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Oviposition and olfaction responses of *Aedes aegypti* mosquitoes to insecticides

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Abstract. Insecticide applications are not particularly effective on *Aedes aegypti* mosquitoes which has been attributed to their 'closet' behaviour, or ability to rest in places that remain unexposed to insecticides. Some researchers have suggested that insecticides repel mosquitoes, which would result in less exposure and increased dispersal. If repellence due to insecticides is a fact, acquiring a vector-borne disease, such as dengue, could legitimately be attributed to local vector control efforts and this would lead to restitution claims. This study thus investigated the effect of insecticide presence on mosquito behaviour indirectly via oviposition and directly via olfactory response. In all experiments, oviposition in each insecticide compared to its water and ethanol controls was not significantly different. This indicates that *Ae. aegypti* mosquitoes are not affected by insecticide presence and that increased dispersal is unlikely to be caused by vector control spraying.

INTRODUCTION

Aedes aegypti's anthropophilic and endophilic nature appears to make it an ideal target for domestic insecticide application programmes. However, fogging and ULV programmes are not effective, which has been attributed to the predilection of the vector for concealed protective spaces (Reiter, 1993). Another factor that may contribute to the failure of spray programmes is the repellent effect of insecticides on aedines, which was suggested as a potentially important consideration in domestic control programmes (McClelland, 1967; Surtees, 1967). Evidence in support of this hypothesis has accrued over time. For instance, Surtees (1967) suspected that insecticide spraying would have the effect of depressing oviposition; Jacob (1969) found that ovicide-treated tire breeding sites repelled gravid females; Von Windeguth *et al.* (1971) found that larvicides induced excito-repellent responses in ovipositing mosquitoes in Florida; Verma (1986) found that several synthetic pyrethroids repelled ovipositing *Ae. aegypti*; and Moore (1977) directly linked repellent effects to insecticide concentration. Contrary results were recorded for insect growth regulators where methoprene briquettes were observed to attract oviposition (Carroll, 1979). Assessing repellence is important because of the possibility that the use of a larvicide with irritant or repellent properties in control operations could result in greater dispersal of the mosquito population through relocation of gravid or ovipositing females to other areas with untreated sites. This would be unfortunate in the case of *Ae. aegypti* and it may even have legal implications because the mosquito typically has a small dispersal range.

One way to assess the response that mosquitoes have to insecticides is to examine oviposition in treated breeding sites. Possible outcomes are that insecticide presence, a) has no effect on mosquito behaviour, (b) attracts mosquitoes to increasing treatment effectiveness, (c) repels mosquitoes to cause further dispersal and control failure, (d) causes behaviour that varies according to the makeup of the insecticide and its volatility, and (e) enables mosquitoes to recognize and avoid insecticides. Despite the latter four items being of great significance, remarkably few researchers have attempted to determine mosquito responses to insecticides. This study was undertaken to determine if any change in mosquito behaviour in the presence of water, ethanol, temephos, malathion or permethrin could be attributed indirectly via oviposition and directly via olfactory response to olfactory cues, insecticide recognition, irritancy or repellence.

MATERIALS AND METHODS

This experimental design employed a two-pronged approach to reveal and explain the cause of mosquito responses to insecticides. The first experiment investigated the aims indirectly by assessing response in terms of oviposition, while the second experiment directly assessed mosquito response to the odours emitted by treated breeding sites.

Oviposition Experiment

Insecticide avoidance behaviour was not measured using standard excito-repellency methods because the aim was to observe the choice of oviposition sites by *Ae. aegypti* females. Tendered oviposition sites contained either an insecticide (malathion, temephos or permethrin), ethanol or water. Ethanol was a control since it was used to dilute the insecticides. Individual mosquitoes were used in preference of groups of mosquitoes. Initially, oviposition responses of individual unfed 2-day-old female *Ae. aegypti* (5 replicates) were investigated for each insecticide. Subsequent tests used gravid and postgravid mosquitoes. Two consecutive tests were conducted for each group, corresponding to the first and second oviposition cycles. Mosquitoes were fed blood at the beginning of each week/cycle.

There were three groups for each of the control and insecticide groups. Group 1: No change in mosquitoes or solutions between week 1 and week 2; Group 2: No change in solutions between week 1 and week 2, but change in mosquitoes in week 2 with new mosquitoes replacing the old ones; Group 3: No change in mosquitoes between week 1 and 2, but change in solutions in week 2 with old solutions being discarded and new solutions being placed in test containers. Thus nine tests addressing combinations of the variables involved were thus undertaken over the testing period.

The tests took place in the Gabrielli Insectary located in Townsville Australia (Latitude: 19.25°S, Longitude: 146.77°E, Elevation: 4 m). The mosquitoes were maintained according to the procedures of Foster (1980) with fairly even light:dark natural lighting. Five oviposition test replicates for each group were carried out in ventilated 2 L test cages in an environmentally controlled experimental room (25° ± 1°C, 60 ± 5% RH).

Oviposition sites were treated with insecticides at the following LC^{99} doses estimated from a Townsville colony established in 1995: malathion (1.042 mg/L), temephos (0.193 mg/L) and permethrin (0.0093 mg/L) (Canyon & Hii, 1999). Malathion, permethrin and temephos were appropriately diluted with 97% ethanol to obtain the desired concentrations. One ml of solution was used to treat each container to standardize on the amount of ethanol being used.

Into each test cage was placed an untreated container of water, a container of water treated with one ml of ethanol and a container of water treated with one ml of insecticide diluted to the correct dose. Pieces of tongue depressor wood were placed in each 50 ml container as an oviposition substrate.

Eggs were collected and counted at the end of each week/cycle. The percentages of all eggs laid in the containers were calculated and were found to have a skewed distribution. Nonparametric tests were thus used. The Mann-Whitney Test was used to assess significant differences between each container type between weeks 1 and 2.

Olfaction Experiment

To confirm whether the response was due or not due to insecticide odours, a Y-tube olfactometer was used to ascertain behaviour in response to variations in insecticide odour from treated and time-altered oviposition sites.

Air was pumped through environments containing the test oviposition solutions, which loaded the moving air with moisture and characteristic odours, into the wind tunnels where mosquitoes were released. Samples were taken from the oviposition sites prepared in the Oviposition Experiment at 4-day intervals and were tested for activity in the olfactometer with ten replicates. Twenty tests were carried out using a total of 200 mosquitoes. Factors relating to the vector-site relationship such as response initiation, speed and odour selection were going to be monitored, however, the mosquitoes moved so quickly through the wind tunnel that the collection of this data would have been meaningless. A limitation of this approach is that organophosphate insecticides are characterized by a relatively high volatility compared to pyrethroid insecticides which are not typically used to treat oviposition sites and primarily act as contact insecticides.

A dichotomous logistic regression analysis with dummy coding for days and insecticides was performed. All models, including models with the interactions between days and insecticide were examined, but no significant relationship was found.

RESULTS

Oviposition Experiment

No significant differences were observed between eggs laid in different scenarios for the same insecticide and its water and ethanol controls. Despite this, several interesting observations were made between the control and insecticide tests. Table 1a displays an initial test with only ethanol and water present indicated no significant difference over time in preference to ovipositing females. It also shows the results obtained from tests with no exchange of either mosquitoes or solutions. Table 1b displays results from the tests in which mosquitoes were replaced in week 2 with new mosquitoes. Table 1c shows the results of maintaining the same mosquitoes and changing the solutions in week 2. No obvious bacterial contamination of the tests site was detected.

Olfaction Experiment

As Figure 1 and Table 2 indicate, there are no obvious patterns and oviposition responses to all substances tested were similar. All the bars ended up with a sum of around 25, the 50% mark, thus indicating that the results are what can be expected by chance alone and that no further interpretation is recommended.

Different logistic regression models did not show any significance. No significance emerged even when the interaction terms were modelled with respect to preference. Even when the different mosquito conditions were compared between week 1 and week 2, no significant differences were observed.

DISCUSSION

A comparison between week 1 and week 2 revealed similar oviposition rates overall with the mean number of eggs laid per mosquito in weeks 1 and 2 being 54±6 SD and 56±8 SD respectively. When no insecticide was present, a mean of 59 eggs were laid, thus no depression of oviposition was observed due to insecticide presence.

It was surprising to produce results that contradicted previous studies (McClelland, 1967; Surtees, 1967; Jacob, 1969; Von Windeguth *et al.*, 1971) and common perceptions so comprehensively. And yet, the results bode well for current control practices. Temephos and malathion were equally preferred with water and water was preferred over permethrin. Overall, ethanol was not preferred in favour of either

Table 1. Oviposition results for controls and insecticides in each experimental setting (Week 1 mean eggs laid (Std Dev): Week 2 mean eggs laid (Std Dev, *p value*)

Insecticide	Water	Ethanol	Insecticide
a. No changes in mosquitoes or container solutions between weeks 1 and 2			
Control	35.4 (28.7): 29 (32.3) *0.540* [a]	23.8 (26.6): 18.6 (18.7) *0.968* [a]	–
Temephos	21 (5.8): 12 (17.0) *0.561* [a]	15.2 (10.8): 7.8 (12.0) *0.649* [a]	12.8 (13.6): 28 (27.1) *0.172* [a]
Malathion	23.2 (22.7): 5.8 (10.9) *0.112* [a]	15.4 (22.8): 6.8 (8.2) *0.929* [a]	20.6 (17.8):46.6 (21.7) *0.122* [a]
Permethrin	33.8 (21.0): 19.8 (29.5) *0.160* [a]	14.4 (25.6): 14.2 (27.0) *0.858* [a]	10.2 (18.9): 27.6 (26.7) *0.339* [a]
b. No change in solutions between weeks 1 and 2, but change in mosquitoes in week 2 with new mosquitoes replacing the old ones			
Temephos	16.6 (22.0): 18.6 (19.5) *0.881* [a]	25.6 (26.0): 1.4 (1.7) *0.236* [a]	16.6 (24.1): 48.8 (29.1) *0.024* [a]
Malathion	21.8 (28.9): 16.4 (21.5) *0.814* [a]	5.8 (7.8): 16.2 (25.8) *0.929* [a]	29.2 (22.6): 19.2 (26.4) *0.26* [a]
Permethrin	40 (15.0): 34.8 (30.7) *0.784* [a]	3.4 (6.1): 4.6 (10.3) *0.488* [a]	5 (6.6): 24.8 (16.9) *0.143* [a]
c. No change in mosquitoes between weeks 1 and 2, but change in solutions in week 2 with old solutions being discarded and new solutions being placed in test containers			
Temephos	20.4 (28.2): 20.8 (28.7) *0.713* [a]	7.2 (14.0): 15.8 (18.6) *0.878* [a]	15.6 (22.1): 18.2 (26.7) *0.874* [a]
Malathion	25.4 (15.1): 17.2 (19.5) *0.685* [a]	8.8 (17.0): 9.2 (8.6) *0.500* [a]	21.4 (13.0): 33.0 (18.7) *0.598* [a]
Permethrin	17.6 (22.1): 3.6 (5.7) *0.023* [a]	17.2 (23.8): 4.8 (8.0) *0.847* [a]	14 (14.4): 34.2 (18.5) *0.001* [a]

a: different letters after the p-value on the same row indicate significant differences

water or insecticides. Contrary to published observations from the 60s and 70s, all tests showed a decrease in preference for water over time and an increase in preference for all insecticides. Although significant differences between data were unavailable, there is evidence to suggest that the attractant response to temephos was based almost entirely on olfactory cues, and that the attractant responses to malathion and permethrin were based almost entirely on prior recognition of the insecticide as opposed to its carrier.

The use of figures employing rate displays is a useful way of revealing patterns of potential interest. Table 3 describes the possible outcomes and study design while the following sections describe responses to each of the insecticides.

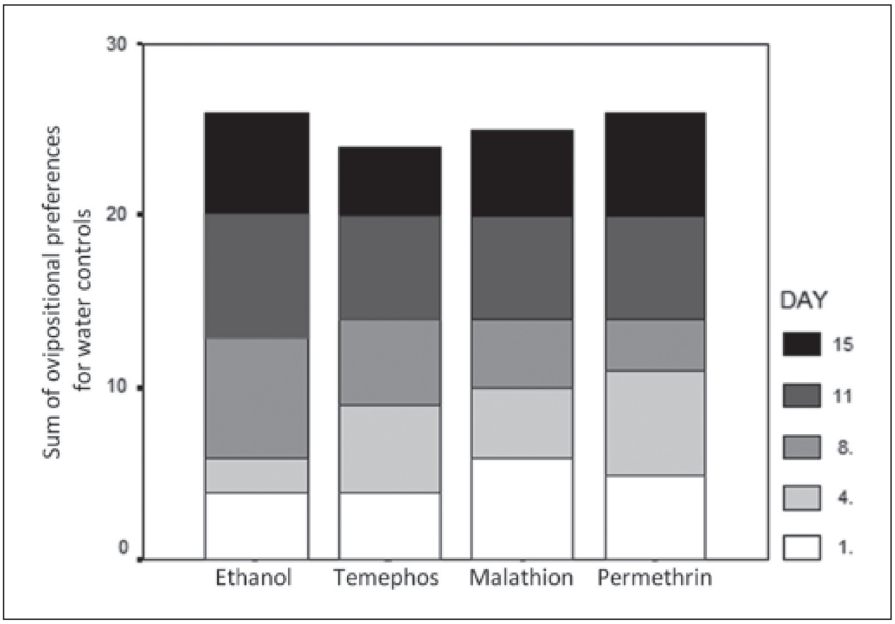

Figure 1. Preferences for water controls are displayed. For instance, in the first bar a total of 26 water preferences were observed over the course of the study

Table 2. Olfaction data in percentage response to aged samples of ethanol and insecticides compared to water. For instance, on day 1, 60% of mosquitoes flew towards the ethanol control and 40% flew towards the water control

Day	1	4	8	11	15
Ethanol	60%	80%	30%	30%	40%
Temephos	60%	50%	50%	40%	60%
Malathion	40%	60%	60%	40%	50%
Permethrin	50%	40%	70%	40%	40%

Table 3. Possible oviposition outcomes

Potential Week 2 outcome	Wk 1	Wk 2 NC	Wk 2 MC	Wk 2 SC
1 None expected	x	=x	=x	=x
2 Olfactory – attractant	x	>x	>x	=x
3 Olfactory – repellent	x	<x	<x	=x
4 Learned – attractant	x	>x	=x	>x
5 Learned – repellent	x	<x	=x	<x

NC – no change (control). MC – only mosquitoes changed in week 2. SC – only solutions changed in week 2.

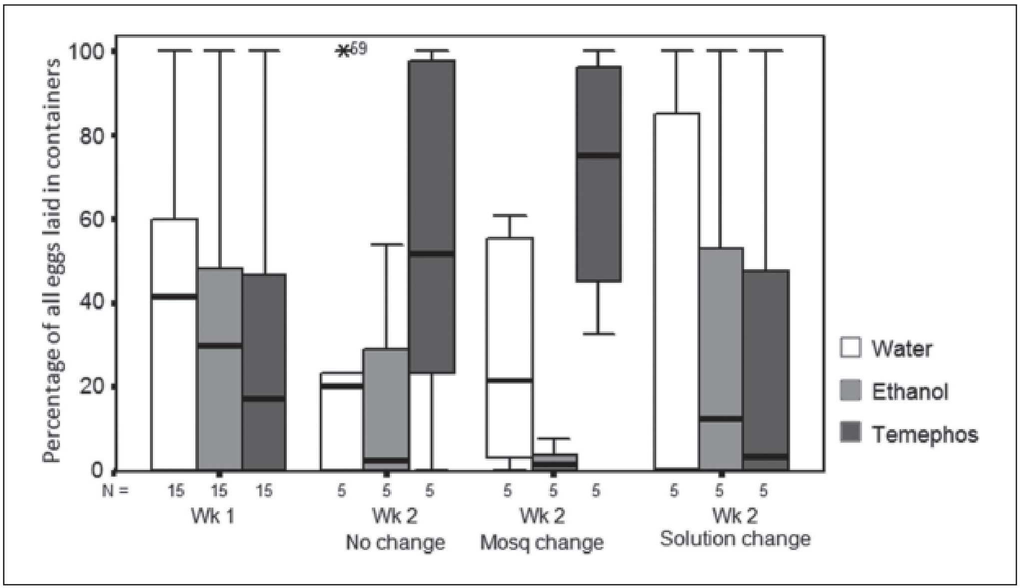

Figure 2. Oviposition preferences in the presence of temephos, ethanol and water

Temephos. In Figure 2, there were no changes in preference for water in all test settings. A possible preference for ethanol declined over time and the introduction of new mosquitoes confirmed this trend. A change in solutions resulted in preference for ethanol returning to original levels. This possibly indicated the return of an olfactory stimulant.

When no changes were made and both mosquitoes and solutions were allowed to age together, a shift in preference from week 1 to week 2 is evident for temephos. The change in mosquitoes accompanied by a similar preference for temephos indicates that the preference was probably not a learned response and thus was olfactory in nature. This was supported when the solutions were changed and the preference for temephos returned to original levels. The profile for temephos fits potential outcome #2 *Olfactory Attractant*.

No initial preference for any particular container was observed in week 1 and in week 2 when the solutions were renewed. The increase in preference for temephos and the decline in preference for ethanol indicated that the response to temephos was due to the insecticide itself and not to its carrier, ethanol.

Malathion. In Figure 3, preference for water remained much the same in all tests with a decline over time for the control mosquitoes. Ethanol did not act as an attractant since preference for this substance was low over all test conditions.

Preference for malathion increased over time with the control mosquitoes. The apparent decrease in preference with new mosquitoes and increase in preference with a change of solutions may have been a return to original levels or may indicate that olfactory cues were less important.

The decline in preference for water over time for the control mosquitoes was due to a large increase in preference for malathion. Similarities between week 1 and changed-mosquito rates and between control and changed-solution rates indicate that preference may increase for malathion treated sites. The malathion profile fits potential outcome #4 *Learned Attractant*, which suggests that the observed response was due to learning and not to olfactory cues.

Permethrin. In Figure 4, preference for water was not entirely consistent due perhaps to large changes in preferences for permethrin. As in the malathion tests, ethanol was not preferred in all situations.

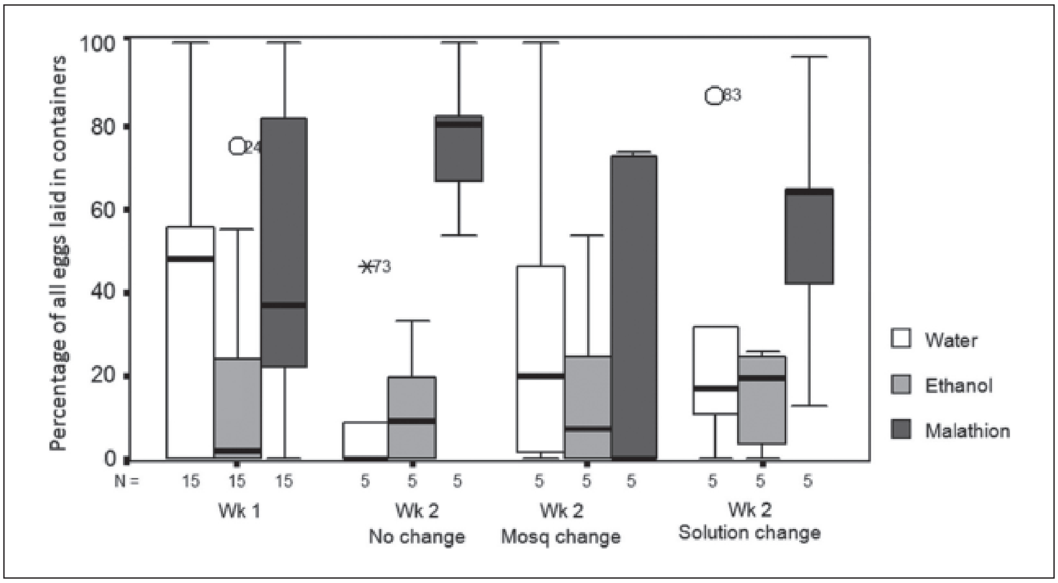

Figure 3. Oviposition preferences in the presence of malathion, ethanol and water

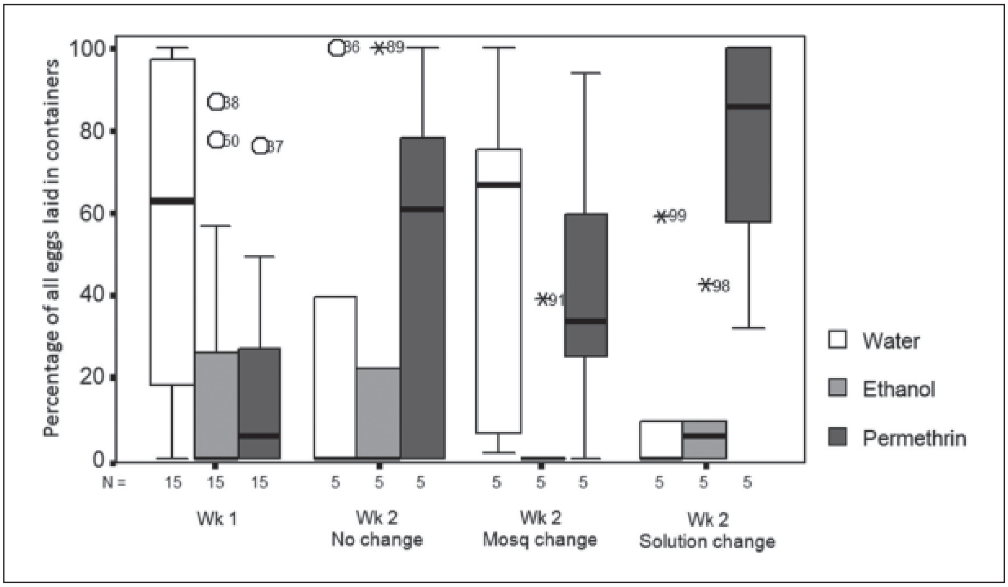

Figure 4. Oviposition preferences in the presence of permethrin, ethanol and water

Preference for permethrin increased over time in the controls and when solutions were renewed preference remained closer to week 2 control levels. A change in the mosquitoes resulted in a marked decline in preference. The pattern in these results is less obvious, however appears to conform with potential outcome #4 *Learned Attractant* in that odour appears to be less important and insecticide recognition may have occurred.

The lack of significant or even trend response data in olfaction trials was initially a setback, however, the results were somewhat in concordance with those obtained in the oviposition tests. The oviposition results indicated an olfactory response to ethanol in the temephos test alone and to temephos. These olfactory results did not support this and indicated that the lack of significance in the temephos oviposition data may be correct. The oviposition results for malathion and permethrin were confirmed in that olfaction is not important to ovipositing gravids when encountering sites treated with these chemicals.

The results for temephos, malathion and permethrin all indicated a preference for treated breeding sites one week after treatment. If these lab results were directly transferrable to the field, current management strategies being used for mosquito control would be considered appropriate and no dispersal of mosquito populations would be expected following treatment of a breeding area. The apparent ability of mosquitoes to recognize the presence of insecticides and to be attracted is not expected to detract from current control strategies, but is hypothesized to enhance them. Field studies are necessary to confirm these lab results.

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