SKIP OVIPOSITION BEHAVIOR OF LABORATORY, FIELD AND TRANSGENIC STRAIN OF *AEDES AEGYPTI* (L.)

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Abstract. The oviposition behavior of 3 strains of *Aedes aegypti* (L.) namely, laboratory-reared, field collected and transgenic strain was studied. Newly emerged mosquitoes mated before first blood feeding. The time for the mating varied from 8 to 11 hours after emerging while time for first blood meal was similar in the three strains. All 3 strains of *Ae. aegypti* females preferred to lay eggs in the afternoon. Their capability of oviposition was 2-3 folds higher than that in the morning. The oviposition behavior response to different conspecific egg densities was similar in the three strains. *Ae. aegypti* preferred to lay eggs in breeding sites with low egg density of 5-10 eggs. When the choice for selecting oviposition site was denied, the skip oviposition behavior disappeared. This study indicated that 95% of *Ae. aegypti* laid all the eggs within 72 hours. There was also no difference in oviposition behavior when a particular strain of *Ae. aegypti* was provided with eggs of a different strain.

Keywords: transgenic *Aedes aegypti,* oviposition behavior, skip oviposition

INTRODUCTION

Dengue is the most common arbovirus infection globally today and transmission is occurring in at least 128 countries and almost 4 billion people are at risks (Brady *et al*, 2012; Stanaway *et al*, 2016). In the continued absence of an effective tetravalent vaccine and specific treatment, dengue can only be controlled via suppression and elimination of the *Aedes* vectors. Present vector control technology, however, appears to be ineffective to control the disease, often resulting in massive outbreaks. There is a need to search for more effective and innovative dengue vector control strategy such as mosquito infected with *Wolbachia*, sterile insect technique using gamma irradiation or the genetically modified mosquito (WHO, 2016). Sterile Insect Technique (SIT) involves releasing sterile males to mate with the wild type females, resulting in sterile eggs , thereby sustained release will be able to suppress the natural population to level below the transmission threshold.

Recent advances in biotechnology have resulted in a strain of *Aedes aegypti* haboring a lethal gene killing the larvae. The technique, known as Release of Insects with Dominant Lethal (RIDL) is based on the development of transgenic

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insects with a genetic construct that confers dominant lethality. The RIDL operates in a non-sex specific manner and kills all insects carrying at least one copy of the construct. The principle of RIDL is simple: if the genetically modified insects are homozygous for a dominant lethal and mate with wild type insects; all the progeny are heterozygous for the dominant lethal and so all will die (Alphey, 2000).

Before releasing genetically modified insects to the wild environment, their oviposition behavior and bionomics should be studied to ensure successful release and suppression of the *Ae. aegypti*. An important aspect is the skip oviposition behavior of transgenic *Ae. aegypti* in comparison to wild type and lab strain of *Ae. aegypti*.

Mogi and Mokry (1980) used the term 'skip oviposition' to describe the behavior of *Wyeomyia smithii* Coquillet females that distributed their eggs from the same batch among several different places. The similar behavior has also been observed in small containers in the laboratory studies with *Ae. aegypti* (Fay and Perry, 1965). This peculiar oviposition behavior of *Ae. aegypti* female mosquito is an important factor that may impact the dispersal (Edman *et al*, 1998), flight range, survival and longevity of this mosquito. Studies carried out by Soman and Reuben (1970) and Allan and Kline (1998) documented that the presence of conspecific eggs and larvae or pupae attracted female mosquito to oviposit. They observed that the oviposition responses by female *Ae. aegypti* were similar between larval water and control water but significantly greater to larval water from *Ae. albopictus*. The authors also stated that significantly more eggs were laid by gravid *Ae. aegypti* females on oviposition paper containing either *Ae. aegypti* or *Ae. albopictus* eggs than on oviposition paper without eggs. However, in contrast, other studies have shown that presence of conspecific larvae repels the *Ae. aegypti* mosquito female from the oviposition and field studies offering substrates already containing conspecific eggs showed greater superoviposition when fewer than 25 eggs were present (Chadee *et al*, 1990). Craig *et al* (2008) showed that *Ae. aegypti* exhibited a strong oviposition preference for substrates with intermediate number of conspecific eggs and indicated that skip oviposition behavior was modulated according to the availability of suitable breeding sites.

The egg laying behavior of *Ae. aegypti* is thus affected by the presence of conspecifics in potential breeding sites. Such behavior can be considered as a strategy for avoiding oviposition in sites where larval nutrition is limited. The presence of conspecifics also seems to encourage the egg laying and may be due to the guarantee of suitability for survival of the larvae. The overcrowding and starvation in the breeding site repel the female mosquito from egg laying and making further search for more suitable breeding sites (Mulla, 1979). Edman *et al* (1998) documented that the availability of outdoor oviposition breeding sites may cause different egg laying behavior than the oviposition site environment found indoors. This study was conducted to determine and compare skip oviposition behavior of a laboratory, field and transgenic (RIDL) strain of *Ae. aegypti*.

MATERIALS AND METHODS

Laboratory strain *Ae. aegypti*

The laboratory- reared *Ae. aegypti* mosquito strain from the insectarium at Institute for Medical Research (IMR), Malaysia was used as the laboratory strain. This colony has been maintained in the IMR insectarium for 1,100 generations

(F=1,100) at 26±2ºC and 70±10% relative humidity and a photoperiod of 12:12 (12 hours light and 12 hours dark).

Field strain *Ae. aegypti*

The field strain *Ae. aegypti* was recently colonized from a collection obtained from an island, Pulau Ketam in the state of Selangor and kept in the insectarium in IMR at 26±2ºC and 70±10% relative humidity. The F1 generation was used in this study.

Transgenic *Ae. aegypti*

The transgenic *Ae. aegypti* used in this study was maintained at 26±2ºC and 70±10% relative humidity in the Arthropod Containment Level-2 insectarium. The RIDL-513A strain was originally generated with a Rockefeller strain genetic background. Rockefeller is a laboratory strain, originally of Caribbean origin, colonized in the early 1930s. After several decades of lab rearing, this strain has adapted well to lab rearing, but conversely is likely to have lost traits related to field performance. The RIDL-513A insertion was therefore introgressed into more recently colonized strains, by backcrossing for at least 5 generations. In each case multiple independent homozygotes were generated and pooled to try to minimize inbreeding/genetic bottleneck effects. The present test strain My-RIDL-513A was generated using a laboratory strain of Malaysian origin. This strain was constructed by Oxitec (Abingdon, UK) and was established in the IMR ACL-2 insectarium since 2006.

Experimental conditions

In all the experiments distilled water was used as the oviposition medium. The same volume of water (225 ml) was used for all ovitrap in each experiment. The experiment was carried out in the insectarium at IMR, temperature and relative humidity were measured during each experiment using a thermo-hydrometer.

Determination of optimum survival time in different size of cages

This study was conducted using 20 mosquito cages as replicates that occupied a large area. Therefore, it was necessary to select mosquito cages of appropriate size that allowed optimum survival of *Ae. aegypti*.

Two types of mosquito cages were used in the insectarium of IMR, namely, large cages (30 cm x 30 cm x 30 cm) and small cages (20 cm x 20 cm x 20 cm). Single *Ae. aegypti* female mosquito was kept inside a large and a small mosquito cage separately to observe their daily survival rate. The experiment was replicated with 10 large cages and 10 small cages for each of the three strains concurrently to avoid any bias from the experiment.

Time of mating and first blood meal of newly emerged mosquitoes

The pupae obtained from the eggs of *Ae. aegypti* were transferred into a bowl and placed in a mosquito cage. A white mouse was introduced into the cage as the food source for mosquitoes that emerged from the pupae. The observation was started as soon as the first adult mosquito emerged. Continuous observations were made to observe the first mating and the time of first blood meal of newly emerged mosquitoes. Three experiments were carried out for each of the three strains.

Determination of optimum time for oviposition in *Ae. aegypti*

Experiment was carried out to determine the peak oviposition time of *Ae. aegypti*. The experiments were carried out at intervals ranging between 09.30 AM and 11.30 AM and 01.00 PM and 04.00 PM. During each specific time, 100 females were allowed to oviposit for 1, 5, 15, and 30

minutes. The experiment was triplicated for all the three strains of *Ae. aegypti*. The mean number of eggs laid in each time period was counted to determine the optimum time egg laying activity of *Ae. aegypti*.

Oviposition response to different egg densities of lab, field and transgenic strain

A single pupa was isolated in a glass vial for emergence. Virgin females were mated prior to providing them with a blood meal. Three days after bloodmeal, gravid females were allowed to oviposit. An ovitrap was introduced into the cage. Eggs were obtained on cardboard paddle (3 cm x 10 cm). Viable eggs from these paddles were counted, while non-viable eggs (hatched or dehydrated) were removed from paddles using a needle under the dissecting microscope. The eggs of all three strains of mosquitoes were separately collected on cardboard paddles.

Egg densities to be used as conspecific eggs were categorized as follows: 0 eggs, 10-50 eggs, 100-150 eggs, and >150 eggs. These were classified as zero egg, low density, medium density, and high density.

Oviposition response to different conspecific eggs density was observed with four ovitraps in small cages. Black colored ovitrap (25 ml) holding cardboard oviposition paddle and 225 ml distilled water was used to provide gravid female mosquitoes with oviposition substrate options.

Ae. aegypti were blood fed on white mouse. The mouse was removed after 24 hours or until the mosquitoes had fully engorged. In addition to blood feeding, mosquitoes were supplied with 10% sucrose solution. The tests were conducted continuously for 72 hours. Forty-one replicates for each strain were carried out for each egg laying experiment. Four ovitraps each with zero eggs, low egg density, medium egg density and high egg density were placed into each mosquito cage with a single gravid female *Ae. aegypti*. The ovitraps were rotated clockwise after 24, 48, and 72 hours to avoid any bias for site preference. The test for each strain was replicated 41 times for oviposition response with the zero, low, median, and high egg densities.

The experiment was carried out for all three strains of *Ae. aegypti*. Their own conspecific eggs were used for each test. After 72 hours, the cardboard paddles were collected, covered with a fine mesh and kept in trays for drying. The number of eggs in each paddle was counted under a dissecting microscope and recorded for different egg density categories for all three strains. The site selecting preference for oviposition in the three strains of *Ae. aegypti* was then compared.

Oviposition response to single pre-fixed egg density

This experiment was similar to the oviposition site selection experiment, which had choices of zero, low, median and high. However in this pre-fixed egg density individual gravid mosquitoes were offered no choice of egg density for oviposition. In the 40 replicated experiments, a range of egg densities ranging from 0 eggs to 185 eggs was offered. At the end of each assay, the number of eggs laid in the ovitrap was determined and mosquitoes dissected to determine the proportion of egg retention.

Observation of egg laying behavior in ovitraps with eggs of different *Ae. aegypti* strains

The mosquitoes were fed with a blood meal from white mouse and allowed to lay eggs 3 days post-feeding. Ovitrap with conspecific eggs of another strain of *Ae. aegypti*

was used. A low number (10-50) of egg densities were used. For each strain three replicates were conducted. The experiment was carried out to observe the eggs laying in ovitraps as follows: Egg laying behavior of lab strain females in ovitraps with eggs of transgenic strain; egg laying behavior of field strain females in ovitraps with eggs of transgenic strain and egg laying behavior of transgenic strain females in ovitraps with eggs of laboratory strain.

Dissection of female mosquitoes for retained eggs

The individual female mosquitoes used in oviposition experiments were dissected for retained eggs. The mosquitoes were first killed using a killing mixture (benzene:chloroform = 1:1) in a paper cup and dissected under a dissecting microscope. The wings and legs of mosquito were removed and the abdomen placed in a saline drop. The 6-7th abdominal segment was cut and the ovaries removed using dissecting needles. The ovaries were carefully examined for retained eggs under a dissecting microscope. The number of eggs retained was recorded.

Data analysis

Data obtained were keyed into a spreadsheet (Microsoft Excel® 2010) and then analyzed statistically using SPSS® (version 15; IBM, Armonk, NY) using appropriate tests.

RESULTS

Determination of optimum survival time in different size of cages

The survival of *Ae. aegypti* females reared in large and small mosquito cages was analyzed by ANOVA (SPSS version 15) and found to be not significantly different in all three strains (p>0.05). The mean survival was 12.1±0.96 days, 12.3±1.05 days, and 12.6±0.96 days in small mosquito cages for laboratory, field and transgenic strain, respectively, while in large cages the respective mean life span for the three mosquito strains was 13.0±1.15days, 13.1±1.10days and 12.3±1.25 days, respectively. Based on these findings, the oviposition studies were carried out in small mosquito cages.

Time of mating and first blood meal of newly emerged mosquitoes

Females of the laboratory strain first mated 8.0±0.15 hours after emergence, while for the transgenic mosquitoes, first mating occurred 7.0±0.43 hours post-emergence. However, the duration was comparatively longer in the field strain, which was 11±0.89 hours.

Laboratory, field and transgenic strain *Aedes aegypti* female mosquitoes started their first blood meal at about 20±1.53, 22±1.53, and 19±1.53 hours, respectively, after emergence which was not significantly different among the three strains (p>0.05) (ANOVA, SPSS version 15).

Determination of optimum time for oviposition in *Ae. aegypti*

There was a significant difference in egg laying between morning and afternoon (p<0.05). Most of the eggs were laid in the afternoon. An average of 46 eggs per 100 females were laid in the morning within a time period of 30 minutes; compared to 173 eggs per 100 females in the evening, also within similar time period (Table 1). Hence, the mean number of eggs laid per female was 4.60±9.00 in the morning and 17.30±8.33 in the evening. The ratio of eggs laid in the morning and evening was 1:3. Eggs were deposited on edge of the paddle stick and were aligned singly on the paddle. Newly hatched eggs were white in color and darkened after 10-15 minutes.

Table 1
Oviposition of *Aedes aegypti* in morning and afternoon.

Time of the day	Time	Replicates (*n*)	Duration (mins)	Eggs laid (*n*)	Mean eggs/100 females, *n*±SD	*p*-value
Morning	09.30 AM - 11.30 AM	3	30	138	46.0±9.00	0.002
Afternoon	01.00 PM - 04.00 PM	3	30	520	173.0±8.33	

Table 2
Classification of egg density.

Time for oviposition (min)	Eggs, *n*±mean	Classification	
1	6±0.40	Zero	0
5	30±0.23	Low	10-50
15	105±0.61	Medium	100-150
30	173±0.12	High	>160

Oviposition response to different egg densities of lab, field and transgenic strain

The eggs were categorized into four groups, namely, zero egg density (0 density, without eggs on the paddle), low egg density (10-50 eggs on the paddle), medium egg density (100-150 egg density on the paddle) and high egg density (>160 eggs on the paddle) (Table 2).

The oviposition behavior of *Ae. aegypti* in different conspecific egg densities was significantly different in all the three strains (p=0.001) (Table 3). *Ae. aegypti* females exhibited high preference for ovitraps with low densities of conspecific eggs (10-50 eggs) for 72-hour observations**.** This pattern was consistent in all three mosquito strains in which the mean number of eggs per female was 100.63, 80.29 and 84.00 in laboratory, field and transgenic strain of *Ae. aegypti*, respectively. The second oviposition preference was for the conspecific eggs with mean densities of between 100-150. The mean numbers of eggs per female were 8.75, 10.48, and 7.85 for lab, field and transgenic strain, respectively, within a period of 72 hours. The oviposition preference was then followed by zero egg density and high egg density (>160 eggs). The least oviposition preference was observed in high conspecific egg density ovitraps, the mean number of eggs per female being less than 1.4 in all the three mosquito strains.

Further analysis using Mann-Whitney test showed that there was no significant difference (p<0.001) in oviposition preference in zero, low, medium, and high egg density classification in all the three strains.

Oviposition response to single pre-fixed egg density

The results of oviposition behavior of *Ae. aegypti* in pre-fixed con-specific egg densities (no choice, with only one ovitrap in the cage) are shown in Table 4**.** The mean number of eggs laid in different

Table 3
The oviposition preference for the three different conspecific egg densities observed for the three mosquito strains.

Mosquito strain	Conspecific egg densities				*p*-value
	(0 eggs) Mean±SD	(10-50 eggs) Mean±SD	(100-150 eggs) Mean±SD	(>160 eggs) Mean±SD	
Laboratory	1.07±1.07	100.63±25.04	8.75±8.46	0.90±2.37	<0.001
Field	1.31±1.31	80.29±15.73	10.48±11.61	1.34±3.35	<0.001
Transgenic	1.24±1.24	84.00±19.10	7.85±10.39	0.97±2.32	<0.001

Table 4
The oviposition preference with pre-fixed con-specific egg densities observed for the three strains (No choice test).

Mosquito strain	Conspecific egg densities (Means)				*p*-value
	(0 eggs)	(10-50 eggs)	(100-150 eggs)	(>160 eggs)	
Laboratory	19.65	23.50±2.00	18.70±3.91	20.15±0.36	0.811
Field	23.10	21.25±4.00	23.65±3.00	14.00±2.11	0.224
Transgenic	17.95	23.35±2.01	15.90±2.41	24.80±0.39	0.264

density classification for laboratory, field and transgenic strain ranged from 18.70-23.50; 14.00-23.65, and 15.90-24.80, respectively. The mean number of eggs laid in different con-specific egg densities was similar.

Observation of egg laying behavior in ovitraps with eggs of different *Ae. aegypti* strains

The results of the egg laying in ovitraps with different conspecific eggs showed that there was no significant difference among the three strains ($p>0.05$). The mean number of eggs laid was 93±7.00, 94±12.2, and 96±5.29 in lab strain containing transgenic strain eggs, field strain containing transgenic strain eggs, and transgenic strain containing laboratory strain eggs, respectively (Table 5).

Dissection of female mosquitoes for retained eggs

The dissection showed that 95% of *Ae. aegypti* females of the 3 strains laid their eggs in the available ovitraps with any number of conspecific eggs.

DISCUSSION

The oviposition behavior of *Ae. aegypti* is an important factor that may impact the dispersal (Edman *et al*, 1998) and this can be directly or indirectly related to their survival, longevity and flight range of this mosquito. Knowledge of oviposition behavior may also be used to improve surveillance and control of this important disease vector.

In this study we were interested to determine the site selecting behavior

Table 5
Egg laying behavior in ovitraps with eggs of different *Aedes aegypti* strains.

Ovipositing female strain	Replicates (*n*)	Mean *n* eggs introduced	Mean *n* eggs laid *n*±SD	*p*-value
Laboratory	3	30 (transgenic)	93±7.00	0.326
Field	3	30 (transgenic)	94±12.2	
Transgenic	3	30 (laboratory)	96±5.29	

for oviposition by *Ae. aegypti* specially related to their 'skip oviposition' habit and super-oviposition behavior. Earlier studies have shown that the mosquito may lay eggs at several sites in a single gonotrophic cycle (Reiter, 2007) and the presence of conspecific eggs encouraged the oviposition of *Ae. aegypti* (Chadee *et al*, 1990). Oviposition in mosquitoes can be regarded as the consummatory act to an appetitive drive involving a hierarchical sequence of behavioral steps linked with their life cycle. This behavior exhibits many characteristic events associated with their life such as egg laying environment, timing of egg laying, mating, blood feeding, and aquatic life.

Generally, male mosquitoes emerge a few days before female mosquitoes. This gives the males the opportunity to mature before the females emerge and ready to mate. After mating, the females look for a blood meal. In this study, the time of mating of newly emerged mosquito showed similar pattern in both lab and transgenic strains; the time being 8±0.15 hours and 7±0.43 hours, respectively, after emergence, but comparatively longer period for the field strain (11±0.89 hours).

Under favorable conditions, a young female mosquito can undertake her once-a-life-time mating and finds a blood-meal within a day, and lays her first eggs a couple of days later. The shorter time may be due to the adaptation of the transgenic and laboratory strains because they were reared in the insectarium for many generations.

The time taken for the first blood meal varies from mosquito species to species. In the present study, the average time required for the first blood meal was 19.7 hours, 20.3 hours and 21.7 hours in lab, field and transgenic strain, respectively. Earlier studies showed that most adult females took their first blood meal when they were 23-26-hour-old (Jones and Pilitt, 1973).

Studies on optimum time for oviposition (Harrington and Edman, 2001; Russell and Ritchie, 2004; Chadee, 2010) found that oviposition in caged populations of *Ae. aegypti* followed a very clear diet periodicity with a peak in the afternoon. Our results on oviposition were in accordance with their findings. This observation was in contrast to previous report by Corbet (1966) who pointed out that the peak was not in the afternoon and recommended a field study to validate this hypothesis.

In the present study the experiment was carried out between 09.30 AM and 04.00 PM. Harington and Edman (2001) showed that the oviposition time gradually increased with the warm and rainy climate. The number of eggs laid at night was very few. This timing effect is likely a result of differences in the day length

and humidity as well as temperature between morning and afternoon. The temperature, relative humidity and other environmental factors in the afternoon seem to be more favorable to *Ae. aegypti* oviposition. Our study showed that the number of eggs laid in the afternoon was 3 folds higher, compared to morning. However, the peak oviposition time may vary from place to place.

In selecting site for oviposition with choices given, low density of conspecific eggs (between 10-50 eggs) was the most attractive to gravid female for egg laying. The results of our study showed that they were not attracted to paddles with zero density (no eggs on the paddle) and high number (>160) of egg densities. They were moderately attracted by the median number of conspecific egg density, which was not the most preferred compared to the low density of conspecific eggs.

Similar study carried out by Craig *et al* (2008) had shown that their prominent acceptable conspecific egg density for egg laying of *Ae. aegypti* was intermediate egg density (11-38). The classification range in our study was different compared to that of Craig *et al* (2008) who used *Ae. aegypti* strain from Cairns, North Queensland, Australia. They obtained the eggs at 28ºC and 80% relative humidity, which were similar to our laboratory conditions. However, there may be inherent egg laying capability of the different strains of mosquitoes which seemed to be lesser compared to the strains we used.

In the absence of choice for oviposition, the present study showed that *Ae. aegypti* laid eggs in whichever available ovitraps with eggs. According to Craig *et al* (2008), *Ae. aegypti* females need to lay their eggs as soon as possible. Our study indicated that 95% of the females deposited all their eggs within 72 hours. It may be assumed that when there are many breeding sites in the close proximity, *Aedes* exhibits skip oviposition. However, skip oviposition will be avoided when there is no choice for selection of breeding sites. This strongly suggests that the presence of multiple breeding sites will increase mosquito density and dispersal.

The attraction of gravid *Ae. aegypti* females to low densities of conspecific eggs may be used as their survival strategy, as the presence of conspecific eggs in a breeding site may indicate that the site is suitable and safe for development of offspring. It also indicates absence of predators and the presence of suitable environment for shelter, nutrition and development of larvae. Also it can be argued that the attraction may be due to the presence of conspecifics that can serve as mates for offspring. It seems possible that the gravid female, which is going to lay eggs, is agreeable with the decision of former females which have already laid their eggs. The guarantee of the breeding site given by the former females that have already laid eggs will be considered by the newly arrived gravid female for egg laying in such breeding site.

In our study, the least preference of egg laying by *Ae. aegypti* was the high number of conspecific egg density (>160). Such behavior can be considered as a strategy for avoiding overcrowding in the same breeding site, which may lead to limitation of larval nutrients. This avoidance effect of high conspecific eggs was also reported by Mulla (1979) who showed that this deterrence of oviposition behavior in site with high conspecific density had evolved to minimize the adverse effect of larval overcrowding.

There was no significant difference if

the transgenic adult females were provided with ovitraps containing laboratory or field strain *Ae. aegypti* eggs. Furthermore, there was also no difference if the field strain was provided with eggs of laboratory strain. Such observations are important in future open field release of transgenic *Ae. aegypti* males to control dengue, since the released transgenic males will mate with the field females and will transfer the lethal genes to the females.

It is expected that the oviposition behavior of the mated field females will not alter. In the unlikely event of the release of small number of transgenic females, such females will also exhibit similar oviposition behavior like their untransformed counterparts. It is obvious that genetic transformation did not alter the oviposition behavior of transgenic *Ae. aegypti* females.

In conclusion, transgenic *Ae. aegypti* females did not exhibit any alteration in oviposition behavior and oviposit in the same manner as the laboratory and field strain in the presence of appropriate density of conspecific eggs.

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