EFFECT OF AXENIC LARVAE ON THE OVIPOSITION SITE SELECTION BY *AEDES ATROPALPUS*

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ABSTRACT. Axenic rearing of *Aedes atropalpus* was performed from previously sterilized eggs to pupae in sterile 2500-ml culture flasks containing 500 ml of sterile culture medium. The larval density in the axenic rearing waters was approximately 500 larvae/liter. A series of experiments was conducted using respectively 6-, 9-, 12- and 19-day old axenic larval rearing water. All these tested waters were significantly preferred by the *Ae. atropalpus* females. Sterile, distilled water was also significantly attractive after only 48-hr immersion of axenic fourth-instar larvae. An axenic larval rearing water with higher larval density (900 larvae/liter) was however repulsive. The last experiment presents evidence for blocking of the attractant effect at high larval densities.

INTRODUCTION

Several authors have emphasized the oviposition preference of mosquitoes for water containing larval mosquitoes. Such an effect was demonstrated for *Culex* (Hudson and McLintock 1967, Rotraut et al. 1973, Dadd and Kleinjan 1974, Suleman and Shirin 1981), for *Anopheles* (Reisen and Siddiqui 1978), and for *Aedes: Aedes atropalpus* (Coquillett) (Kalpage and Brust 1974, Maire 1984), *Ae. aegypti* (Linn.) (Roberts and Hsi 1977), *Ae. triseriatus* (Say) (Bentley et al. 1976, McDaniel et al. 1979) and *Ae. togoi* (Theobald) (Trimble and Wellington 1980).

In spite of aseptic conditions, Trimble and Wellington (1980) could not distinguish if the oviposition stimulant associated with *Ae. togoi* 4th-instar larvae was: 1) bacterial metabolites, 2) larval excretory products that mimic naturally occurring stimulants produced by plants or animals, or 3) compounds of larval origin that are specifically produced to aid gravid females in identifying pools suitable for preadult development. This paper presents the results of experiments conducted with *Ae. atropalpus* larvae reared in complete axenic conditions to resolve the question about whether the preference shown by mosquito females to larval holding waters is or is not of semiochemical (pheromone) origin.

MATERIALS AND METHODS

COLONY MAINTENANCE. The *Ae. atropalpus* colony, originally from La Gabelle, near Trois-Rivières, Québec (46°18'N, 72°37'W), has been maintained in our laboratory for several years. Environmental conditions were: 23°C temperature, 17L:7D photoperiod and 75% RH for adults. Immature mosquitoes were reared in stainless steel pans filled with tapwater. They were fed a mixture of yeast extract (10%) and Purina chow (dog, kittten or mouse chow). Pupae were removed every 2 days and placed in cages 30×30×37 cm at a density of approximately 400 per cage. An aqueous honey solution (1:1 volume) was provided for adult nutrient. Mice were also presented three times a week for blood feeding, even though this mosquito species is known to be autogenous. When the colony was blood fed, females did not exhibit any reduction in fecundity from one generation to another as previously observed when only honey was available as a food source to females throughout the year.

In the subsequent oviposition bioassays, two oviposition dishes (petri dish glass covers) containing test solutions were randomly placed in each cage six days after the first adults emerged. Eggs laid overnight were removed daily and counted. Fresh test solutions were also placed in each cage every morning. Each experiment was conducted over 5 days (first gonotrophic cycle) and was started the second day after the first eggs were laid.

EGG STERILIZATION. Eggs were surface-sterilized using the procedure of Lang et al. (1972) with minor modifications. There are two stages for this sterilization: the first when eggs are collected from oviposition filter papers (24–48 hr after deposition) and the second before their hatching and axenic rearing. Prior to sterilization, the eggs were stored for a variable period (approximately 1–3 weeks) at 23°C, 80–90% RH and 16:8 L:D photoperiod.

Eggs were first removed from the oviposition papers with a washing bottle and deposited on a Nitex® nylon screen (500 μm), washed with tapwater, filtered on a second screen (100 μm), placed in 70% ethanol for 30 sec and rinsed with distilled water. They were then soaked in a 2% sodium hypochlorite solution for 3 min and rinsed again with sterile distilled water. The eggs were then carefully collected with a sterile brush and pushed into a 15-ml disposable sterile conical tube filled with 0.1% benzalkonium chloride (Zephiran chloride). The capped tube was agitated on a Vortex® mixer for about 3 min. The eggs were removed with a

sterile Pasteur pipet and sequentially washed in three sterile conical tubes filled with phosphate buffer, pH 6.5. Each tube was agitated for 15 sec on a Vortex mixer. As many as 12,000 eggs were thus sterilized per operation. These eggs were deposited on white 9 cm SxS Blue Ribbon® filter paper discs (approximately 500 to 1,000 eggs per disc). Each disc was previously sterilized and placed in an autoclaved petri dish. The petri dishes were sealed with masking tape and stored for 1 to 3 weeks.

Just before the axenic rearing, the second stage of the egg sterilization was conducted as described by Lang et al. (1972). The eggs were removed from the petri dishes with a sterile brush and placed into a 15-ml disposable sterile conical tube containing 8 ml of 0.1 ml benzalkonium chloride. They were kept in this solution and agitated on a Vortex mixer for exactly 2.5 min. Then, the eggs were sequentially washed in three baths with sterile phosphate buffer, each bath being agitated during 10 sec on a Vortex. The eggs began to hatch during the first bath and virtually all the 1st-instar larvae emerged.

All procedures were performed in a class II, vertical laminar flow containment hood, previously irradiated with UV light for at least 30 min.

PREPARATION OF CULTURE MEDIUM. The culture medium was slightly modified from that of Lang et al. (1972). Solution A consisted of minerals, bovine albumin and cholesterol, while solution B contained vitamins, buffer and RNA (Table 1). Each solution was autoclaved 20 min at 121°C. An equal quantity of solution B was poured into solution A just before starting the axenic rearing.

AXENIC REARING. Recently hatched 1st-instar larvae were carefully transferred into three autoclaved 2500-ml culture flasks (low form Pyrex® brand) containing 500 ml of sterile culture medium. The average larval density was 500/liter. Two other flasks with only sterile culture medium served as controls. The axenic rearing of mosquitoes can be performed from a few days up to three weeks, i.e., from 1st-instar larvae up to pupae and even adults. This permits testing at any stage of development.

STERILITY TESTS. Systematically, several kinds of sterility tests were conducted with nutrient agar (Gibco), mycophil agar with low pH (BBL), incubated at 35°C for 48 hr, and Sabouraud medium, incubated at 25°C for one week.

Table 1. Composition of the axenic culture medium for rearing mosquito larvae and pupae (after Lang et al. (1972), slightly modified).

Solution	Amount	Solvent	Amount for 2500 ml
Solution			
1) NaCl	300 mg		
$MgSO_4$ 7 H_2O	5 g		
$FeSO_4$, 7 H_2O	300 mg		
$MnSO_4$, H_2O	200 mg	250 ml H_2O	25 ml
$CaCl_2$, 2 H_2O	397 mg		
$ZnCl_2$	25 mg		
2) Cholesterol	90 mg	30 ml of absolute ethanol	5 ml
3) Bovine albumin*			14 ml
Solution B			
4) Ca Pantothenate	125 mg	200 ml of 25% ethanol	20 ml
Pyridoxine HCl	50 mg		
5) Thiamin	25 mg		
Biotin	1.25 mg	200 ml 0.02 N acetic acid	20 ml
Nicotinamide	125 mg		
Choline. Cl	1.25 g		
6) Riboflavin	80 mg	200 ml H_2O	50 ml
7) Folic acid	13.5 mg	45 ml 1.0 N $NaHCO_3$	5 ml
8) K_2HPO_4	6 g	200 ml H_2O	5 ml
KH_2PO_4	6 g	(ph 6.5)	
9) RNA (sodium)	25 g	250 ml H_2O	2500 mg equiv** (21.75 ml approx.)
10) Sterile distilled H_2O			to complete (2,289.25 ml)

* Bovine albumin, fraction V, 7.5% in Phosphate buffered saline (Gibco).

** The exact volume added varied depending on the concentration of the stock solution which was standardized spectrophotometrically.

These tests were carried out at different stages; during the egg sterilization, when solutions A and B were prepared, before the axenic rearing and before filtration of the axenic medium with or without larvae.

When the two solutions are mixed, the culture medium is composed of a clear yellowish solution and, at the bottom of the flask, a white precipitate due to autoclaved albumin. Any flask with turbidity after a few days was considered as non-axenic and was discarded. With time, axenic culture could be performed with at least a 50% success rate.

OVIPOSITION BIOASSAYS. A first experiment tested the activity of water with larvae which were never in contact with bacteria or fungi (axenic conditions). Axenic 4th-instar larvae of *Ae. atropalpus* were transferred by Pasteur pipette from their initial axenic culture medium to 1000 ml of sterile distilled water at a density of about 1 larva/2 ml. This larval holding water (LHW) after 48 hr of larval immersion was then filtered through sterilized Whatman #2 filter paper and the filtrate was used as the test solution.

A second series of experiments detected both the threshold and the cumulative effect of larval pheromone activity during the mosquitoes' aquatic phase. Axenic rearing was conducted as previously described. Respectively, 6, 9, 12 and 19 days after the beginning of the larval rearing, each sample pair (one flask with larvae, another without) was filtered and the filtrate or larval rearing water (LRW), stored as the test solutions.

A third experiment tested the effect of high larval density on oviposition site selection. A 6-day old and a 12-day old axenic larval culture medium with 700 and 900 larvae/liter respectively, were used as the tests solutions.

STATISTICAL ANALYSIS. Five cages per experiment were replicates and each experiment was conducted during 5 days. The unsexed adult population was maintained at ca. 300 per cage. The total number of eggs laid per dish and per cage for the 5 days was expressed in percentage and then arcsine transformed before statistical analysis. Results were submitted to a paired *t*-test analysis. For each experiment, only the oviposition response of the female population was deliberately measured. Even though, incidentally, individual gravid females were observed first to lay some eggs in one solution and then the remaining of their egg batch in the other solution. This individual ovipositing behavior was not measured in this series of experiments.

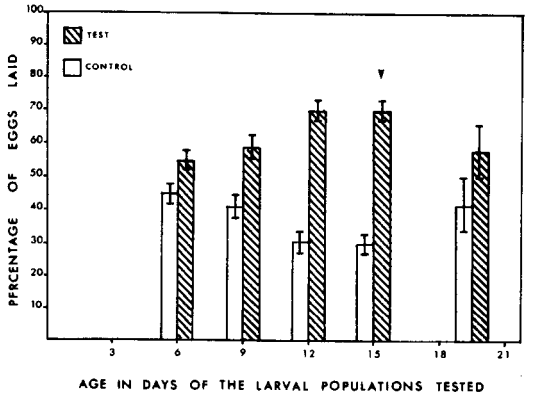

Fig. 1: Average percentage (± SE) of eggs laid by *Ae. atropalpus* females in axenic larval rearing waters (cross hatched) and their respective controls (white) according to age of axenic reared larvae. The larval density is approximately 500 larvae/liter (arrow: larval holding water after a 48-hr larval immersion of 14-day old 4th-instar larvae).

RESULTS AND DISCUSSION

When *Ae. atropalpus* females were offered a choice between sterile distilled water and sterile LHW, there was a significant difference in the respective number of eggs laid; 70.4±3.3% of 9,517 eggs were deposited in LHW (t=10.16; 0.01>P>0.005). One may conclude that 4th-instar even when absolutely free of any microorganisms, are responsible for the attraction of ovipositing females by LHW.

The results of the second experiment are shown in Fig. 1. All the tested larval rearing waters (LRW) were significantly preferred by female mosquitoes. This attractiveness was evident as early as 6 days after the axenic eggs hatched, when larvae were in the 2nd-instar. There were 55.2±2.9% of the 16,448 eggs laid in the 6-day old LRW, (t=3.52; 0.02<P<0.05). Attraction was more significant when the 9-day old LRW (3rd-instar larvae) was the test solution; 57.8±3.7% out of 47,440 eggs were laid in this LRW (t=4.741; 0.01>P>0.005). The highest values were observed when the 12-day old LRW was tested (both 3rd- and 4th-instar larvae) with 69.0±2.7% of the 26,925 eggs laid (t=14.80; P<0.001). The 19-day old LRW (4th-instar larvae with a few pupae) was also significantly preferred but showed greater variability. This may be due to the cumulative excretion of active metabolites by larvae over three weeks in a very confined milieu (flasks). The results of experiment 1 indicate that the LHW was as significantly attractive after only 48-hr of larval immersion (14-day old 4th-instar larvae) as was the case of the 12-day old LRW (Fig. 1).

In the third experiment, a 6-day old LRW and a 12-day old LRW were tested with higher

larval densities (700 and 900 larvae/liter, respectively, that being *in natura* exceptionally high densities for most of Culicidae). Under such conditions, ovipositing *Ae. atropalpus* females did not show any difference between the 6-day old test solution and its control (45.3±4.0% of the 24,818 eggs laid were in the LRW with a non-significant $t=2.64$). As for the 12-day old LRW, it was significantly repulsive with 68.6±7.9% of the 50,293 eggs laid in the control ($t=5.126$; $0.01>P>0.005$).

The third experiment was not initially designed to include an analysis of the overcrowding effect. The information, although fragmentary, is of interest for future investigation. Several factors could be set forth to explain such results. The production of an inhibitor factor by the larvae of *Ae. atropalpus* when overcrowded cannot be excluded. The repellent effect of an excessive excretory production by overcrowded larval populations also cannot be excluded either. Thus, the overcrowding effect, directly or not, could act as a regulating factor disguising the simultaneous production of oviposition attractants. In natural conditions, such a regulating mechanism could lead ovipositing females to lay their eggs in other more propitious sites.

From these results, I conclude that the larvae of *Ae. atropalpus* produced oviposition attractants. Thus, there is production of pheromone(s) by the larvae and this production begins with the youngest instars. Also, the effect of these pheromones is sufficiently high to be significantly attractive, even when the control is a complete culture medium with all minerals and vitamins needed for optimal breeding conditions. It is necessary now to conduct the same set of experiments with other species of *Aedes* before making generalizations. The evidence that pheromone produced by the larvae acts as an oviposition attractant for female mosquitoes should actively stimulate many further investigations to detect what kind of chemical communication occurs between aquatic and terrestrial stages.

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