PUBLIC HEALTH

Calibration and Evaluation of Field Cage for Oviposition Study with *Aedes* (*Stegomyia*) *aegypti* Female (L.) (Diptera: Culicidae)

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Neotropical Entomology 37(4):478-485 (2008)

Calibração e Avaliação de Semi-Campo para Estudos de Oviposição para Fêmeas de *Aedes* (*Stegomyia*) *aegypti* (L.) (Diptera: Culicidae)

ABSTRACT - Differences among results gathered from insect behavior studies conducted in laboratory and field situations are due to ambient variables that differ greatly between both environments. In laboratory studies the environmental conditions can be controlled whereas in field temperature, humidity and air velocity vary uncontrollably. The objective of this study was to calibrate and evaluate an experimental area (field cage) (14 x 7 x 3.5 m) subdivided into eight test cages (2.5 x 2.5 x 2 m) for use in behavioral oviposition tests of *Aedes aegypti* (L.) mosquitoes for developing a new methodology to assess attractants and oviposition traps. Test cage calibration involved: (1) minimal experiment duration tests; (2) optimal female release number per traps test and (3) trap placement tests. All tests used gravid *A. aegypti* females; 3-4 days post blood meal and the sticky trap MosquiTRAP® to catch adults. Ninety percent of the females released were recaptured 2h after the beginning of the experiment, and this allowed up to 32 test repetitions/day to be conducted in the field cage. The minimum number of females necessary to conduct statistical analyses was 20 females/trap/test per cage. No significant difference was found in the behavioral response of gravid females to four different trap positions within test cages. Field trapping results with attractant were similar to those in the field cage. Therefore, the field cage could replace field trapping for evaluating at least mosquito traps and oviposition attractants for *A. aegypti*.

KEY WORDS: Oviposition behavior, attractant, semi-field, MosquiTRAP

RESUMO - Discrepâncias entre resultados de estudos de comportamento de insetos em laboratório e em campo ocorrem devido a variáveis encontradas entre os ambientes. Enquanto em laboratório as condições são controladas, em campo os insetos são avaliados em condições variáveis de temperatura, umidade e velocidade do ar. O objetivo deste trabalho foi calibrar e avaliar uma área experimental (14 x 7 x 3,5 m) contendo oito gaiolas de teste (2,5 x 2,5 x 2 m), para estudos comportamentais de oviposição para *Aedes aegypti* (L.) visando desenvolver uma nova metodologia para avaliar atraentes e armadilhas de oviposição em semi-campo. A calibração consistiu na (1) determinação do tempo de realização dos experimentos e no (2) número de fêmeas liberadas por teste e na (3) avaliação das posições das armadilhas no interior das gaiolas de teste. Fêmeas de *A. aegypti* de 3-4 dias após repasto sangüíneo e MosquiTRAP® foram utilizadas nos experimentos. Noventa por cento das fêmeas liberadas foram recapturadas após 2h do início dos experimentos, permitindo a realização de até 32 repetições/dia. A amostra mínima de fêmeas por experimento para a realização de análises estatísticas foi limitada a 20 fêmeas/armadilha/gaiola. Não foi observada diferença na resposta comportamental das fêmeas nas diferentes posições avaliadas. Resultados com MosquiTRAP em área urbana foram semelhantes aos do semi-campo. Portanto, a metodologia pode substituir os testes de campo para avaliar pelo menos armadilhas e atrativos de oviposição para *A. aegypti*.

PALAVRAS-CHAVE: Comportamento de oviposição, atrativo, semi-campo, MosquiTRAP

The population density and the physiological state of the *Aedes aegypti* (L.), the mosquito vector of dengue and yellow fever, oscillate during the year and the increase in population occurs especially during the rainy season (summer), the hottest months of the year (Consoli & Lourenço-de-Oliveira 1994). Consequently, behavioral studies of *A. aegypti* in urban areas impose a few constraints such as: (1) the population size is unknown; (2) the physiological state is variable; (3) there is the risk of contracting dengue during the experiments; and, (4) operational difficulties for the execution of tests (excessive demand for labor, availability of vehicles to go to the study area, need for authorization of the local residents, the ethics committee and the city hall). Due to these limitations there was a need to develop an alternative methodology to carry out behavioral studies of the *A. aegypti* mosquito.

The semi-field methodology consists on the utilization of an experimental area built in the open-air (external area) and exposed to variations in temperature and air relative humidity, showing, therefore, characteristics closer to those found in the field (urban area).

Semi-field studies have been successfully conducted with *Anopheles gambiae* (Giles), the malaria mosquito vector. In these studies it was possible to study the biological cycle (Knols *et al*. 2002), determinants of mating and the reproductive success of the genetically modified mosquitoes (Okanda *et al*. 2002), trap evaluation and development (Mathenge *et al*. 2002, Njiru *et al*. 2006), influence of sugar availability and indoor microclimate on survival (Okech *et al*. 2003), and feeding and survival in the absence of blood meals (Impoinvil *et al*. 2004). However, before using the semi-field in oviposition studies, it is necessary to identify/establish the variables which can influence the response of the evaluated insects. Physical factors such as temperature, humidity, luminosity and wind can interfere in the response of the evaluated insect. It is also important to identify the minimum time necessary to recapture the females released; the minimum sample of females released so that a significant difference can be observed and; to check if the evaluated females show preference for any of the installation sites of the trap. The main advantages of semi-field tests is the absence of risk of infection, the standardization of the physiological state of the test mosquitoes, and it is very practical and rapid tests results (Seyoum *et al*. 2002a, b).

This study aimed at calibrating and evaluating an experimental area (semi-field) for the development of oviposition attractants and traps for the capture of the adult *A. aegypti* mosquito. The calibration process consisted on determining (1) the time necessary to carry out the experiments (2) the number of females released by trap and (3) the response of the pregnant females to the different positions of the traps inside the test cages. The experimental area was assessed by comparing a new formulation of the synthetic oviposition attractant in semi-field and in field conditions (urban areas).

Material and Methods

Breeding and rearing of *A. aegypti* in laboratory. The species is being reared in the Laboratório de Ecologia Química de Insetos Vetores, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, (ICB-UFMG) since 2000 and the generation used in the tests was the F9 one. The rearing room was kept at 27 ± 1^{o}C, 75-80% of humidity and 12L:12E at photoperiod (Eiras & Jepson 1991). The larvae were kept in plastic bowls with water and fed with ornamental fish food (Goldifish®). The adult mosquitoes (males and females) were kept in cages covered with fine mesh cloth (30 x 30 x 30 cm) and fed with a glucose solution (10% sucrose). Only adult females aged 10 to 20 days received blood meals. Anesthetized rats (*Rattus norvegicus,* Berkenhout) were used to feed the evaluated females. The females used for the blood meal were separated by a selection cage, consisting of an acrylic box (50 x 50 x 50 cm), with a small electrical fan at the back (Silva 2003). The electrical fan sucks the attractant odor (technician's hand) to the interior of the breeding cage (placed inside the acrylic box), selecting into one jar (250 ml), attached to the front of the selection cage, only the females which responded to the attractant odor. Females with 3-4 days post blood meal (time necessary for the maturation of the eggs) were used in the experiments.

Trap to collect adult mosquitoes. The MosquiTRAP® is a black opaque container of approximately one liter, 24 cm deep and 14 cm of diameter (Eiras 2002, Favaro *et al*. 2006, Gama *et al*. 2006). Inside the trap were placed: (a) a sticky card (38 x 19 cm) on which the pregnant *A. aegypti* females are captured after touching the internal surface of the trap (b) a protection screen (12 cm of diameter) which avoids the oviposition of the mosquito females directly on the water, (c) a synthetic oviposition attractant (Atr*Aedes*) identified from grass infusion (*Panicum maximum,* Jacq.) fermented to 15-20 days (Eiras & Sant'Ana 2001, Sant'Ana 2003) specific for *A. aegypti* females, and (d) tap water (500 ml).

Experimental area (field cage). An area of 14 x 7 x 3.5 m was built in the open-air for carrying out the behavioral tests of the oviposition of the *A. aegypti* mosquito (Fig. 1) at ICB-UFMG in Belo Horizonte, MG (20^{o}10'S; 44^{o}00'W). The experimental area was covered on the sides and top (ceiling) with a polyethylene mesh (mesh 72; 1.0 mm) to prevent the entry of insects and/or to prevent test mosquitoes to escape to the external area. The ceiling was covered with transparent PVC plastic, allowing natural light and preventing rainwater from entering. Transparent PVC plastic (1 m high) was also placed along the whole extension of the experimental area to reduce the influence of air drafts inside the test cages. The floor was entirely covered with small black stones (brita) (Fig. 1).

Eight test cages (2.5 x 2.5 x 2 m) were placed inside the experimental area (Fig.1). The test cages walls and ceiling were made of white fabric (voile) and a zipper was placed at the door (entrance) of each test cage to facilitate the entrance and exit of a person and prevent the insects from escaping. A potted plant (*Spathiphyllum* - Schot in Schott & Endlicher) was placed at the center of each test cage, to function as a resting place for the evaluated mosquitoes.

A thermo-hygrometer was placed in the center of the experimental area in order to monitor temperature and humidity throughout the experiments. The temperature and relative humidity of the air inside the experimental area were recorded at the beginning and the end of each replication of all experiments, aiming at evaluating its effect in the capture of pregnant females.

The experiments were carried out in the afternoon (1 p.m. to 5 p.m.) from January to April 2006.

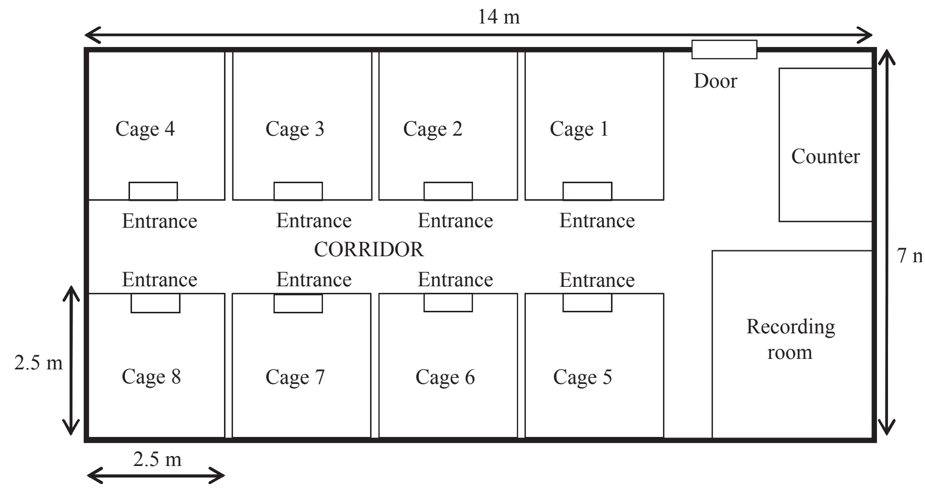

Fig. 1. Schematic drawing of an experimental area (field cage) showing the disposition of test cages and annexes (recording room and counter).

Experiment 1: Recapture rate of the females released in semi-fields. The objective of this experiment was to determine the minimum duration of the tests. Two MosquiTRAPs and two black opaque containers were placed 1 m equidistant from each other on the ground inside each test cage (Fig. 2). The traps were baited with tap water (500 ml) and Atr*Aedes,* whereas the black containers were empty, used only as a resting place. Twenty gravid *A. aegypti* females, by treatment, were released in the center of each test cage. The females were kept in plastic jars (250 ml) with sealed lids and the jars were placed on the ground, in the center of each test cage. The lid of the jar was opened, after the person had left the test cage, by a thread fixed at the jar's lid. Once the test began, the number of recaptured females (stuck on the sticky card) was evaluated in the two traps after 30, 60, 90, 120, 150 and 180 min. The recapture rate (number of females of captured/total of females released*100) was calculated for each treatment. Eight replications were carried out.

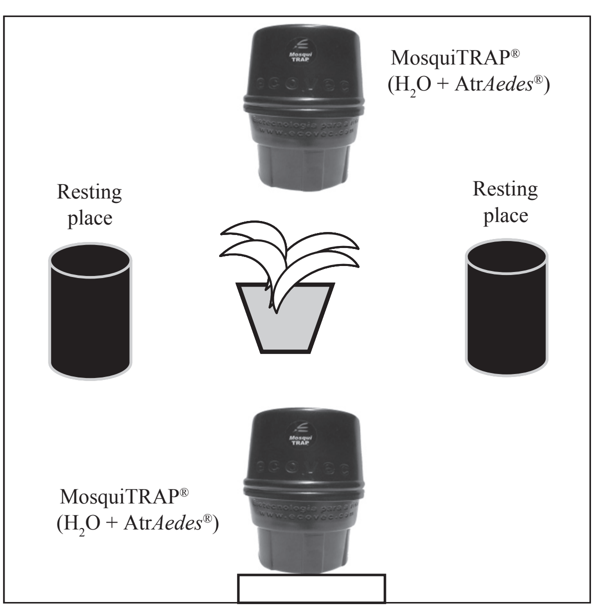

Fig. 2. Distribution of traps to capture gravid *A. aegypti* females inside the test cages, in order to determine the duration of the behavioral tests (n = 8).

Experiment 2: Number of females released per trap. The aim of this experiment was to determine the minimum sample of released females in each repetition which allows statistical analysis. The time of the experiments which captured the highest rate of gravid *A. aegypti* females in the experiment 1 was used in this experiment. Four MosquiTRAPs containing only tap water (500 ml) were placed in the same positions as the previous experiment. Sixteen replications were carried out for each treatment.

Experiment 3: Effect of traps position inside the test cages. This experiment aimed at verifying if *A. aegypti* females showed preference for any of the installation positions of MosquiTRAP® inside test cages. The number of released females was the one that presented less variation in the rate of gravid females recaptured in the previous experiment. Four positions (two longitudinal and two transversal ones) were evaluated separately inside each test cage. The transversal positions were identified as A and B (Fig. 3a) and the longitudinal ones as C and D (Fig. 3b). The MosquiTRAPs were baited with AtrAedes and 500 ml of tap water whereas the black containers were empty and considered only as a resting place. Five replications were carried out for each transversal and longitudinal position in each test cage.

Experiment 4: Validation of the experimental area. The response of the gravid *A. aegypti* females to a new formulation of the synthetic oviposition attractant was evaluated in semi-field conditions and in urban areas. The Atr*Aedes* was associated to two synthetic compounds identified in the infusion of grass infusion of *P. maximum* (Eiras & Santana 2001) aiming to verify the synergistic and/or additive effects resulting from the association. MosquiTRAPs containing only tap water and Atr*Aedes* were used as control

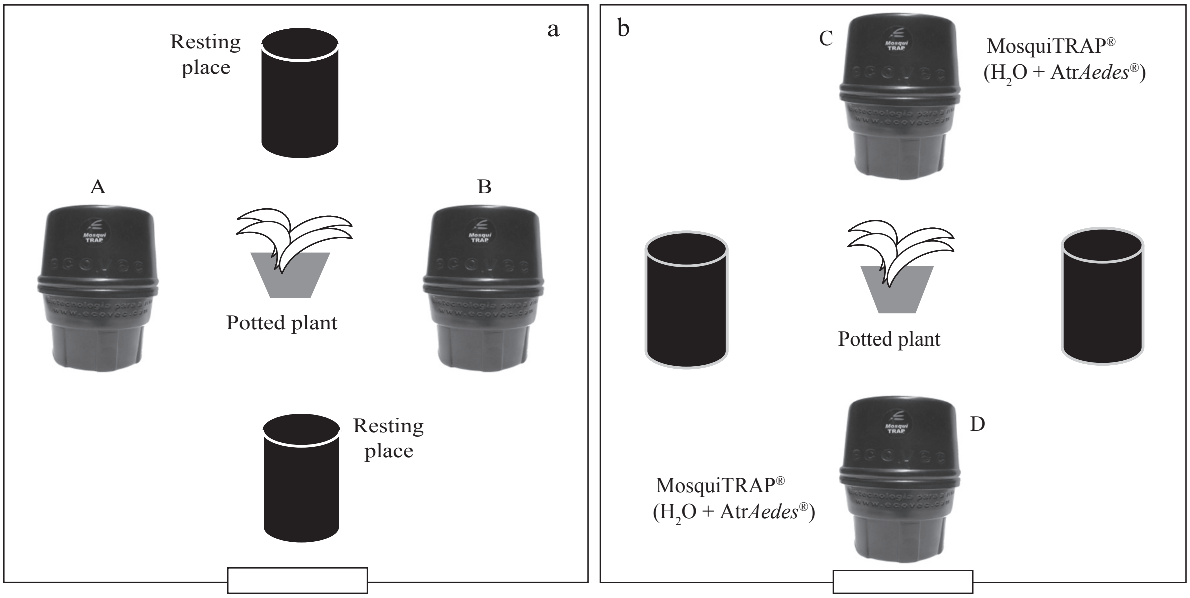

Fig. 3. Transversal (3a) and longitudinal (3b) positions of MosquiTRAP® inside the test cages (n = 5).

in all repetitions, whereas traps baited with Atr*Aedes* and 50 µl of the solution of the synthetic compound A and B (Eiras & Santana 2001) at the concentration of 10^2 ng / 50 µl hexane were used as test. The experiment set up was a double choice.

The field tests were carried out in the Pampulha Campus of the UFMG in two previously selected locations. Both field sites were similar with people walking around, many vegetation (small bushes, grasses, and especially potted plants) and high populations of *A. aegypti* mosquitoes.

In the field, the MosquiTRAPs were placed on the ground, 3 m equidistant from each other, in the buildings external area, but protected from the rain. The traps were placed in the morning (8 a.m. to 10 p.m.) and checked after 24h (Gomes *et al*. 2006). The water and the attractant solutions in the traps were replaced daily during the reading of the MosquiTRAP. The treatments were the same used in semi-field conditions, and the experiment was carried out using randomized in block design. In the experimental area the traps were placed on the ground, 1 m equidistant from each other and the experiments lasted 2h. Eight replications were conducted, and in each replication 40 gravid *A. aegypti* females were released, total of 360 females.

Statistical analysis. The Systat program for Windows version 8.0 was used in the analysis of the results. The data (rate of females recaptured) were transformed arcsine and submitted to the normality test of Kolmogorov-Smirnov at 5% probability. Normal distribution data were submitted to the one way analysis of variance (ANOVA) and the averages were compared by the Tukey test (α = 5%). The test t of Student was used when only two treatments were evaluated. When the data distribution was not normal, the Kruskal-Wallis nonparametric test was used and the Mann Whitney test was used to compare averages with a significance level of 5%. Linear regression was calculated to check if the temperature and relative humidity of the air influenced the capture of gravid *A. aegypti* females.

Results

During the experiments in the semi-field a variation in the temperature (26.0 ± 0.21ºC) and relative humidity (68.6 ± 0.77%) occurred due to natural environmental variations (Figs. 4 a and b). No significant relationship was observed between the recapture rate and the humidity inside the experimental area (r = 0.012). However, a positive (r = 0.277; P = 0.002) and significant correlation was observed between the temperature and rate of females recaptured. A 100% recapture rate was observed when the experiments were done at temperatures between 25 ºC and 30ºC and humidity between 65% and 70% (Figs. 4 a and b).

Experiment 1: Recapture rate of the females released in semi-fields. The results showed that approximately 96.7 ± 1.22% of the gravid *A. aegypti* females were recaptured in the trap up to 180 min after they were released. The lowest recapture rate was observed after 30 min test period (57.0 ± 3.92%) and it was significantly lower to 60 min (80.2 ± 0.96%). Recaptures higher than 90% were observed only after 120 min and they were not significantly different among themselves. Based on this experiment results, the 120 min interval was chosen, as it corresponded to the highest rate of recaptured gravid females (Fig. 5)

Experiment 2: Number of females released per trap. The total number of females released influenced the recapture rate in the four traps. A negative relationship was observed when an increase on the number of females released led to a reduction in the variation of the recapture rate in the traps (Table 1). A negative relationship was also observed between

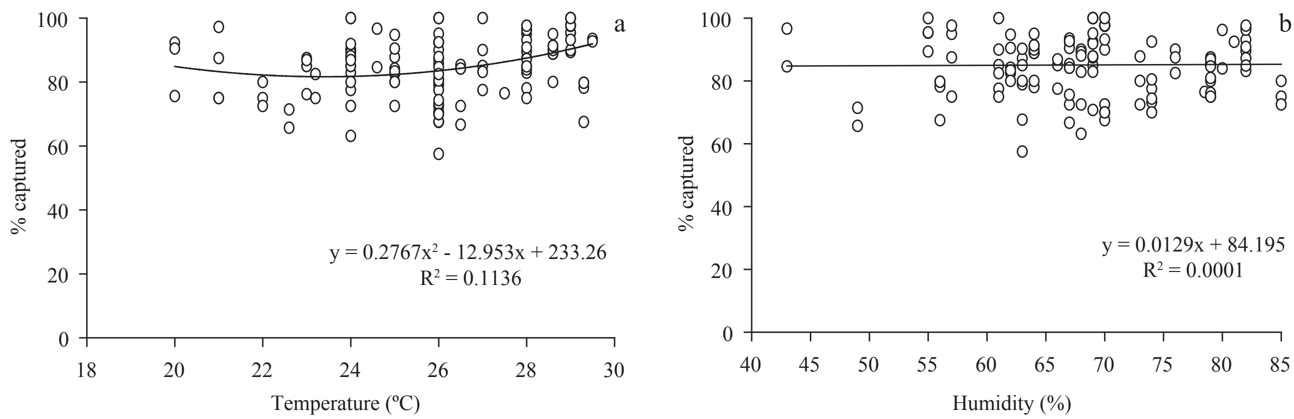

Fig. 4. Effect of temperature and humidity in the capture of gravid *A. aegypti* females in field cage (n = 130).

the coefficients of variation (CV) (standard deviation/ average*100) and the number of females released, which had their number reduced as the number of females/trap increased.

No significant difference was observed between the treatments containing 5, 10 and 15 females/trap/cage ($P > 0.05$). However, a significant difference was observed for 20 released females/trap/cage when compared to 5, 10 and 15. Since no significant difference ($P > 0.05$) was observed between 20, 25 and 30 females/trap/cage, 20 females/trap were used in the experiment 3 (Effect of the traps installation position inside the test cages) (Table 1).

Experiment 3: Effect of traps position inside the test cages. The result of the experiment which assessed the position of the MosquiTRAPs placed in four different positions inside the test cages 1, 2, 4, 5, 6, and 8 showed that the gravid females recapture rate were similar, regardless of the positions (Test t Student, gl = 4, $P > 0.05$) (Table 2). However, in cages 3 and 7 the trap placed at the position C captured a significantly higher percentage of gravid females than those placed at the position D ($P = 0.049$ and $P = 0.016$; respectively, test t of Student) (Table 2). No tendency was observed in the responses of the *A. aegypti* females in these two cages, when the transversal positions (A and B) were compared ($P > 0.05$).

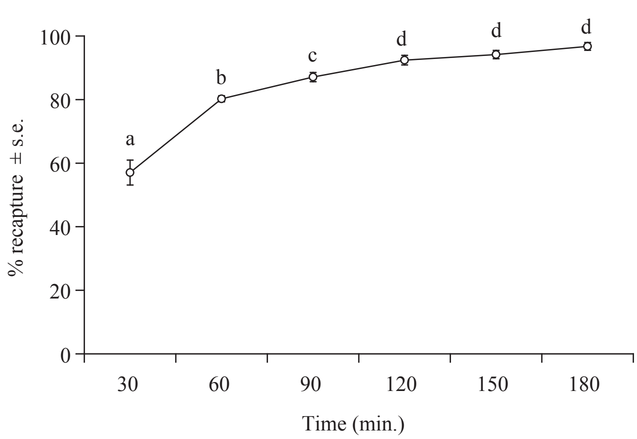

Fig. 5. Evaluation of the time spent to capture gravid *A. aegypti* females using MosquiTRAP in field cage (n = 8). Different letters are statistically different by Mann Whitney test ($P < 0.05$).

Experiment 4: Validation of the experimental area. The number of gravid *A. aegypti* females captured in the field 2 was higher than in the field 1, with 288 and 146, respectively. The Atr*Aedes* formulation in combination with the two synthetic candidates to oviposition attractants was significantly more attractive to gravid *A. aegypti* females than the control (Atr*Aedes*) in the field and semi-field ($P < 0.05$) (Table 3). Therefore there is a similarity between the results obtained in the two environments (field and semi-field).

Discussion

Environmental factors such as temperature, relative humidity of the air, light, and wind may influence mosquitoes' oviposition behavior (Bentley & Day 1989, Consoli & Lourenço-de-Oliveira 1994, Clements 2000). The variation of these factors may lead to a misinterpretation of the results, since what is being assessed is not the treatment itself, but also the conditions to which the insect is being submitted.

Temperature and relative humidity average values similar to the ones used in this study were observed in the experimental areas (semi-field) built in Kenya to evaluate the behavioral tests of the *A. gambiae* mosquito (Knols *et al.* 2002, Impoinvil *et al.* 2004). Okech *et al.* (2003) compared the temperature and relative humidity of the air in semi-field conditions with those obtained in natural environment. They did not notice any significant difference between the average values obtained in both environments and the *A. gambiae* behavioral responses. In the present study we observed an interval of variation between temperature (25ºC to 30ºC) and humidity (65% to 70%) at which 100% of released females were recaptured. Based on the results, we decided to conduct experiments in the semi-field only when the temperature and humidity in the experimental area were within this interval.

The air turbulence has great influence in the dispersion of the odor plume formed from its source. Thus, understanding

Table 1. Variation of the capture rate of gravid *A. aegypti* females by MosquiTRAP®, according to the number of females released in field cage (n = 16).

# gravid females *A. aegypti* released/trap	Recapture (%)	Standard deviation (s.d.)	Variation coefficient (VC)
5	96.3	3.77 b	78.4
10	90.0	3.83 b	42.6
15	100.0	2.83 ab	18.3
20	95.6	2.32 a	12.1
25	97.8	2.29 a	9.4
30	97.1	1.96 a	6.7

Different letters indicate significant differences (P < 0.05, ANOVA).

Table 2. Percentage (± S.D.) of gravid *A. aegypti* females recaptured by MosquiTRAP® installed at four different positions, inside the test cages (n = 5).

Test cage	Trap position inside the test cage			
	A *versus*	B	C *versus*	D
1	41.6 ± 4.52	39.0 ± 4.26	46.5 ± 5.70	34.6 ± 5.12
2	38.5 ± 4.26	41.9 ± 4.60	52.7 ± 5.73	36.8 ± 5.23
3	48.2 ± 6.05	29.3 ± 5.50	51.8 ± 5.86	24.1 ± 4.18*
4	41.6 ± 3.52	32.0 ± 5.72	40.4 ± 3.36	37.7 ± 3.31
5	42.0 ± 3.33	45.5 ± 6.01	43.8 ± 3.28	41.7 ± 5.12
6	41.1 ± 4.23	42.1 ± 4.22	35.5 ± 0.94	42.0 ± 1.66
7	33.6 ± 5.36	46.7 ± 7.80	52.7 ± 4.68	34.5 ± 3.63*
8	38.0 ± 4.66	38.4 ± 4.85	46.6 ± 1.74	34.3 ± 3.43

*Significantly different at P < 0.05 by , Student t-test.

Table 3. Mean of gravid *A. aegypti* females captured by MosquiTRAP® baited with Atr*Aedes* and in combination with two potential oviposition synthetic attractants in the field and the field cage.

Formulation	Field site 1 (mean ± s.e.) (n = 88)	Field site 2 (mean ± s.e.) (n = 40)	Field cage (mean ± s.e.) (n = 8)
*Atr*Aedes + compounds (A + B)	7.3 ± 1.22 b	10.3 ± 2.62 b	23.9 ± 1.68 b
*Atr*Aedes	3.3 ± 0.56 a	5.1 ± 1.62 a	14.3 ± 1.32 a

Means of the same column followed by the same letter are not significantly different (Student t-Test and Kruskal Wallis test, P > 0.05).

the odor plume structure during the behavioral bioassays is important because the test insects have to be exposed within the odor plume and the behavioral bioassay has to evaluate how the insects locate the odor source (Geier *et al*. 1999, Eiras & Mafra-Neto 2001). When olfactory stimuli is dispersed outside the test area in a confined area, the insect will not be able to fly towards the odor source. On the other hand, when the odor plume is disrupted and the air is shifted to one direction, one odor treatment can benefit the other, leading to misinterpretation of the insect response to a specific odor stimulus.

The understanding of the time required to evaluate the insect responses to a specific stimulus in semi-field conditions allows establishing the duration of the experiments and the number of replications that can be conducted per day. In our study, after 120 min. of experiment, 90% of the females were recaptured within traps. Since no significant difference (P > 0.05) was observed during the time periods of 120, 150 and 180 min, it was established that 120 min. would be enough to carry out the behavioral tests inside the experimental area. Consequently, it would be possible to carry out four tests per day per cage, and a total of 32 tests per day in the experimental area.

Regarding the number of mosquito per test, a reduction in the coefficient of variation (CV) of the percentage of recaptured females was observed in the four traps installed inside the test cages when the number of released females increased. The CV measures the percentage variation of a response; in this case, the difference between the percentages of recaptured females in each trap. High values for the standard deviation and the CV indicate a high variation in the percentage of females recaptured among the four traps. When this occurs, a significant difference would be difficult to be detected between treatments, since the high values of standard deviations would be bias for the insect tests responses to specific treatment. However, as the number of released females increased, the standard deviation value and the CV reduced.

The results obtained in this study suggest that in the cages 3 and 7 the positions C and D should not be used to evaluate the responses of the gravid *A. aegypti* females. Only the positions A and B should be used, since significantly different responses were not observed in these positions. Regarding the other cages (1, 2, 4, 5, 6, and 8), the four positions can be used as no significant difference was observed in the females captured in the different positions.

The experimental area with eight test cages developed in this study is a new tool for carrying out behavioral tests using *A. aegypti* mosquito and can be used to evaluate prototype traps, releaser devices and the development of new formulations of synthetic oviposition attractants. This tool offers intermediate conditions between the laboratory and the field, as the behavioral tests are conducted with insects from laboratory. In this case, the physiological status and age of test insects are known, and also they are free of viruses and bacterial contamination. One disadvantage of the field cage is that the temperature and humidity are not controlled and the experiments should be done only when the conditions of temperature and humidity are, at least, 25ºC and 65%, respectively. Among the advantages of this experimental area, are: (a) the fact that many replications can be carried out per day (up to 32); (b) agility, practicality and rapidity to obtain the results; (c) the possibility of carrying out experiments almost during the entire year, except in the winter in tropical countries; (d) the study are conducted with insects of known physiology status, thus avoiding dissection of female mosquitoes to know the parity and (e) that the gravid females mosquito population in the study are is well established.

In this study, after the calibration of an experimental area with eight test cages, we observed similar responses in field cage and in field tests in urban areas, confirming that the methodology employed in this study is reliable. Therefore, the field cage can be used to evaluate oviposition responses of gravid *A. aegypti* female mosquitoes to develop traps and new formulations of synthetic attractants.

Acknowledgments

We are grateful to Professor Ivan Barbosa Machado Sampaio for statistical support, to Adriana dos Santos Gomes (*in memoriam*), Isabella Matozinhos and Liliane Ramos dos Santos Machado for the help with the experiments, to Jivago Leite Proti for the preparation of the insects used in the experiments. Financial support by CAPES, CNPq and Ministério da Saúde is acknowledged.

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Received 16/V/07. Accepted 28/V/08.