DIFFERENTIAL PREFERENCES OF OVIPOSITION BY *AEDES* MOSQUITOS IN MAN-MADE CONTAINERS UNDER FIELD CONDITIONS

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Abstract. A study was made of the oviposit behavior of gravid female *Aedes* mosquitos in man-made habitats under field conditions. The study showed that the gravid female *Aedes* mosquitos preferred containers with relatively easy access but not too open to external environmental influence. The dark surface of the containers served as the initial and long-range attractant to the breeding sites. Volatile chemicals generated by the decaying vegetation in the container may serve as a close-range attractant. Finally, the water quality and the quantity of 'food' derived from decaying vegetative matter in the water determined the amount of eggs deposited in each container. The study confirmed previous findings that each gravid female *Aedes* mosquito had the tendency to lay her eggs in more than one container. However, the results of the study suggests that under favorable conditions, each gravid female *Aedes* mosquito could be encouraged to lay all her eggs in a single breeding site.

INTRODUCTION

In terms of both morbidity and mortality, dengue is the most common and widespread human arboviral infection in the world today (Gubler and Meltzer, 1999). The geographical distribution, spread, incidence as well as severity of dengue fever (DF) and dengue hemorrhagic fever (DHF) are increasing in the Americas, Eastern Mediterranean, Caribbean, Southeast Asia, and Western Pacific. Some 3 billion people live in areas potentially at risk for exposure to dengue virus infection and transmission. Annually, it is estimated that globally, there are 20 million cases of dengue infection, resulting in around 24,000 fatalities (WHO, 1966; 2000).

Both DF and DHF are due to infections by dengue viruses. Dengue virus belongs to the family Flaviviridae, under the genus *Flavivirus*. There are four serotypes of dengue virus (DEN-1, DEN-2, DEN-3, and DEN-4). They are antigenically very similar to each other but different enough to elicit only transient partial cross-protection after infection by each one of them (Henchal and Putnak, 1990; Monath, 1990). Dengue viruses are transmitted to humans through the bite of infected *Aedes* mosquitos, principally *Aedes aegypti*. *Ae. aegypti* is a tropical and subtropical species of mosquito found around the globe, usually between the latitudes 35ºN and 35ºS, approximately corresponding to a winter isotherm of 10ºC. *Ae. aegypti* is one of the most efficient mosquito vectors for arboviruses, especially dengue virus. It is highly anthropophilic and thrives in close proximity to humans. Dengue outbreaks have also been attributed to *Ae. albopictus*, *Ae. polynesiensis*, and several species of the *Aedes scutellaris* complex. Each of these species has its particular geographical distribution. So far, they have been less efficient epidemic vectors than *Ae. aegypti* (WHO, 2000). Both *Ae. aegypti* and *Ae. albopictus* are found in Malaysia, though *Ae. aegypti* is not an indigenous species (Rudnick *et al*, 1965).

Oviposition is an important component of most mosquito-borne diseases (Bentley and Day, 1989). A tremendous amount has been written concerning the oviposition of mosquitos including *Aedes* species (Bates, 1940; Angerilli, 1980; Bailey, 1981). Selection of oviposition sites by

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gravid female mosquitos is a crucial event for the survival of their species. Gravid females follow visual or olfactory cues to appropriate water collections and guided by chemical cues and physical factors in the water, assess the quality of the water before making a decision to lay their eggs (Bonnet and Chapman, 1956; Muir, 1988). The presence of aquatic predators of mosquito eggs, larvae, or pupae is a risk that some species take into account before oviposition (Lowenberger and Rau 1994; Ritchie and Laidlaw-Bell 1994; Zahiri and Rau, 1998). A large proportion of the studies have dealt with laboratory and field investigations of mosquito responses to the chemical and physical aspects of the oviposition site (Ingram, 1954; Wallis, 1954; Fay and Perry, 1965; Kitron *et al*, 1989; Chardee *et al*, 1993; Allan and Kline, 1995); but relatively few field studies have been attempted locally. This study reports the differential preferences of oviposition in man-made habitats under field conditions by *Aedes* mosquitos found in an urban area of peninsular Malaysia.

MATERIALS AND METHODS

Place and duration

An on-going project was begun to study the oviposit behavior of *Aedes* mosquitos (*Ae. aegypti* and *Ae. albopictus*) under field conditions starting on the 1st of March 2002. The study was carried out in an established residential housing estate in the city of Petaling Jaya, peninsular Malaysia (GPS co-ordinate: 03º 05.649N, 101º 37.045E; elevation: 62M).

Components of study

The study consists of three parts. The first part was to establish which was the most-preferred feature of man-made containers for oviposition in the field by the *Aedes* mosquitos found in an urban area of peninsular Malaysia. The second part was to compare the field efficiency of oviposition rate by local female gravid *Aedes* mosquitos in the identified most-favored feature of the container with the ovitrap introduced recently by the Kuala Lumpur Municipal Council. The third part of this study was to determine the influence of the container's color on the oviposition rate of these mosquitos using the identified most-favored type of container as the index container.

Man-made containers

Gallon size empty plastic containers commonly used in Malaysia to hold medicinal syrup were sourced from a private pediatric clinic in Kuala Lumpur. The containers were used to create the required man-made *Aedes* breeding habitats for this study. The containers were labeled A1, A2, B1, B2, C1, C2, D, E, F1, F2, G, H, I, J, and K as shown in Fig 1a. The containers were thoroughly washed with either rain or tap water. A variable number of openings of various shapes and sizes were made on the upper side of these plastic containers (Fig 1). A1 and A2 both had a 12 x 8 cm oval shape opening on each upper lateral part. B1 and B2 both had a similar type of opening in the same position but only on one side. C1, C2 and H all had the upper portion totally truncated. D and E both had a similar 8 x 8 cm square opening, except D had the cut piece of plastic remaining as an overhanging roof. F1 and F2 had an opening similar to B1 except the opening was directly opposite the handle of the container. G had a 10 x 8 rectangular opening on one side and I had a 22 x 3 cm opening at each upper corner. J had an opening similar to I, except only at one upper corner and K had one side of the container totally removed. A1, B1, C1 and F1 were the same type and shape of containers as A2, B2, C2 and F2 respectively, except A1, B1, C1, and F1 each contained a dried *Ptychosterma macathurii* palm leaves as shown in Fig 1a. In the second part of the study, three ovitraps (DBKL) purchased from the Municipal Council of Kuala Lumpur, Malaysia (Fig 1b) were used to compare the oviposition rate with A1 (now designated as P) and A2 (now designated as Q). During the third part of the study, the containers labeled A1 had their external surfaces painted black with black glossy paint, except the base (now designated as BLACK). These were compared with unpainted A1 (now designated as WHITE) in the oviposition rate of gravid female *Aedes* mosquitos (Fig 1c).

Approaches in the study

The clean containers were each filled with 1.5 liters of clear rainwater and placed in shaded areas within a house garden. The shaded area could either be under some garden plants or in the patio of the house. To reduce the possibility of orientation bias due to position, the containers were randomly placed together except that A1 was always placed beside A2. B1 and B2, C1 and C2 and F1 and F2 were paired, respectively. All the containers were placed in a chosen location for 2 cycles of study before a new site was chosen for the next 2 cycles. All locations chosen in this study were outside the house and within the same garden.

Examination of containers for the presence of mosquito larvae was carried out daily. The end-point for harvesting the immature mosquitos (larvae and pupae) from all containers was taken whenever an early mosquito pupa was noted in any of the containers. The larvae in each of the trapping containers were transferred into a smaller transparent plastic container fitted with a perforated screw-cap for ventilation and enumerated accordingly. The larvae in the holding transparent containers were fed with fish pellets (Azoo Co, Malaysia) until the mature adult mosquitos emerged. Preliminary identification for the species of the collected larvae was made based on the morphological characteristics of the larvae. Final identification of the species of mosquitos collected was based on the morphological characteristics of the adult mosquitos. Though both *Ae. aegypti* and *Ae. albopictus* were collected in this study, no attempt was made to count them separately. They were all counted together as *Aedes* species.

After each cycle of ovitrapping, the containers were thoroughly washed and scrubbed with mild detergent, especially the inner surface to ensure no residual eggs adherent to the surface were carried forward to the next cycle of study.

Fig 1–Plastic containers with variable numbers of openings of various shapes and sizes were used for oviposition by gravid female *Aedes* mosquitos. Fig 1a shows the types habitats for oviposition by gravid female *Aedes* mosquitos used in Study One. Fig 1b shows one of three similar sets of containers [P(A1), Q(A2), and DBKL] used in Study Two. Fig. 1c shows one of the eight similar sets of containers (BLACK and WHITE) used in Study Three.

The containers were then rinsed with clean rain or tap water and subsequently refilled with rainwater before being placed back in the respective locations but at a different orientation to reduce possible bias due to the relative position of the containers with each other. The figures on the number of immature mosquitos collected in each cycle from the respective type of containers were pooled together for analysis. Three sets of containers, each consisting of P(A1), Q(A2), and DBKL, were placed at 3 different fixed locations within the garden in the second part of the study. In the third part of the study, 8 sets of containers, each consisting of a painted black A1 (BLACK) and an unpainted white A1 (WHITE), were placed at 8 different fixed locations within the same garden. Similar procedures for ovitrapping and data analysis was carried out as in the first part of the study.

Statistical analysis

The derived data was tabulated in appropriate worksheets using the Microsoft Excel program. The differential preferences of ovipositon in various types of man-made containers by the gravid female *Aedes* mosquitos were evaluated by the chi-square test, pair *t*-test using the Statistical Program for Social Science (SPSS) and Epi Info 6 (Center for Disease Control and Prevention, Atlanta) free computer program. A p-value of 0.05 or less was taken as the level of significant difference.

RESULTS

Study one

The first part of the study took place from 25th March 2002 to 7th November 2002. Thirteen cycles of ovitrapping in 7 different sites within the same garden were carried out and the number of immature *Aedes* mosquitos collected in each respective type of container at each cycle is shown in Table 1. Statistically, A1 was the most preferred container by the gravid female *Aedes* mosquitos to deposit their eggs (Fisher exact, $p < 0.0001$). Analysis of the data based on the grouping of containers showed that containers with leaflets of *Ptychosterma macathurii* palm leaves (A1, B1, C1 and F1) were favored for oviposition compared to the containers without leaflets (A2, B2, C2 and F2) ($\chi^2 = 28.21$, $p < 0.0001$). Among the containers with leaflets (A1, B1, C1 and F1), A1 was the container of choice for the gravid female *Aedes* mosquitos for oviposition ($\chi^2 = 9.28$, df = 2, $p = 0.0258$). There was also a significant difference in the preference of the type of containers for ovipositon among containers without leaflets (D, E, G, H, I, J, and K) ($\chi^2 = 15.60$, df = 6, $p = 0.0159$).

In the analysis of the types of containers, there was a statistically significant difference in the mean number of larvae collected from the differed types of containers, where A1 had the highest mean number (Kruskal-Wallis test: $\chi^2 = 77.21$, df = 14, $p < 0.001$). There was no significant difference in the mean number of immature mosquitos among B1, C1 and F1 (Kruskal-Wallis test: $\chi^2 = 0.747$, df = 2, $p = 0.688$) but there was a significant difference in the mean number of immature mosquitos between A1 and (B1+C1+F1) (Mann-Whitney test: $z = 2.697$, $p = 0.007$). There was a significant difference in the mean number of immature mosquitos between containers with leaflets (A1+B1+C1+F1) and those without leaflets (A2+B2+C2+F2) (Mann-Whitney test, $z = 6.002$, $p < 0.001$). Among other containers without leaflets, there was no difference in the mean number of immature mosquitos among containers E, H, I, J, and K (Kruskal-Wallis test: $\chi^2 = 5.264$, df = 4, $p = 0.261$) but there was a significant difference in the mean number of immature mosquitos between containers (D+G) and (E+H+I+J+K) (Mann-Whitney test: $z = 2.754$, $p = 0.006$).

Study two

In the second part of the study (3rd October 2002 to 23rd April 2003), three different fixed sites within the garden were chosen. In each cycle of ovitrapping, different orientations for P, Q and DBKL were adopted to avoid positional biasness. The number of immature *Aedes* mosquitos collected in each container at each ovitrapping cycle is shown in Table 2. There was a highly significant difference in the preference for containers P(A1) and DBKL for oviposition compared to containers Q(A2) ($\chi^2 = 44.97$, df= 2, $p < 0.0001$). Though DBKL containers had a slightly higher number of times with immature mosquitos collected as compare to P, the difference between

Table 1
Comparison of the number of immature *Aedes* mosquitos collected in each type of man-made container in Study One.

Cycle	Date set	Date count	Days	Type of man-made container for oviposition by gravid *Aedes* female mosquitos															
				A1	A2	B1	B2	C1	C2	D	E	F1	F2	G	H	I	J	K	Total
1	25/3/2002	4/4/2002	10	18	0	17	0	11	0	0	0	4	0	0	2	0	0	0	52
2	5/4/2002	14/4/2002	9	6	2	0	4	9	5	0	3	3	0	0	2	0	5	1	40
3	18/4/2002	27/4/2002	9	2	0	0	0	12	0	0	0	10	0	0	0	0	0	0	24
4	27/4/2002	11/5/2002	13	7	0	0	0	7	0	0	0	0	0	0	0	0	0	0	14
5	12/5/2002	30/5/2002	18	18	0	16	0	0	0	0	0	14	0	0	18	40	0	31	137
6	5/6/2002	17/6/2002	12	38	3	12	3	0	0	0	0	0	0	0	0	5	11	17	89
7	27/6/2002	14/7/2002	17	11	0	13	2	0	0	0	0	10	0	0	0	0	0	0	36
8	27/7/2002	14/8/2002	17	5	0	8	0	0	0	0	0	0	0	0	0	0	0	0	13
9	25/8/2002	2/9/2002	8	29	2	0	0	31	0	0	0	0	0	0	0	0	0	0	62
10	4/9/2002	13/9/2002	9	7	0	2	2	0	3	0	0	1	3	0	3	0	3	1	26
11	14/9/2002	8/10/2002	24	3	0	3	0	4	0	0	0	6	0	0	0	0	0	9	24
12	10/10/2002	23/10/2002	14	11	0	5	0	0	0	0	0	4	0	0	0	0	0	0	20
13	27/10/2002	7/11/2002	11	16	0	6	0	0	0	0	0	19	0	0	0	0	0	1	42
Total number of immature mosquitos				171	7	82	11	74	8	0	3	71	3	0	25	45	19	60	579
Number of cycle with immature mosquito				13	3	9	4	6	2	0	1	9	1	0	4	2	3	6	

Days = Total number of days taken for the first appearance of pupa in any of the container in each study cycle.

the two was not significant (Fisher exact, $p = 0.4364$). In terms of the quantity of immature mosquitos collected in each group of containers, there was a significant difference in each group (Kruskal-Wallis test: $\chi^2 = 38.71$, df = 2, $p < 0.001$). The mean for group P was higher than the mean for group DBKL (Mann-Whitney test: $z = 3.447$, $p < 0.001$) or the mean for group Q (Mann-Whitney test: $z = 4.984$, $p < 0.001$)). The mean group DBKL was higher than the mean for group Q (Mann-Whitney test: $z = 4.986$, $p < 0.001$).

Observation showed that whenever immature mosquitos were found in container P, similar immature mosquitos would be found in paired DBKL containers but not vice versa. Similarly, not a single occasion was noted where an immature mosquito was found in container Q without the presence of immature mosquitos in the paired containers DBKL and P.

Study three

The third part of the study was carried out from the 3rd of December 2002 to the 23rd of April 2003, with twelve cycles of ovitrapping. The number of immature *Aedes* mosquitos collected in the respective BLACK and WHITE containers is shown in Table 3. BLACK containers were preferred more than WHITEs by the gravid female *Aedes* mosquitos for oviposition (Mantel-Haenszel: $\chi^2 = 5.42$, $p = 0.0200$) though both were of the same shape and contained leaflets of *Ptychosterma macathurii* palm leaves. A significantly higher mean number of immature *Aedes* mosquitos were collected in the BLACK containers than the WHITEs (Mann-Whitney test: $z = 3.878$, $p < 0.001$). Whenever immature mosquitos were found in container WHITE, similar immature mosquitos would be found in the paired container BLACK but

Table 2
Comparison of number of the immature *Aedes* mosquitos collected in each type of man-made container in Study Two.

Cycle	Date Set	Date Count	Days	Number of immature mosquitos			
				P (N)#	Q (N)	DBKL (N)	Total
1	3/10/2002	12/10/2002	9	49 (3)	14 (3)	34 (3)	97
2	12/10/2002	23/10/2002	11	54 (2)	8 (2)	52 (3)	114
3	27/10/2002	7/11/2002	11	51 (3)	0 (0)	29 (3)	80
4	9/11/2002	19/11/2002	10	83 (3)	19 (2)	53 (3)	155
5	20/11/2002	29/11/2002	9	110 (3)	4 (2)	52 (3)	166
6	3/12/2002	12/12/2002	9	69 (3)	12 (2)	63 (3)	144
7	14/12/2002	22/12/2002	8	85 (3)	3 (1)	79 (3)	167
8	23/12/2002	1/1/2003	9	92 (3)	19 (3)	70 (3)	181
9	2/1/2003	11/1/2003	9	96 (3)	12 (2)	52 (3)	160
10	12/1/2003	22/1/2003	10	40 (1)	0 (0)	38 (2)	76
11	26/1/2003	5/2/2003	10	36 (2)	0 (0)	20 (2)	56
12	8/2/2003	19/2/2003	11	99 (3)	0 (0)	32 (3)	131
13	21/2/2003	2/3/2003	9	85 (3)	12 (1)	20 (3)	117
14	5/3/2003	16/3/2003	11	101 (3)	6 (2)	44 (3)	151
15	17/3/2003	29/3/2003	12	107 (3)	3 (1)	44 (3)	154
16	2/4/2003	11/4/2003	9	132 (3)	10 (1)	49 (3)	191
17	13/4/2003	23/4/2003	10	61 (2)	5 (1)	38 (3)	104
Total				1,350 (46)	127 (23)	769 (49)	2,246

Days = Total number of days taken for the first appearance of pupa in any of the containers in each study cycle.
(N)# = Number of containers with immature mosquitos collected.

Table 3
Comparison of the number of immature *Aedes* mosquitos collected in each type of man-made container in Study Three.

Cycle	Date Set	Date Count	Days	Number of immature mosquitos		
				BLACK (N)#	WHITE (N)	Total
1	3/12/2002	12/12/2002	9	101 (8)	72 (8)	173
2	14/12/2002	22/12/2002	8	142 (8)	84 (8)	226
3	23/12/2002	1/1/2003	9	104 (7)	53 (6)	157
4	2/1/2003	11/1/2003	9	164 (8)	61 (6)	225
5	12/1/2003	22/1/2003	10	175 (7)	52 (5)	227
6	26/1/2003	5/2/2003	10	141 (7)	48 (4)	189
7	8/2/2003	19/2/2003	11	204 (8)	121 (7)	325
8	21/2/2003	2/3/2003	9	125 (5)	26 (5)	151
9	5/3/2003	16/3/2003	11	151 (7)	51 (5)	202
10	17/3/2003	29/3/2003	12	178 (5)	49 (4)	227
11	2/4/2003	11/4/2003	9	168 (5)	52 (4)	220
12	13/4/2003	23/4/2003	10	116 (4)	24 (3)	140
Total				1,769 (79)	693 (65)	2,462

Days = Total number of days taken for the first appearance of pupa in any of the containers in each study cycle.
(N)# = Number of containers with immature mosquitos collected.

not vice versa.

The results of this study showed that the mean number of days from the day of setting up the ovitraps to the day of the first appearance of pupae was 13.2 days (range 8 to 24, SD = 4.71), 9.8 days (range 8 to 12, SD = 1.07) and 9.8 days (range 8 to 12, SD = 1.14) for Study One, Study Two and Study Three, respectively. Observations showed that the newly formed pupae were of light brownish color and they took at least another day to turn dark brown before the adult mosquitos emerged from the pupal casings. Both *Ae. aegypti* and *Ae. albopictus* were obtained in this study but no attempt was made to enumerate each species separately.

DISCUSSION

It is presently accepted by most researchers that ovipositing mosquitos do not lay their eggs indiscriminately and that restriction of breeding habitats of a particular species to a given habitat is largely a result of selection preference guided by the instinct of the particular ovipositing gravid female mosquitos. Research into the manner by which culine and aedine mosquitos find the proper sites for oviposition has indicated that one or more responses may be chemically regulated (Dethier *et al*, 1960). Demina and Nikolskii (1928) reported that breeding areas containing high calcium content were preferred by the ovipositing females of *Anopheles maniculipennis*. Bates (1960) obtained similar results with calcium when tested with several species of *Anopheles* mosquitos. He also found that dark background colors were preferred by anophelines. Woodhill (1941), and Wallis (1954) reported that salinity and odor influenced the oviposition of *Ae. aegypti*. O'Gower (1963) using infusions of horse manure and dark surfaces reported that the *Ae. aegypti* tactile and visual responses were the most important stimuli for oviposition. Gjullin *et al* (1961) showed that grass infusions and log pond water increase the oviposition of both *Ae. aegypti* and *Culex pipiens quinquefasciatus* in comparison with distilled water. Their evidence indicates that chemical agents influence oviposition. Hazard *et al* (1967) demonstrated that bacteria in the hay infusions are responsible for the production of these chemicals. Torres-Estrada *et al* (2001) reported gravid *Ae. aegypti* females were attracted to oviposit in sites that contained or previously held *Mesocyclops longisetus* and suggested that volatile compounds, monoterpene and sesquiterpene, derived from these copepods were the more likely attractants.

It is evident from our study that a number of factors and stimuli are responsible for the selective preferences of gravid female *Aedes* mosquitos in the quantity and location of oviposition. Evidence from previous studies and our present study indicates that *Aedes* mosquitos most likely use visual stimuli in initially seeking breeding sites. Containers with a dark surface preferred. As they move closer to breeding containers, olfactory stimuli may come into play in their selection of oviposition. Volatile chemical agents released from decaying vegetation or bacteria contained in the water act as the attractant (Hazard *et al*, 1967; Saxena and Sharma, 1972; Ritchie, 1984; Reisen and Meyer, 1990; Reiter *et al*, 1991). After they have landed on the breeding sites, tactile and other stimuli that are able to assess the quality of the water and the quantity of food that will be available to support their progeny determine the number of eggs deposited in each container (Bentley and Day, 1989). In our Study One, the type, shape and number of openings of the containers affected the choice of oviposition. This may be related to the accessibility and protective effect conferred by each form of container. Containers with limited access, such as D+E (A1 more than B1 or F1) and those that are too open (C1, C2, H) to environmental influences with the tendency to be overfilled during heavy rainfall, were not preferred. The function of *Ptychosterma macathurii* palm leaves used in containers A1, B1, C1 and F1 possibly provided not only initial landing sites for the gravid female mosquitos to deposit their eggs, but the decaying leaves also provide a necessary chemical attractant and 'food' that supports the development of immature forms. In Study Two, immature mosquitos were collected slightly more frequently in DBKL containers than P(A1) containers (though statistically not significance). The black colored caps of the DBKL containers may have played on initial role in attracting the gravid females to them but the olfactory and tactile stimuli subsequently determine the number of eggs to be deposited in P(A1) containers. The above hypothesis is supported by the findings obtained in Study Three.

Previous studies showed that each gravid female *Aedes* mosquito has the tendency to lay her eggs in more than one container or breeding site (Kitron *et al*, 1989; Reiter *et al*, 1995; Zahiri and Rau, 1998). The results of this study supported these findings. The probable reason is that the breeding habitats of *Aedes* species, unlike *Anopheles* and *Culex* mosquitos, whether in nature (tree holes, leaf axils, curled-up dry leaves, etc) or in man-made containers (tires, plastic containers, vases, etc) are not large enough to hold sufficient quantities of water and food to sustain a single brood of progeny. However, if this condition is rectified, each gravid female *Aedes* mosquito may be encouraged to lay all her eggs in a single favorable site. This may have been the case in Study Two where no immature mosquitos were collected in a total of 26 (49-23) Q(A2) containers (Table 2) when placed together with DBKLs and P(A1)s. Likewise, in Study Three, a total of 14 (79-65) WHITE containers did not have any immature mosquitos while immature mosquitos were collected in their respective paired BLACK containers. A gravid female could have been attracted to the BLACK container, finding it suitable to lay all her eggs in it rather than moving to another container.

In conclusion, for those who are interested in designing effective ovitraps for *Ae. aegypti* and *Ae. albopictus*, many factors need to be taken into consideration such as color, size, shape and the opening of the ovitraps. For efficient optimal ovitrapping, factors need to be taken into account include the quality of water, type and quantity of 'food' in it that will attract the gravid female *Aedes* mosquitos to deposit as many eggs as possible in the ovitrap.

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