# Influence of breeding site availability on the oviposition behaviour of *Aedes aegypti*

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*Despite the importance of the mosquito* Aedes aegypti *in the transmission of arboviruses, such as yellow fever, Chikungunya fever and dengue fever, some aspects of their behaviour remain unknown. In the present study, the oviposition behaviour of* Ae. aegypti *females that were exposed to different densities of breeding sites (2, 4, 8 and 16) was evaluated in laboratory and semi-field conditions. The number of breeding sites that were used was proportional to the number available, but tended towards stabilisation. Females used four-six breeding sites on average, with a maximum of 11. A high percentage of eggs was observed in the water, along with the presence of a breeding site termed "favourite", which received at least 40% of the eggs. The results are discussed in ecological, evolutionary and epidemiological approaches.*

Key words: *Aedes aegypti* - oviposition - dengue - animal behaviour

The mosquito *Aedes (Stegomyia) aegypti* (Linnaeus, 1762) is the primary vector of urban yellow fever and Chikungunya virus in Brazil (MS/SVS 2009, 2014) and of dengue virus throughout the world (WHO 2009). Dengue fever is a major public health problem worldwide and approximately 2.5 billion people are living in areas at risk of infection. Because no specific antiviral therapy or vaccines are commercially available, the only applicable dengue prevention strategy is vector control (WHO 2009).

Oviposition site selection by mosquitoes is a critical factor in their life history (Bentley & Day 1989) because their immature forms are unable to move to other breeding sites if the conditions become unfavourable (Onyabe & Roitberg 1997, Spencer et al. 2002). Thus, the females of the Culicidae family may enhance the development and survival of their immature forms by selecting adequate breeding sites through the perception of physical and chemical stimuli (Bentley & Day 1989, Navarro-Silva et al. 2009).

The mosquito *Ae. aegypti* usually lays eggs on the oviposition site wall, just above water level, generally in man-made containers that are located around cities (Fay & Perry 1965, Reiter 2007). The preferred containers for the deposition of eggs are of large volume (Harrington et al. 2008), dark coloured and contain stagnant water with a low concentration of decomposing organic matter, although the infusion of some plants may have attractive effects (Consoli & Lourenço-de-Oliveira 1994, Colton et al. 2003, Wong et al. 2011). In addition, *Ae. aegypti* females

can enhance the development and survival of their immature forms by selecting egg-laying sites that reduce exposure to parasites (Zahiri et al. 1997), predators (Pamplona et al. 2009) and competition (Chadee et al. 1990, Zahiri & Rau 1998, Seenivasagan et al. 2009) or increase access to food (Allan & Kline 1995, Ponnusamy et al. 2008).

The females of this vector also exhibit the "skip oviposition" behaviour, which comprises the distribution of the eggs at several breeding sites. Such findings have already been observed under both laboratory (Fay & Perry 1965, Chadee et al. 1990, Corbet & Chadee 1993, Chadee 2010) and field conditions (Chadee & Corbet 1987, Apostol et al. 1994, Reiter et al. 1995, Colton et al. 2003). Although this behaviour has been identified in *Ae. aegypti*, it is not known whether this behaviour is a strategy to avoid high densities of immature forms at breeding sites where food can be limited or to minimise the risks that are associated with temporary breeding sites (Reiter 2007).

The skip oviposition behaviour can result in vector dispersal when appropriate breeding sites are not found (Reiter et al. 1995, Edman et al. 1998). Thus, the removal of oviposition breeding sites, a common practice in dengue control programs, may not be the most appropriate strategy. When highly productive containers are no longer available, female mosquitoes choose suitable alternative receptacles based on the availability of food or sun exposure (Wong et al. 2011) and the search for new oviposition sites may result in the female's dispersion and the subsequent spread of disease (Reiter et al. 1995, Edman et al. 1998).

Although knowledge about the oviposition habits of *Ae. aegypti* is important for adequate control strategies, many aspects of this life history trait are still poorly understood (Madeira et al. 2002, Wong et al. 2011). The present study investigates some aspects of the skip oviposition behaviour of *Ae. aegypti* females that can contribute to the planning of vector control strategies, as it expands the knowledge of the dispersion mechanisms of the species. To do so, we investigated the number of

breeding sites that each female uses to lay the eggs and how this distribution occurs with different amounts of available oviposition sites and in different conditions: laboratory and semi-field.

## MATERIALS AND METHODS

*Maintenance of the Ae. aegypti mosquito in the laboratory* - A colony of *Ae. aegypti* was maintained in the Laboratory of Chemical Ecology of Vector Insects of the Federal University of Minas Gerais from 2007. The tests were conducted between 2008-2009.

The breeding room was kept at a temperature of 27 ± 1°C, 75-80% relative humidity (RH) and a 12L:12D photoperiod (Eiras & Jepson 1991). The larvae were kept in plastic tubs containing water (approximately 5 cm deep) and fed reptile food (ReptoLife®, Alcon, Brazil) until they reached the pupa stage. The pupae were collected daily and transferred to adult screened cages (Bugdorm-1, Mega View Science Education Services, Taiwan) (30 × 30 × 30 cm) and fed 10% sucrose solution. A selection cage was used to separate the gravid females that were used in the assays. Blood meal was prepared using chicken blood (*Gallus domesticus*), which was obtained from a slaughterhouse, including the anticoagulant heparin (Gomes et al. 2006). *Ae. aegypti* females (F6 and F7) of five-10 days of chronological age and three days after blood meal (Gomes et al. 2006) were used in the experiments.

*Experimental area* - The tests were performed in laboratory and semi-field conditions. In laboratory conditions, four transparent acrylic boxes (150 × 50 × 41 cm) were kept in a room with monitored temperature and humidity conditions (27 ± 3°C, 60-90% RH) and a photoperiod of 12L:12D (Eiras & Jepson 1991). The semi-field tests were performed in an experimental area of 14.0 × 7.0 × 3.5 m, where eight cages (2.5 × 2.5 × 2.0 m each) were installed (Roque & Eiras 2008). The temperature, RH and the photoperiod ranged according to the external environment and were monitored with a Thermo-Hygrometer. The tests were considered valid only when the temperature reached a minimum of 25°C (Roque & Eiras 2008).

*Bioassays* - The tests evaluating the pattern of distribution of eggs by *Ae. aegypti* females were divided into two experiments. The females were evaluated in laboratory conditions in experiment 1 and in semi-field conditions in experiment 2.

Experiment 1 (laboratory) was performed in the four acrylic boxes in which four different densities of breeding sites (2, 4, 8 and 16) were simulated. In each box, a single gravid female of *Ae. aegypti* was released and allowed to remain for 96 h in the interior of the box. The breeding sites were ovitraps that were set with 300 mL of tap water and a rectangular wooden paddle (12 cm × 3 cm) (Fay & Perry 1965, Fay & Eliason 1966). For each density level, 12 repetitions of the experiment were performed. The positions of the breeding sites were kept constant (Fig. 1). Sugar solution (10%) absorbed into cotton was provided as food to the insects during the experimental processes.

After 96 h, the paddle and the water that were present in each trap were collected, labelled and taken to the laboratory. To verify the distribution of eggs at each breeding
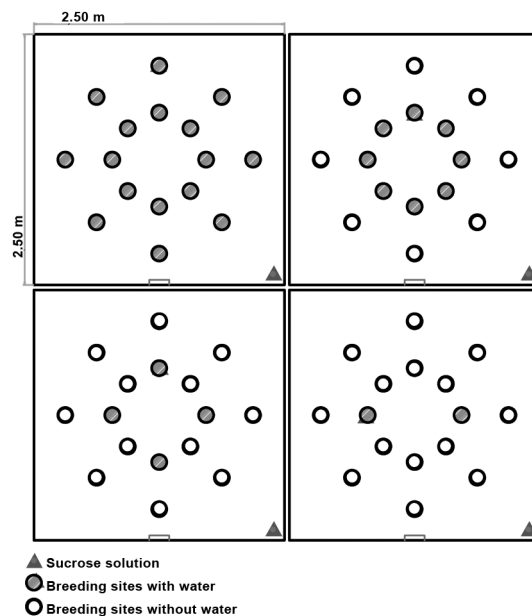


Fig. 1: representation of the distribution of the different densities (2, 4, 8 and 16 breeding sites) and the positions of the breeding sites in the interior of the acrylic boxes. In the centre, position of the sugar solution.
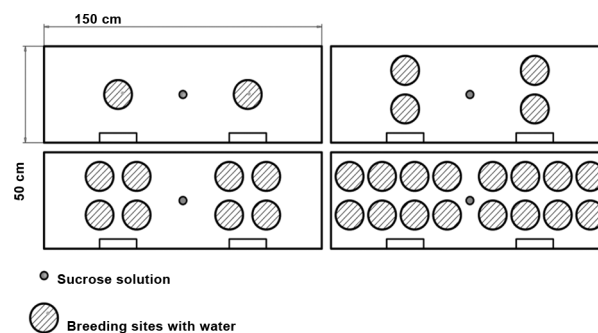


Fig. 2: diagram of the distribution of the ovitrap with and without water of each treatment/density of breeding sites.

site, the number of eggs that were present in the water and in the paddle of each ovitrap was counted using a manual counter and a stereoscopic microscope (20 ×).

Experiment 2 (semi-field) was conducted in the interior of the four fabric cages (2.5 × 2.5 × 2.0 m) (Roque & Eiras 2008). The same densities (2, 4, 8 and 16 ovitraps) that were evaluated in the laboratory conditions were evaluated in this environment. For each density, 15 repetitions of the test were performed. The positions of the breeding sites were kept constant and the same methodology as used in experiment 1 was followed. However, to avoid visual effects of the treatment itself, each cage received all 16 of the ovitraps, but only the test traps (densities 2, 4, 8 or 16) received water (Fig. 2).

*Statistical analyses* - For statistical analyses, the specific locations of egg deposition (water and paddle) were called habitats and the local of performance of the experiment (laboratory or semi-field) was called environment.

To compare the number of eggs that were deposited in each environment/density, data were submitted to a normality test at a significance level of 95%. Means with a normal distribution were compared by ANOVA. When the distributions were not in accordance with the criteria of normality, medians were compared using the Kruskal-Wallis test.

The proportions of eggs that were deposited on water by each single female (nº eggs on the water/nº total eggs) at the different densities of breeding sites both in laboratory and semi-field conditions were compared by two-factors ANOVA. Before analyses, the data were transformed in arcsin(sqrt) to normalise the distribution and stabilise the variances.

The number of colonised breeding sites in relation to the amount of available ones, both in laboratory and semi-field conditions, was compared by the Kruskal-Wallis test.

By investigating the female groups above and below the regression lines, we obtained two groups: those that colonise various breeding sites and those that colonise few sites. We compared these two groups by the Mann-Whitney *U* test to determine whether there was any difference between the groups with higher and lower frequencies of colonised breeding sites and the proportion of eggs that were deposited in the preferential breeding site. The R program (available from: R-project.org) was used to analyse the results.

### RESULTS

*Environment (laboratory or semi-field) of the experiment and egg deposition* - The oviposition behaviour of *Ae. aegypti* was maintained in both environments. Under laboratory conditions, the mean number [± standard deviation (SD)] of eggs that were laid by one *Ae. aegypti* per repetition was 73.9 (± 25.64), ranging between 40-142 eggs. Similarly, under semi-field conditions, the mean number (± SD) of eggs that were laid by one *Ae. aegypti* per repetition was 72.2 (± 20.57), ranging between 41-123 eggs.

On average, females in both the experimental environments distributed the same number of eggs and colonised the same number of breeding sites (ANOVA p > 0.05). Therefore, these oviposition behaviours do not seem to be influenced by changes in the tested environments.

*Oviposition on the water* - The tested females exhibited a strong tendency to deposit their eggs on the water of the ovitrap. The mean proportion of eggs that were laid on the water surface tended to be higher than that on the paddle when the number of available breeding sites was greater (Fig. 3). Furthermore, the "water" habitat received significantly more eggs in semi-field than in laboratory conditions (ANOVA p < 0.05) (Fig. 4).

*Breeding sites colonised in relation to available ones* - The availability of breeding sites directly influenced the dispersion of eggs by females. There was a significant difference (p < 0.05) between the densities
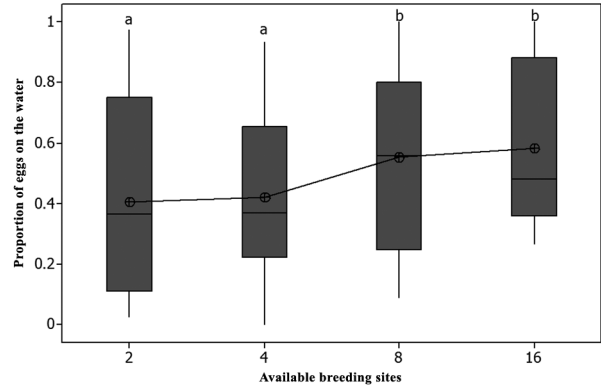


Fig. 3: box plot of the proportion of eggs on the water in different density of oviposition breeding sites. Different letters mean statistical difference (ANOVA p < 0.05).
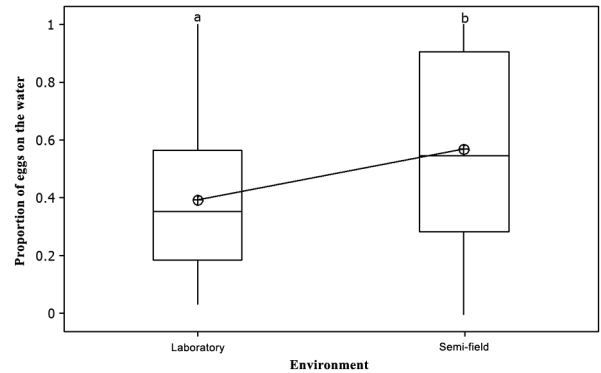


Fig. 4: box plot of the number of eggs laid on water and paddle in semi-field and laboratory conditions. Different letters mean statistical difference (ANOVA p < 0.05).

2*4, 2*8, 2*16 and 4*16 both in laboratory and semi-field conditions (Fig. 5). Most insects colonised four-six breeding sites whenever available. Furthermore, females rarely used more than seven breeding sites. One female dispersed the eggs among 11 breeding sites, the highest number observed in the study. Skip oviposition behaviour, although widely used, may not occur, as observed in eight (7.4%) females.

*Breeding sites colonised and number of eggs deposited on the "favourite" one* - By counting the number of eggs at each breeding site, we noticed that one of the sites usually received most of the eggs (40% or more of the total deposited eggs). Although all the breeding sites were identical and in semi-field and were not statistically different from each other, it one site generally received more eggs than the others. This breeding site was noted as the favourite breeding site.

The amount of eggs that were deposited at the "favourite" site was similar in both laboratory and semi-field

Fig. 5: mean and standard deviation of breeding sites colonised on the basis of available ones. Different letters mean statistical difference (Kruskal-Wallis $p < 0.05$).

conditions. The average percentage of eggs that were deposited in the favourite site was significantly higher with a density of two breeding sites and did not vary among the other densities (Fig. 6), showing that females tend to aggregate more eggs when there are only two available breeding sites. This number can be reduced by an increase in the dispersion among several breeding sites. There was a negative relationship between the number of colonised breeding sites and the percentages of eggs that were laid at the favourite breeding site (simple linear regression $y = -0.0682x + 0.9256$, $F_{1,106} = 65.9$, $R^2 = 0.38$, $p < 0.001$) (Fig. 7). However, even those females that used many breeding sites seemed to deposit at least 40% of their eggs at the favourite breeding site, as shown by the dashed line (Fig. 7). Nevertheless, comparing the percentages of eggs that were laid by the *Ae. aegypti* females of the two groups (above and below the regression lines, Fig. 8), a significant difference was observed in both semi-field ($t = -6.62$, $p < 0.001$) and laboratory ($t = 2.87$, $p = 0.001$) (Fig. 8) conditions.

In other words, both environments (laboratory and semi-field) showed higher percentages of eggs laid at favourite breeding sites by females those that distributed their eggs in small numbers of containers. Although all the breeding sites were identical and in semi-field they were not statistically different from each other, one site generally received more eggs than the others. This breeding site was denominated as the favourite breeding site.

## DISCUSSION

The results that were obtained under laboratory and semi-field conditions were similar, showing that the general aspects of the oviposition behaviour were maintained despite the individual variations that were observed between the tested females (Wong et al. 2012). The variation in the number of eggs per female is common in ex-

periments that assess a single gravid *Ae. aegypti* female (Chadee et al. 2002). These variations are probably due to the blood-feeding efficiency (Xue et al. 2008) and the size of the adults, which may interfere with the reproductive capacity of males and females (Blackmore & Lord 2000, Ponlawat & Harrington 2009).

The average number of eggs was similar to that found by other authors (Christophers 1960, Madeira et al. 2002, Rey & O'Connell 2014). As expected, the average number of eggs that were laid did not significantly differ between densities (2, 4, 8 and 16 breeding sites) or between environments (laboratory or semi-field). This result is probably due to the duration of the tests, which were performed between the third-seventh day after the consumption of blood meal, during which the females laid almost all of the eggs that were produced (Gomes 2006, Reiter 2007, Chadee 2010).

It was demonstrated for the first time that the behavioural response of females depended on the resources (number of breeding sites) that were provided to them. In an experiment with eight available containers, Chadee (2010) noted that 87% of females used one to four breeding sites, with a maximum of seven. In our study, the number of containers that were used by the females increased following the increased availability of breeding sites under both laboratory and semi-field conditions. However, the number of breeding sites that were colonised seemed to stabilise at around five, even when there were 16 breeding sites available and reached a maximum of 11 under semi-field conditions. This result has epidemiological importance, as the search for breeding sites seems to be a crucial factor in the dispersal of the females and hence the diseases that they transmit (Edman et al. 1998, Honório et al. 2003, Costa-Ribeiro et al. 2007, Wong et al. 2012). The determination of the average and maximum number of breeding sites that
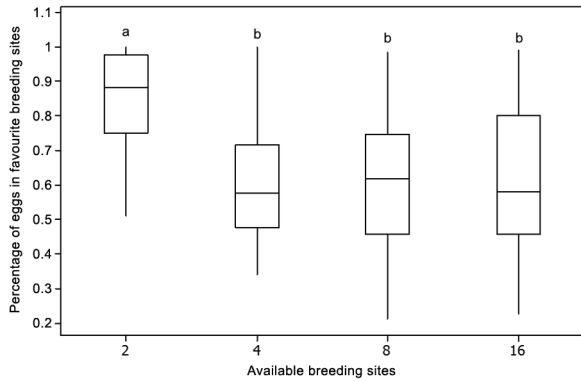
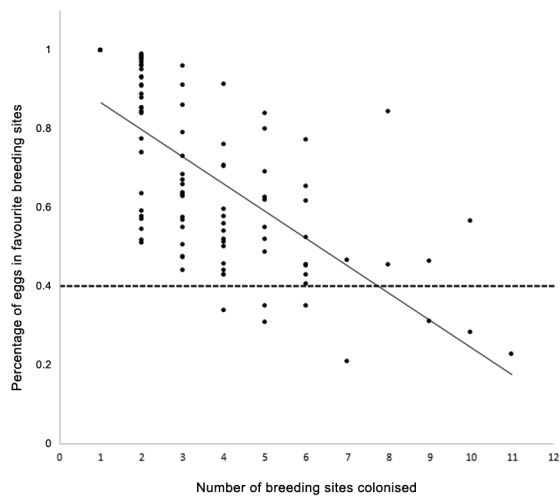Fig. 6: box plot of the percentage of eggs laid in the favourite breeding.



Fig. 7: percentage of eggs laid on favourite breeding site in relation to the number of colonised breeding sites under laboratory and semi-field conditions.
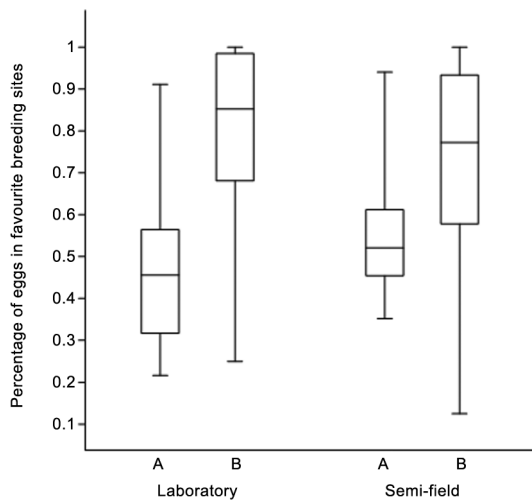


Fig. 8: percentage of eggs laid in the favourite breeding site in laboratory by *Aedes aegypti* female's who had higher frequency of breeding site colonisation (A) and smaller frequency (B). Percentage of eggs laid in the favourite breeding site in semi-field by *Ae. aegypti* female's who had higher frequency of breeding site colonisation (A) and smaller frequency (B).

were used by each female can aid in the development of methodologies for monitoring and controlling the vector. Therefore, further studies should be conducted to verify this behaviour in other environments and with different densities of breeding sites, given that few females colonised more than eight ovitraps.

The results of the present study support the existence of the "skip oviposition" behaviour in *Ae. aegypti* females, as previously observed (Christophers 1960, Fay & Perry 1965, Corbet & Chadee 1993, Apostol et al. 1994, Reiter et al. 1995, Honório et al. 2003, Reiter 2007, Chadee 2010). The ability of *Ae. aegypti* females to distinguish potential breeding sites that will sustain the survival of their offspring during their development is a crucial factor in the life cycle of mosquitoes (Zahiri & Rau 1998). The selective pressure in favour of the females that make choices that may maximise the survival of their offspring (Reiter 2007, Harrington et al. 2008) justifies the existence of "skip oviposition" behaviour. Nevertheless, this behaviour does not occur sometimes, as observed in a few females in this study (7.4% of females) and by other authors (Harrington & Edman 2001, Chadee 2010).

The large proportion of eggs that were laid on water contradicts the findings of most authors, who reported that the number of eggs that were deposited on water is negligible compared to that deposited on the walls, filter paper or paddles of the breeding site (Chadee & Corbet 1987, Chadee et al. 1995, Silva et al. 2003). However, a study in Brazil demonstrated a large number of eggs that were deposited on water by females of two populations and at different humidities. The obtained figures were 42.9% and 57.3% (80% RH) and 61.4% and 63.2% (51% RH) for populations L and B, respectively (Madeira et al. 2002). These findings were similar to the observations of the present work and almost 10 times higher than those reported by other authors (Chadee & Cobert 1987, Chadee et al. 1995, Silva et al. 2003, Rey & O'Connell 2014, Soares et al. 2015). Furthermore, Madeira et al. (2002) observed behavioural plasticity in the oviposition of *Ae. aegypti* because females of the same population distributed their eggs in different available proportions in oviposition substrates, as observed in our study.

Considering the behavioural plasticity of *Ae. aegypti*, it is possible that the oviposition in both environments (water or paddle/side walls of the breeding site) can be advantageous depending on the circumstance.

In anthropic environments, females can lay eggs in a large range of ephemeral containers that are very susceptible to disturbance (Reiter 2007). This behaviour can make the choice of laying most of their eggs out of water relevant in places that can only be achieved by increasing the level of the water and can offer more chances of the larvae hatching and reaching adulthood. Furthermore, deposition on the walls of the breeding sites may be an example of "germ banking" for the future of the *Ae. aegypti* population (Tsunoda et al. 2010). In unfavourable conditions, the maintenance of "germ banking" until the return of favourable periods may be more advantageous to the offspring than the immediate eclosion of the larvae.

On the other hand, the behaviour of laying eggs directly on the water is also relevant to the vector from an epidemiological perspective, as it helps to maintain their

populations when there is no rainwater to supply breeding sites, thus allowing the existence of floating populations of mosquitoes during the dry season (Gomes et al. 2006, Soares et al. 2015). The deposition of eggs on water can also prevent predation by ants and cockroaches and the reduction of viability due to delays in hatching (Madeira et al. 2002). This result could perhaps explain why the number of eggs on the water was higher in semi-field conditions, where the eggs would be more exposed to these attacks. The increase in the number of eggs on water as observed in this study could also be explained by the abundance of resources (breeding sites). The quick hatching of the larvae would increase the chances of the offspring finding the same available resources.

However, to be considered a viable strategy, the eggs that were laid on the water surface must have hatching rates similar to those that were laid outside of the water. Unfortunately, this study did not evaluate these rates. The literature is controversial on this issue because some authors have reported an extremely low hatching rate (2%) (Silva et al. 2003), while others have found higher rates, such as from 47-53% (Madeira et al. 2002) and from 73-80% (Rey & O'Connell 2014). Therefore, we believe that the deposition of eggs on the water may be, at least in certain cases, a good choice for the survival of *Ae. aegypti*.

Our results are also relevant in the context of the strategies of monitoring vector populations. In Brazil, ovitraps can be used to obtain the indices of infestation (ovitrap positivity index = number of ovitraps positive × 100/number of ovitraps inspected and egg density index = number of eggs/number of positive ovitraps) by counting the number of eggs on the paddle of the installed ovitraps (Gomes 1998, MS/SVS 2009). In these surveys, the liquids (water or infusion grass) that are present in the ovitraps are discarded without performing any type of analysis. However, Madeira et al. (2002) and the present study, both conducted in Brazil, found high amounts of eggs in the ovitrap water. If these results are similar to other Brazilian populations of *Ae. aegypti*, this may contribute to the generation of lagged indices. Additional studies should be performed to determine if this trend is maintained in other populations of the country and under field conditions.

The present work demonstrated for the first time the existence of a favourite breeding site and showed that egg distribution was not equal. The term favourite breeding site was used to denote the breeding site that received the highest percentage of eggs. This site was observed in semi-field and in laboratory conditions and with different amounts of available breeding sites.

The *Ae. aegypti* females usually choose the most productive containers to deposit most of their eggs. These containers are usually large, dark and unmanaged (Maciel-de-Freitas & Lourenço-de-Oliveira 2011, Wong et al. 2012). In the present study, this behaviour was maintained even when all of the breeding sites were identical. There are two possibilities to explain this result. Either the females were able to recognise clues that were insignificant to us or this was such an intrinsic behaviour that it was maintained even when there was no advantage among the breeding sites.

It is not known if the females lay their eggs in a single visit to the breeding site or if the breeding site receives a portion of the eggs and then the female returns to the same place and deposits eggs again.

The observations on the proportion of eggs demonstrated the existence of two distinct patterns: (i) females lay many eggs at one favourite breeding site (40% or more) and spread the remainder at other breeding sites and (ii) females lay few eggs (less than 40%) at the favourite breeding site and display a greater potential for spreading the eggs remaining over other breeding sites. These results are similar to those found by Wong et al. (2012), who observed an increase in the distribution of eggs in semi-field conditions when the highly productive containers were removed. The first pattern was more frequently observed in our study, although these two strategies can be important, depending on the condition of the breeding site and the environment in which the mosquitoes live.

The behaviour of females laying most of their eggs at the favourite breeding site may favour the species in periods during which temporary breeding sites are scarce. This behaviour can also be advantageous when a female finds high-productivity breeding sites containing ideal conditions, such as large volumes and diameters, dark colouring and the presence of co-specific larvae. This behaviour would indicate the capacity to support large quantities of immature forms (Harrington et al. 2008, Maciel-de-Freitas & Lourenço-de-Oliveira 2011, Wong et al. 2012).

Females that choose other strategies, spreading the higher proportion of their eggs and depositing some at the favourite breeding site or spreading their eggs without considering the favourite breeding site, can favour their offspring during the rainy season, when there is a wide variety of breeding sites that are constantly supplied by rain water.

Thus, the plasticity of behaviour that was observed in this study and in previous studies (Madeira et al. 2002, Wong et al. 2012) shows that populations of *Ae. aegypti* display different oviposition behaviours. The choice of a favourite breeding site and the deposition of large percentage of eggs in water were remarkable behaviours in the assessed population. However, changes in behaviour can also be natural in populations of *Ae. aegypti* (Madeira et al. 2002, Paduan et al. 2006, Hiragi et al. 2009) because they inhabit a variety of environmental conditions and present short life cycles.

The existence of multiple behaviours may indicate that *Ae. aegypti* mosquitoes contain populations of individuals of various origins that have experienced different selective pressures. Mosquito populations show great adaptability, primarily because their larval stages develop in different seasons and under different environmental conditions (Becker 1989) and because different selective pressures of the environment can lead to populations presenting distinct characteristics and great genetic and behavioural plasticity (Begon et al. 2007, Brown et al. 2011).

To determine whether the oviposition behaviour is the result of phenotypic plasticity or intraspecific differences between different populations, studies are required. However, regardless of the origin of behavioural plasticity in the oviposition of *Ae. aegypti*, this feature is important from an epidemiological perspective.

Therefore, studies that elucidate the vector behaviour are important to the definition of control measures and despite efforts, little is known about the oviposition behaviour. Thereby, it is extremely important that further studies investigate these aspects, especially under field and different conditions. Finally, it is also important to understand the behaviour of mosquitoes that are used in the most modern and promising control techniques, such as those infected with the bacteria *Wolbachia* and genetically modified mosquitoes.

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