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Modifying oviposition behaviour of the Oriental fruit fly, *Bactrocera dorsalis* (Hendel) to obtain uniform G_0 stage eggs for microinjection

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ABSTRACT: The CRISPR/Cas9 technology has opened up newer avenues in insect pest management like precision guided sterile insect technique (pgSIT) which achieves a highly specific mutation in the target genes such as spermatogenesis, sex determination related genes etc. In this regard, validating the loss-of-function of the target gene/s is a prerequisite before the final application. This is easily achieved through DNA-free editing by embryonic delivery of the cognate ribo nucleo protein complex (RNP) into G_0 stage eggs. Obtaining uniform G_0 stage eggs is necessary to offset the microinjection injury and have high heritability of the genomic edits. We to optimized a method to obtain intact eggs of Oriental fruit fly, *Bactrocera dorsalis* (Hendel) without injury for microinjection by modifying the oviposition behaviour of the gravid female of *B. dorsalis* to retrieve intact eggs and to obtain large number of G_0 eggs for genome editing. This paper describes a method to obtain required number of eggs for such studies. Out of two egg laying methods, the one with a small container with water covered with parafilm and topped with a thin banana pulp slice provided intact eggs. Maximum oviposition was observed between 20-60 days after eclosion. By the present finding we can obtain sufficient eggs for microinjection at 15-minute interval.

Keywords: G_0 phased embryo, egg laying, gene editing, oviposition parameters, Tephritidae

INTRODUCTION

Bactrocera dorsalis (Hendel) is a destructive polyphagous pest of horticultural crops with a wide host range which includes commercially cultivated crops like Mango, Guava, Custard apple and papaya (Clarke *et al*., 2005; Liquido *et al*., 2017). Infestation by this fly can hamper domestic and export market as it is a notorious quarantine pest resulting in substantial economic losses (Alvarez *et al*., 2016; Ndlela *et al*., 2022). Though different control modules are employed to combat fruit fly infestation most of the options have some disadvantages related to efficiency, time-consuming, mammalian hazard and residual effects. This necessitates the new interventions for effective pest management. Modern advancements in the area of pest management and genetic biological control methods such as the precision guided Sterile Insect Technique (pgSIT) and CRISPR-Cas9 mediated gene drive (Kandul *et al*., 2019; Buchman *et al*., 2018) are making the steady inroads and are emerging as a sound alternatives strategy in managing the insect pests. Despite its potentiality, pest control using CRISPR-Cas9 face numerous challenges including, harvesting numerous undamaged eggs within a narrow time (G0 stage) after egg laying. In *Pyrrhocoris apterus* bug, more mutants were obtained when germ line transformation was carried 12 h after egg laying than eggs injected within 2 h of laying (Kotwica-Rolinska *et al.,* 2019). In lepidopterans, the critical stage lapses after 4 h from egg deposition (Zhang and Reed, 2017). In, sand fly the time window for conducting microinjection was between 1.3 h to 3 h and this time window is even very less for mosquitoes. Generally, the G_0 stage for dipteran insect is less than 2 h so it is very crucial to get sufficient number of viable embryos within this timeframe (Campos and Hartenstein, 1985). Thus, it is essential to decipher information on fecundity and oviposition behaviour of gravid *B. dorsalis* females which are governed by several factors including multiple mating (Shelly, 2000), age of the female fly (Huang and Chi, 2014; Jaleel *et al*., 2018; Choi *et al*., 2020) and host substrate available for oviposition (Huang and Chi, 2014; Kalia and Yadav, 2005). In the present investigation we have carried out a detailed study of the egg laying behaviour of *B. dorsalis* flies, in order to achieve enhanced and scheduled egg laying for laboratory studies. The following parameters,

Table 1. Oviposition frequency of gravid females in different time intervals

Time interval (Minute)	Total	*Mean ± SD
15'	47	4.70 ± 4.32
30'	108	10.80 ± 7.89
45'	61	6.10 ± 6.93
60'	75	7.50 ± 6.50

**Note: ***Mean of five individuals

with regards to oviposition behaviour, have been examined (i) Laboratory host preference (to identify the best host which can elicit enhanced ovipositional response), (iii) Daily egg laying pattern (to decipher the correct age group of the flies for oviposition), (iv) period of time and oviposition response (to schedule timing for oviposition) and (v) Egg laying behaviour in time intervals (to plan number of flies required to harvest desirable number of eggs).

MATERIALS AND METHODS

Insect colony maintenance

Adult flies of *B. dorsalis* were recovered from the infested mango fruits collected from Fruit orchards of Indian Institute of Horticultural Research, Bangalore. Since mango is a seasonal fruit, in order to maintain fly population for extended study period, ripe banana fruit (cv Ney Poovan, commonly called as 'Yelakki') was used. The adults were enclosed in a wooden cage of size 30 x 30 x 30 cm. The cage had a wooden framework fitted with mesh on all sides except for the wooden floor and a glass cover on top for observation and illumination (Figure 1). Yeast extract, sugar and water were provided inside the cage for adult development and survival. Flies were housed in these cages for the entire length of the study. All the experiments were conducted under standard laboratory conditions (temperature: 28°C ± 3°C; RH: 70 ± 10%).

Laboratory host preference

These experiments were performed to identify the most suitable elicitor for egg laying. The following elicitors were tested for oviposition preference by the flies: Banana pulp slice, Banana peel, Guava skin (colour break stage) and Guava skin (ripe, yellow colour). The above-mentioned testing materials were placed on 0.5% agar plates and placed in a cylindrical plastic box [14 cm (height) X 11 cm (diameter)] containing gravid females, for oviposition (no choice experiment) (Figure 2). The agar plates were collected after 1 hour of exposure to the gravid flies. The eggs oviposited on agar plates were counted. The most efficient elicitor was identified by comparing the number of eggs oviposited per hour, beginning with equal number of flies. As Banana and Guava are available throughout the year, only these two fruits were tested as an elicitor for oviposition by female.

Oviposition experiment and egg collection method

The aim of this experiment was to disentangle the effect of oviposition source to assure more egg deposition which can render quality egg collection. In the first experiment (Method A) 0.5% agar was brought to boil with distilled water and poured into small plastic cups and allowed to solidify. In the second experiment (Method B) expanded wax Paraflim was spread over a plastic cup containing sterile water (Figure 3). A thin slice of banana (cultivar Yelakki) of thickness approximately 2-5 mm was placed over the solidified gel/expanded paraflim sheet to elicit oviposition response. The eggs were collected using a brush after distorting the agar gel in method A. Eggs in oviposition cups in method B were harvested by empting the oviposition cup onto a clean cloth and subsequently collected using a brush. Further the quality/viability of eggs collected were assessed. The collected eggs from each method were placed individually in small plastic wells containing mashed banana mixed with preservatives Methyl Paraben (0.1%) and sodium benzoate (0.1%) (Figure 4). The set up was kept in a container floored with water-soaked cotton to avoid moisture loss in mashed banana and to facilitate eclosion. The number of eggs hatched was noted after 24hrs till the fourth day. Mortality at egg stage, were documented and data were subjected to student's t test.

Daily egg laying pattern

These experiments were performed to understand the variability in egg laying during the female lifespan, in order to determine the best age group of the flies for

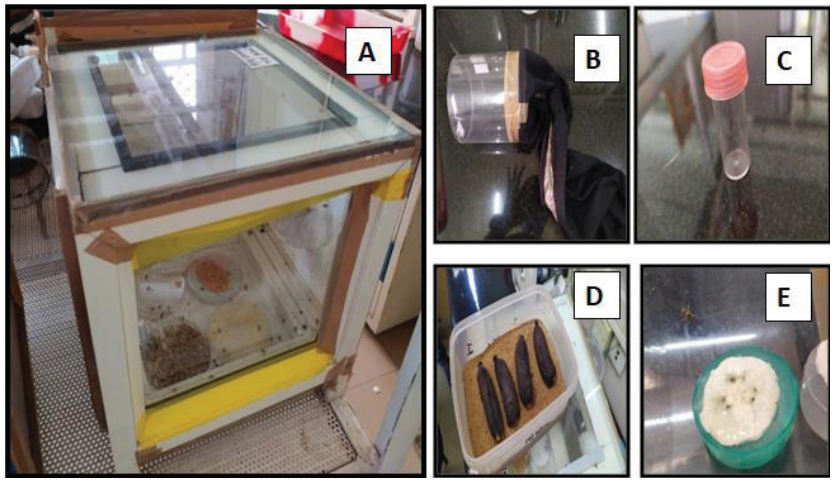

Figure 1. The rearing of fruit fly- a) wooden rearing cage b) Cylindrical box (14 cm height X 11 cm diameter) c) cylindrical cap d) pupation box e) Oviposition cup

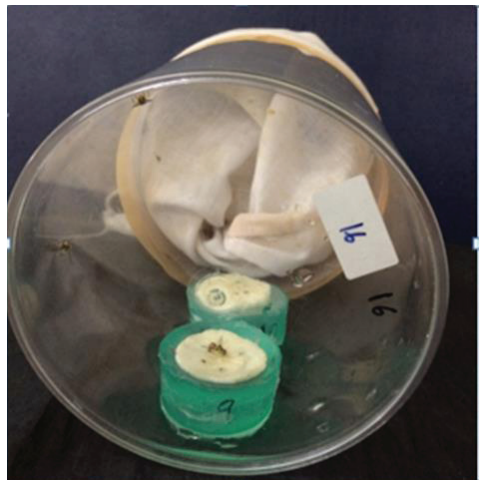

Figure 2. Oviposition substrate provided to the gravid females

maximum egg collection. Flies reared on banana in the laboratory were used for these experiments. After emergence from the pupa, the adults are sexed and paired immediately (1 female and 2 males) in individual cylindrical plastic boxes [14 cm (height) X 11 cm (diameter)]. Yeast extract, sugar and water were provided in the boxes, along with an oviposition source (a slice of banana placed on solidified agar). This oviposition source was replaced at 24h intervals until the female was spentor dead. The number of eggs laid was documented at 24h intervals.

Determination of number of gravid females required for optimal egg harvest

The goal of this experiment was to determine the number of flies per cage to obtain more eggs per fly in a given time. As a prerequisite, the flies reared in banana were maintained in different cages according to their age. Five treatments, with varying number of female flies (1,2,3,4,5) between age group 20-35 days old were released into a plastic container of size 14 cm (h) x 11 cm (r) and provided with oviposition source (Method B). For each treatment, females were selected randomly among the individuals in the same cage. The observations were made three times in a day *viz.,* 1st h- morning (0800 -1000), 2nd h- afternoon (1100- 1300) and 3rd h- evening (1600-1800). Oviposition source were removed after each hour and replaced with fresh oviposition cups until three successive hours. The total number of eggs laid in each oviposition sources were counted. The number of eggs laid per female in each treatment was determined by dividing the total number of eggs laid by the number of females present. The data were subjected to Analysis of variance followed by post hoc test. All the experiments were conducted in laboratory where temperature is 28°C ± 3°C with relative humidity of 70%.

Period of time and oviposition response

These experiments were performed to find the most suitable time of the day to elicit oviposition. Three time points in a day were chosen- Morning (08:00 -10:00), Afternoon (12:00- 02:00) and Evening (04:00 – 06:00). Total of 15 replicates were performed for each period of the day (each replicate consisted of two boxes, one with a single gravid fly and another with 5 gravid flies). Each box was provided with an oviposition cup (0.5% solidified agar with a slice of banana on top). The cups were removed after two hours of exposure and the number of eggs on the oviposition cups was counted. The experiments were conducted in a well-ventilated room on a sunny day.

Oviposition time intervals

G_0 egg stage, *i.e.* intervention within a few minutes of the egg laying in dipterans, is crucial for a successful germline transformation. Experiment was planned to know the number of eggs that can be oviposited to the oviposition source during 15 minutes of exposure period. For this experiment five gravid females aged between 25-40 days were caged in a box and provided with an oviposition cup at time zero, this oviposition cup was removed after 15 minutes of exposure and replaced for the next 15 minutes by fresh oviposition cups. Oviposition cups were removed in this manner for every 15 minutes for 4 times (1h). The total number of eggs laid over the period of every 15 minutes was counted.

RESULTS

Laboratory host preference

Under laboratory conditions banana pulp (45.8 ± 15.75 eggs) was the most preferred host for oviposition followed by banana peel (16.8 ± 10.28), Guava skin (green, colour break stage) (12.6 ± 6.5) and Guava skin (Ripe, yellow colour) (4.2 ± 5.14) (Figure 5). The difference between the treatments were statistically significant (Kruskal wallis test: H =13.07, p=0.004) clearly exhibiting the preference.

Oviposition experiment and egg collection method

In method (A) the average number of eggs produced per female was 26.2 ± 11.20 where as in method (B) the average egg produce per female was 24.4 ± 9.15. There was no statistically significant difference in egg laying among method (A) and Method (B) t(16)=0.5528, p=0.5881, n1=10, n2=10. However, the survival percentage of eggs harvested from the two oviposition methods were 58% and 74% in method A and method B respectively (Figure 6). Clearly the survival of eggs collected from method B oviposition experiment was more and was statistically significant (U=25377, Z=3.066, P=0.0002 n1=210 and n2=288).

Daily egg laying pattern

A total of 18 female flies were observed from the day of emergence till mortality. In plastic containers flies were released in the ratio of one female to two males and were provided with water food and oviposition source. The earliest egg laying was observed on 11th day from emergence and the last batch of eggs were deposited by flies as old as sixty days. The average egg laying in the first twenty days was 2.67 ± 5.13. In the subsequent twenty days it was 19.94 ± 5.013 eggs per female and final twenty days the average egg production per female was 21.79 ± 8.94. The average egg laying per day per gravid female during its life span was 15.2 ± 11.98 in banana (Fig. 7).

Determination of number of gravid females required and period of time for optimal egg harvest

During the first hour of oviposition (Introduction of oviposition source to 1 hour) the mean number of eggs collected per box was 82.4, 28.4, 59.6, 65.8, and 85.8 corresponding to treatments T1 to T5 respectively. For the first hour of oviposition ANOVA test confirms the variability in the egg laying pattern by gravid females in different treatments F (4, 20) = 4.291, P= 0.01114). Similarly, during the second hour the mean egg laying

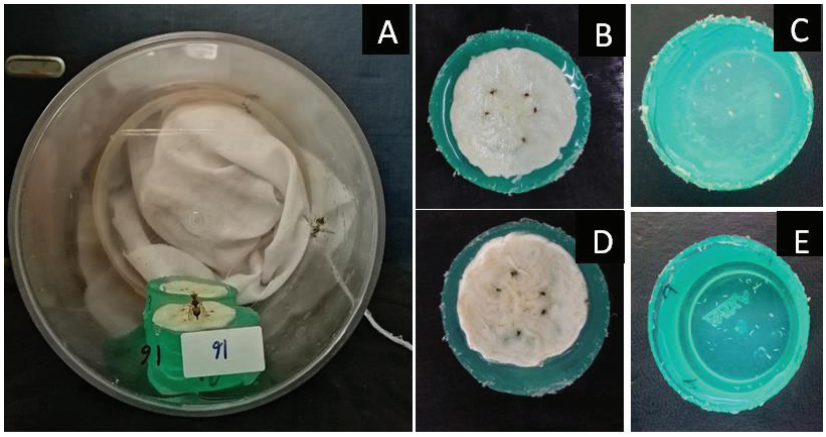

Figure 3. A. Oviposition cage with five gravid females and two oviposition cups **B**. Oviposition cup with 0.5% agar and topped with slice of banana (Method A); **C**. Eggs laid in oviposition cup (Method A) **D**. Oviposition cup with water covered with parafilm and topped with slice of banana (Method B) **E**. Eggs laid in oviposition cup

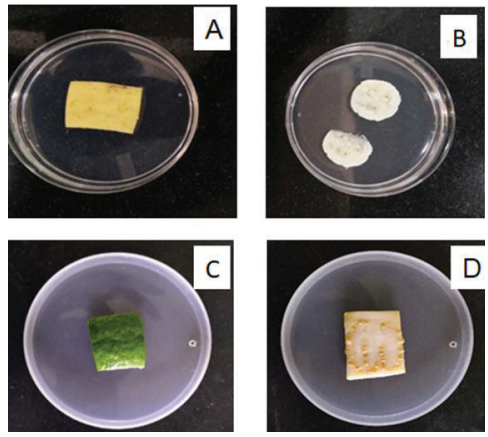

Figure 4. Substrate preference in *Bactrocera dorsalis* under laboratory conditions, **A**. Banana peels **B.** Banana pulp **C.** Guava green **D.** Guava yellow.

was 8.61, 29, 37.5, 43.5, and 60.3. During the third hour the mean egg laying was 25.8, 40, 40.6, 72.2 and 36.8 for the treatments T1 to T5 respectively (Figure 8). The ANOVA result was F (4, 20) =2.331, p=0.0911 and F (4,20) =2.585, p=0.0683 respectively for the second and third hour. The mean number of eggs laid per gravid fly in different treatments from T1 to T5 was 4.68, 4.87, 9.18, 8.3 and 5.74 respectively. In treatment T1 and T2 only 20-46% of flies responded by ovipositing in the oviposition source. In the treatments T3 to T5, 62-100% of flies responded by ovipositing during the three consecutive observation hours. The flies were also tested for their oviposition response following exposure to the oviposition source in three different time windows, during the day.

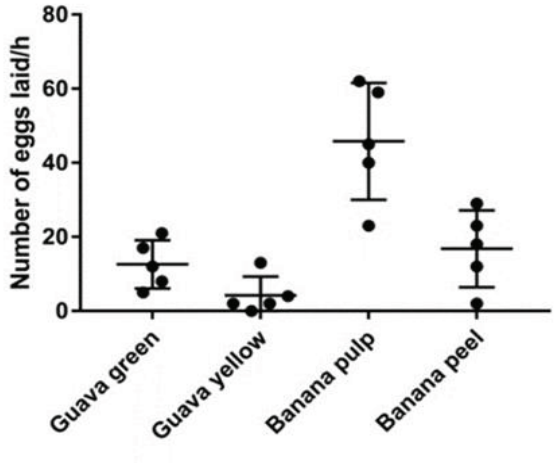

Figure 5. Host preference by B. Dorsalis: guava green, guava yellow, banana pulp, banana pulp (Kruskal wallis test: H =13.07, p=0.004)

Period of time and oviposition response

In the boxes with a single gravid female, 13.6%, 20.0% and 53.3% of the flies responded by ovipositing during the morning, afternoon, and evening hours, respectively. In boxes with five gravid females, 86.6%, 73.3% and 93.3% of the oviposition cups had eggs during morning, afternoon, and evening hours, respectively (Fig. 9). Hence, when single flies were caged, during the two hours of exposure, only half or less than half of the population responded by ovipositing and the oviposition response was greater during the evening hours. However, when a group of gravid females was housed together, the oviposition response of the flies was greater than that which was seen when the flies were housed singly. At any given time, the oviposition response was more than 70% when the flies were housed together.

Oviposition in time intervals

Oviposition in first 15 (15') minute was 47 eggs likewise 108 eggs were laid in next 15 (30') minutes interval, 61 eggs in next 15 (45') minutes interval and 75 in next 15 (60') minutes interval. The observation was

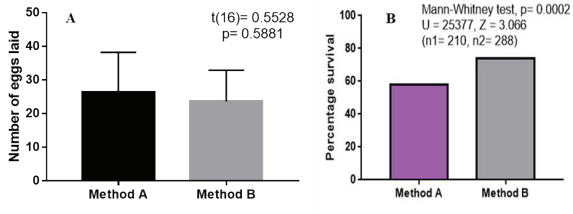

Figure 6. Effectiveness of two different methods in harvesting the eggs. **A** Average number of eggs laid by a gravid female in two different oviposition sources. **B** Survival of eggs after collection from two different oviposition cups.

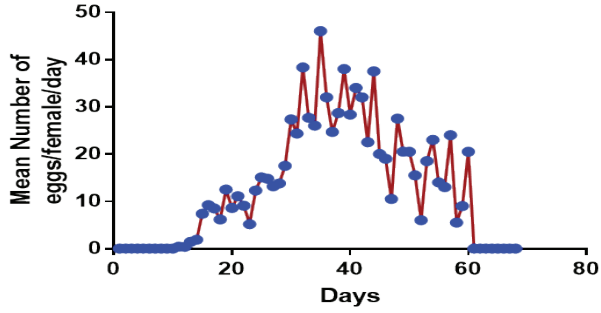

Figure 7. Oviposition pattern in *Bactrocera dorsalis* during their life span (n=18).

done within 1h. The results obtained were signifying that enough numbers of eggs were available for germline transformation at each 15-minute interval to get viable numbers of G_0 phased embryo or eggs. In corroboration to G_0 stage of embryo Wu *et al*., (2018) explained that there was more than 90 per cent mutation, when the Cas9 and sgRNA injected at G_0 phased embryos (Table 1)**.**

DISCUSSION

Bactrocera dorsalis is highly polyphagous in nature and has been recorded on about 478 different host plants (Clarke *et al*., 2005; Liquido *et al*., 2017). Selection of an appropriate host is a very critical decision for the fly as this behaviour should ensure proper egg hatching and facilitate larval development (Joseph *et al*., 2009). This host selection may be mediated by olfactory cues (Jayanthi *et al*., 2014) or by visual stimuli like the colour, size, shape of the fruit (Pinero *et al*., 2017). This host selection may be mediated by olfactory cues (Jayanthi *et al*., 2014) or by visual stimuli like the colour, size, shape of the fruit (Pinero *et al*., 2017). Thus, to harvest the required amount of eggs for the germline transformation through CRISPR – Cas9 the oviposition eliciting substrate becomes crucial.

From the present study we found that banana pulp is a most preferred substrate among the different substrate that were tested. But for experimentation purposes, recovery of the oviposited eggs from the infested whole fruits is difficult and any such attempt may damage the eggs while handling. Hence, a proper egg collection device carrying the food source or oviposition elicitor is necessary for collecting eggs quickly, without damaging them. Previously, agar gel has been used as an egg collection medium in different species such as *Drosophila melanogaster* (Lihoreau *et al*., 2016) and *Anastrepha obliqua* (Fontellas - Brandalha and Zucoloto, 2004). However, in these examples the food material was added into the agar medium prior to solidification. In our current work in order to collect eggs from the

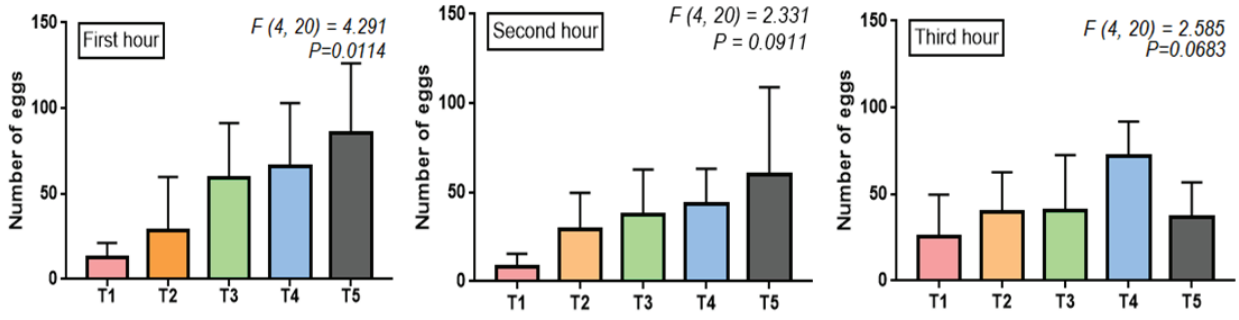

Figure 8. Egg laying in different treatments during three consecutive hours. The total number of eggs laid in 10 boxes for each treatment is plotted with its standard deviation (T1 – One female + One oviposition source; T2 – Two female + one oviposition source; T3 – three female + Two oviposition source; T4 – Four female + one oviposition source; T5 – Five female + One oviposition source).

oviposition cups/sources different methods are followed based on the object of the study. The fast collection was facilitated using method B. Collection from method A involves disruption of agar gel and picking eggs individually which involves more handling error, proportion of eggs prone to damage is high resulting in significantly high egg mortality and subsequently is time consuming. Hence, method B will be advantageous and will aid in our proposition. In nature, flies thrust their ovipositor through the skin of the fruit and deposit their eggs inside the fruit. Replicating this through a slice of banana which is less than 2mm thick allows the flies to insert the ovipositor through the slice and lay the eggs on the parafilm, rather than deep into the agar medium (method A). This enables the easy harvesting of the eggs from the parafilm surface as it is not required to dig the eggs from pulp, a process which can easily damage the eggs and render them unfit for further processes such as microinjection.

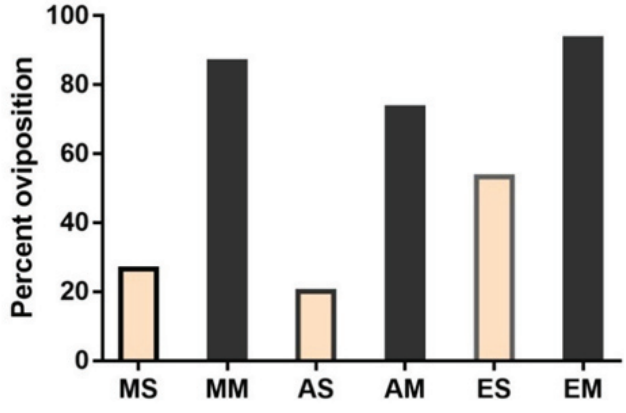

Figure 9. Period of time and egg laying behaviour in the corresponding period (First alphabet: M – Morning, A –Afternoon, E – Evening; Second alphabet: S – Single female, M – multiple female).

Further we have found that maximum egg deposition period was in the age group 20 – 40 days old which account for about 50.51% of the total egg produced during the life span of the fly. Similarly in the study conducted by Xu *et al*., 2012 maximum egg production was observed in the first eight days of egg laying when the first 27 days of egg laying period was considered. Nevertheless ovary maturation and egg development depends on the nutritional status of the flies Pelisse *et al*., 2011.Also when a group of gravid females were housed together the oviposition was accelerated, this shows the plausibility of the effect of conspecifics on the oviposition response. Similarly, Xu *et al*., (2012) found that that when the number of gravid females increased per given enclosure, the fecundity was found to decrease after a critical number of flies. The objective was to get a greater number of eggs; it was evident from our findings that five female flies per box were sufficient.

The present study results show that Yelakki banana is a good elicitor for timely collection of eggs at will, which is economical and available throughout the year. Egg collection for genetic manipulation experiments would require a minimum of around 50 gravid females, in the age group of 20-40 days old, to be housed together for oviposition. A time period of 15 min is enough to collect sufficiently large number of eggs, following which eggs can be collected repeatedly from the same set of flies, if required. In the cage housing the flies, at-least two oviposition sources should be placed, to avoid crowding and facilitate efficient oviposition by the flies.

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