’Agostino–Pearson normality test with the Chi-square distribution (right-tailed) to determine

skewness (to quantify the asymmetry of the distribution) and kurtosis (to quantify the shape of the distribution). Since the normality assumptions were satisfied, we used one-way analysis of variance (ANOVA) to determine the mean variations within and between populations (SPSS ver. 15).

Then, the mean numbers of eggs laid and eggs hatched were represented in the Excel bar diagram with 5% error bars. The numbers of eggs laid and hatched by a single female were calculated from the total number of eggs laid and hatched and drawn as a line graph. Linear regression analysis was also conducted to determine the statistical significance of the study. The beta coefficient, *P*-value, and *R*-squared values were determined.

Results

Out of 1000 pupae kept for emergence, a total of 523 females and 477 males emerged at the end of 24 h and were used as described in the previous section for one set of experiments. The mean of triplicate analysis showed that the females that mated before the blood meal in control 1 laid 2266 eggs, compared with control 2, where unmated females laid only 676 eggs, which is around 70% less than the mated females. In the experimental set, the cage marked D0, where the females were allowed to mate with males immediately after the blood meal, i.e. mating

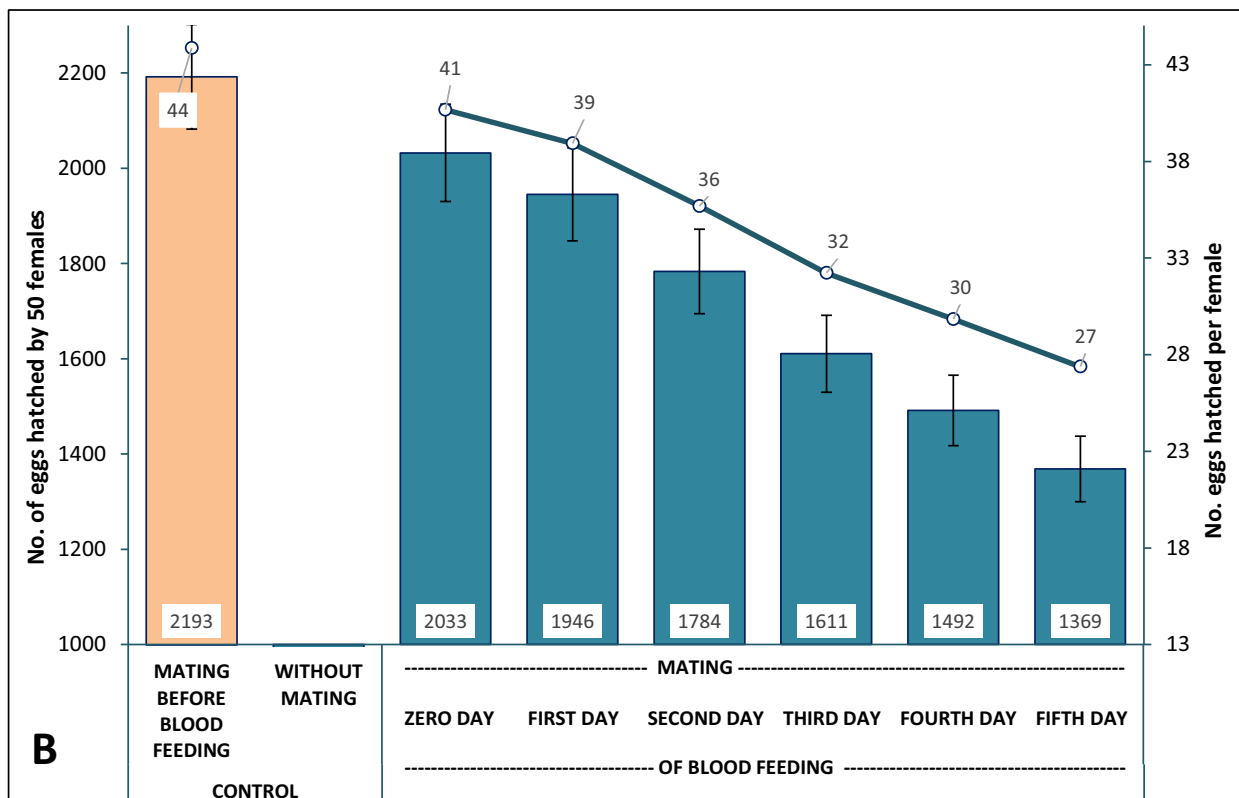
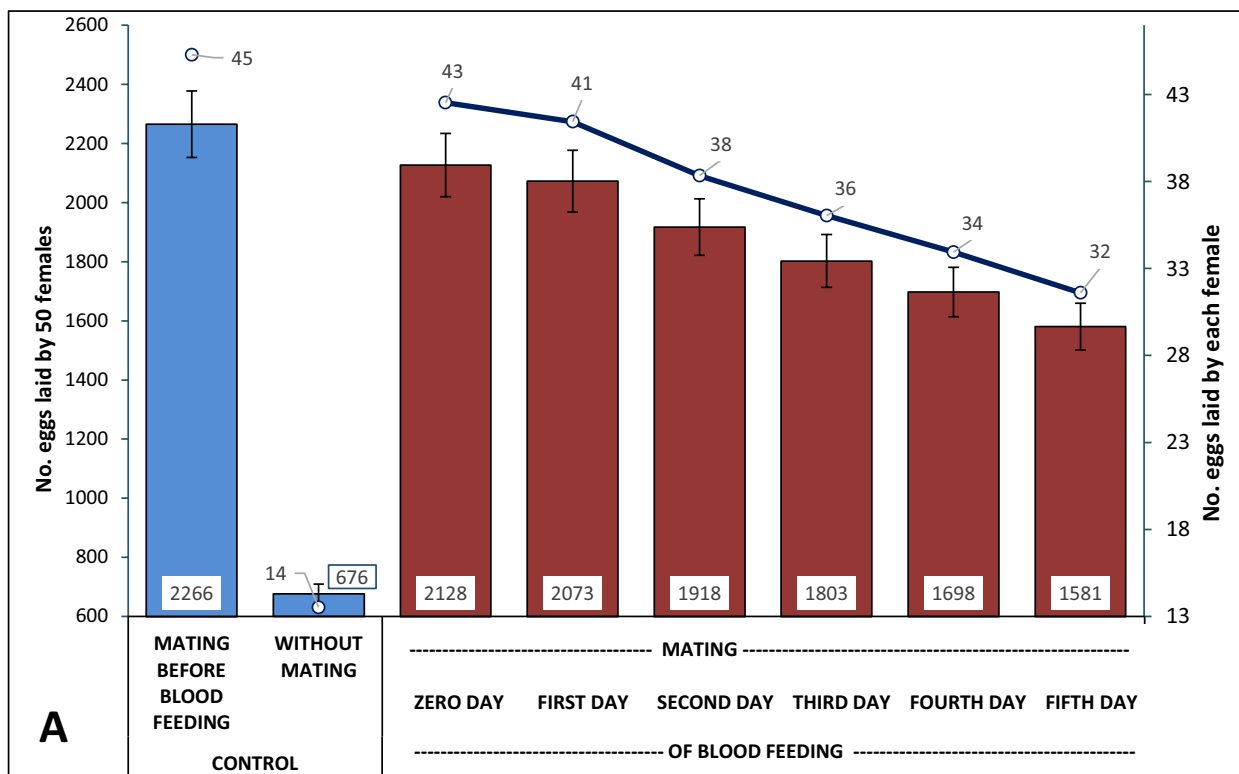


Fig. 2 Mean number of eggs laid (A) and hatched (B) by the female *Ae. aegypti* mosquitoes mated on day 0 of blood-feeding to day 5 of blood-feeding with 5% error bars. Line graph shows the number of eggs laid or hatched by a single female

occurred on day 0 (day of blood-feeding), females laid an average of 2128 eggs, followed by 2073, 1918, 1803, 1698, and 1581 eggs by females mating on the first, second, third, fourth, and fifth days, respectively. The number of eggs laid decreased from day 0 to day 5 of mating when compared with the control, in which males were allowed to mate before blood-feeding (Fig. 2A).

The mean value of the number of eggs hatched showed that, in control 1, 2193 eggs were hatched (96.78%), which was the highest, where male mosquitoes were released and allowed to mate with a female before blood-feeding. None of the eggs laid by unmated females in control 2 hatched. In the experimental set, in the D0 cage, 2033 eggs were found hatching (95.54%), followed by 1946 (93.87%), 1784 (93.01%), 1611 (89.35%), 1492 (87.87%), and 1969 (86.59%) eggs found hatching in the cages where males were allowed to mate on the first, second, third, fourth, and fifth days of blood-feeding. Similar to the sequential reduction of the number of eggs laid, the number of eggs hatched also decreased from day 0 to day 5 of mating when compared with control 1 (Fig. 2B). It was also observed that when the gravid females from D3–D5 cages after mating on the third to fifth days were released back into the respective cages for oviposition, more than 90% of the females laid eggs within the first 30 min. On day 0, a single female laid 43 and hatched 41 eggs, followed by 41:39, 38:36, 36:32, 34:30, and 32:27, respectively, for the first to the fifth day of mating after blood-feeding. These numbers were 45:41 for control 2, and only 14 eggs were laid by a single female in control 1 without hatching.

For the number of eggs laid, the D’Agostino–Pearson test showed a significant difference from the normal distribution, and skewness was -0.05297 ($P=0.95$) (potentially symmetrical). The excess kurtosis was -1.531 ($P=0.379$)

(potentially mesokurtic normal-like tails). The one-way ANOVA showed a significant difference in the number of eggs laid each day ($F=42.69$, $df=17$, $P<0.05$) from day 0 to day 5 using three replicates (3×6 days). Similarly, for the number of eggs hatched, the D’Agostino–Pearson test showed a significant difference from the normal distribution. The skewness was 0.00855 ($P=0.992$) (potentially symmetrical), and the excess kurtosis was -1.7022 ($P=0.328$) (potentially mesokurtic normal-like tails). The one-way ANOVA showed a significant difference in the hatchability of each day’s eggs ($F=31.47$, $df=17$, $P<0.05$) from day 0 to day 5 using three replicates (3×6 days). The linear regression analysis also showed that the number of eggs laid and the number of eggs hatched significantly decreased when days were increased, with $P<0.001$ (Fig. 3). The beta coefficient and R^2 values were 113.75 and 0.92, and 138.89 and 0.94, respectively, for the number of eggs laid and hatched.

Discussion

This study demonstrated that mating can occur before or after blood-feeding. One of the major findings of the study is that mating a few minutes before egg-laying was sufficient to produce 1581 eggs (70% when compared with control) and fertilize 1369 eggs (85%), which is far higher than the 676 non-hatching (unfertilized) eggs (30%) laid by unmated females. This shows that although mating is not essential for producing eggs, even brief exposure to the semen and seminal fluids greatly enhanced the number of eggs and hatching efficiency, even if the mating occurred just before oviposition. The retention time for seminal fluids in the spermathecae of females seems to determine the number of eggs laid and hatched. The seminal fluid retention time was higher in control 1 compared with the females that mated on subsequent days, and hence, the

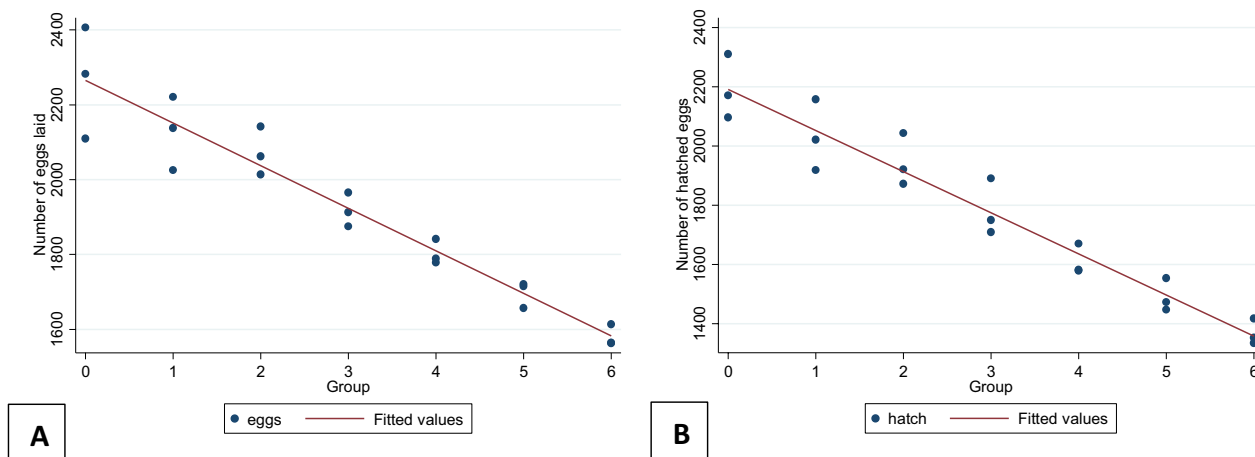


Fig. 3 Linear regression analysis of number of eggs laid (A) and number of eggs hatched (B) by 50 female *Ae. aegypti*

females in control 1 that mated before blood-feeding produced more eggs and showed a higher percentage of hatching than those females that mated on subsequent days of blood-feeding. Females that mated on the fifth day may have had very little time to benefit from the seminal fluids other than fertilization; hence, only 1581 eggs were laid and only 1369 (86.59%) eggs hatched, compared with control 1, where 2266 eggs were laid and 2193 eggs hatched (96.78%). The females that mated on day 0 laid 2128 eggs and hatched 2033 eggs (95.54%). As the females in control 1 were not able to have mated with males and did not have the chance to benefit from the seminal fluids, their egg production was only 30% when compared with control 2. It was also clear that, even at the fully gravid stage, the spermathecae were capable of receiving sperm and seminal fluids from the males. In addition, it was demonstrated that after blood-feeding, if there are no males available to mate, the blood-fed female can delay mating up to minutes before oviposition and still produce viable progeny.

In control 1, females without mating also took blood meals and produced non-viable eggs, showing that mating is not a requirement for blood-feeding, which is similar to the view of Ramirez-Sanchez [28]. Although a few studies have reported post-mating responses (PMR) such as enhanced vectorial capacity [41], increased female host-seeking behaviours [42, 43], blood-feeding frequency [44], longevity [45], induction of transcriptional changes in female reproductive tract tissues [31], increased blood meal size [44], enhanced egg-laying rates [46, 47], and inhibition of re-mating [29, 30], the influence and outcome of mating before blood-feeding versus mating just before oviposition have not been reported elsewhere. Contrary to previous reports [36], which led to the assumption that blood-fed females have a lower success rate for copulation and/or take longer to copulate than their unfed counterparts, our observations clearly showed that the egg production was reduced by only 6% and viability was reduced by only 1.2% in comparing the females that mated before and after the blood meal. Wright and Venard [27] reported that there was no difference in the level of insemination between unfed and blood-fed females. This indicates that males had no preference either for females who had taken a blood meal or for those that had not. However, the decrease in the number of eggs laid and the number of eggs hatched when mating was delayed after blood-feeding indicates the importance of male factors in the fecundity of the females and the influence of semen and seminal fluids on the PMR. With respect to a single female, even a short course of exposure to semen and seminal fluids was sufficient to produce almost double the number of eggs, with 32 eggs produced by the female that mated just before

(1 h) oviposition on the fifth day of blood-feeding, compared with only 14 eggs laid by the unmated female.

In light of these results, the present study may aid in gaining a clearer understanding of the reproductive biology of *Aedes* mosquitoes, which will further strengthen the knowledge in this field to develop novel control methods based on mating behaviour and reproduction. Understanding the mating behaviour in *Ae. aegypti* is of paramount importance for developing control techniques such as *Wolbachia*-based methods or genetic-based control methods [48]. Moreover, understanding the mating and blood-feeding behaviour in relation to the resting and gonotrophic cycles is important, as they can influence or even determine the impact of any intervention strategies. Reliable information on these behaviours in the vector population and on the behaviour of the human population is essential before the selection and implementation of any control method [15].

Conclusions

This study demonstrates the possibility for mating of gravid *Ae. aegypti* mosquitoes minutes before oviposition and still producing viable eggs. Even brief exposure to the semen and seminal fluids of the male minutes before oviposition was found to be sufficient for female *Ae. aegypti* to produce viable eggs and also for producing a greater number of eggs when compared with unmated females.

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Author contributions

MM conceived and designed the study and wrote and edited the manuscript. SS, AT, AM, and NS maintained the mosquito insectary and performed experiments. AV, RV, and VB analysed the data. RR provided design advice and edited the manuscript. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare no competing interests.

Consent for publication

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