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**BACTERIA AS OVIPOSITIONAL ATTRACTANTS FOR *CULEX
PIPIENS* (DIPTERA: CULICIDAE)**C. Lee Rockett¹

The study of factors and mechanisms involving ovipositional attractants in mosquitoes is still a relatively new area of research. Some early workers in mosquito biology suggested that gravid mosquitoes simply scattered their eggs indiscriminately on available aquatic sites; however, numerous field studies have failed to provide any real evidence for indiscriminate oviposition. The current idea is that oviposition involves not only locating an aquatic site, but the selection of an environment containing the necessary physical, chemical, and biotic factors required for larval development. It is desirable for female mosquitoes to be able to detect a breeding site that can successfully provide an environment suitable for development of their own species. According to Kramer and Mulla (1979), it is most likely that gravid females of different species use different factors in cueing in on and selecting ovipositional sites.

It is common knowledge that physical factors such as shade, water color, and water movement play a role in ovipositional site selection by mosquitoes. While our knowledge is incomplete concerning all the physical factors affecting oviposition, our understanding is even less complete in areas dealing with the chemical and biological factors affecting oviposition site selection.

Various investigators such as Gjullin (1961) and Ikeshoji (1975) have studied the effects of chemicals on ovipositional behavior. Numerous chemical factors such as calcium, salinity, carboxylic acids, phosphorus, ammonia, tannic acid, pH, ferric chloride, aluminum chloride, sucrose, decanoic acids, and creosote have been investigated. Among the chemicals influencing mosquito oviposition are certain pheromones associated with eggs, larvae, and pupae which serve as ovipositional attractants. These pheromones have been associated with egg rafts of *Culex pipiens* L. (Starratt and Osgood, 1973). According to Kramer and Mulla (1979), pheromones associated with immature mosquitoes do not appear to operate in the same way for all mosquito species. For example, Bentley et al. (1976) did not find any ovipositional attractants to be associated with *Aedes triseriatus* (Say) eggs but did get a positive effect from larval waters.

Attractant pheromones are not always (as might be expected) species specific. Maire (1984) determined that *Ae. atropalpus* (Coquillett) did not differentiate between *Ae. atropalpus* and *Ae. communis* (DeGeer) larval holding waters. Starratt and Osgood (1973) indicated that different mosquito species may use closely related or identical pheromones. Some mosquito species do appear to exhibit species-specific characteristics in regards to immature pheromones. According to Hudson and McLintock (1967), *Cx. tarsalis* (Coquillett) females selected its own breeding waters over those of *Culiseta inornata* (Williston), *Ae. aegypti* (L.), and *Cx. pipiens*. It would appear that much remains to be learned about the role of pheromones as ovipositional attractants.

Investigators such as Suleman and Shirin (1981) have noted that the presence of organic matter and associated microorganisms, particularly bacteria, cause an aquatic environment to be more attractive for gravid females. Kaul et al. (1977) mentioned a distinct relationship between organic levels and larval density in *Cx. quinquefasciatus* (Say). They suggested that the high organic levels are associated with significant bacterial activity.

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Maw (1970) indicated that bacteria are ovipositional attractants but did not identify any bacteria. Gerhardt (1959) could not correlate mosquito breeding in his test sites with pH, carbon dioxide, chlorides, sulfates, acid carbonates, and ammonia. He did suggest that mosquitoes are attracted to products of decay from organic materials.

While the general consensus among oviposition researchers is that bacteria may serve as ovipositional attractants, few individuals have attempted to identify specifically the bacterial species which serve as ovipositional attractants. Common practice has simply been to say that "bacteria" serve as attractants or that bacteria "X" are found in high numbers in a particular breeding site.

Hazard et al. (1967) concluded that bacteria in hay infusions produce chemicals that are ovipositional attractants and that the primary bacteria in these hay infusions were *Aerobacter* (now *Enterobacter*) *aerogenes*. He further reported that bacterial attraction by ovipositing mosquitoes was of adaptive value for the mosquitoes since bacteria are reported to stimulate the growth of mosquito larvae. Steelman and Colmer (1970) were unique in that they did attempt to identify specific bacteria as attractants but only looked at coliform bacteria. Steelman and Colmer (1970) did determine that one coliform (*Escherichia coli*) was more attractive to ovipositing female mosquitoes than another coliform (*Enterobacter aerogenes*). Both of these coliform species are commonly found in polluted waters. Many other bacteria also commonly occur in mosquito breeding waters. Ikeshoji et al. (1975) observed that *Pseudomonas aeruginosa* (Schroeter) could produce an ovipositional attractant if cultured on fatty acid substrates.

The purpose of this work was to investigate further the specific role of bacteria as ovipositional attractants for a single species of mosquito, *Culex pipiens pipiens* L. (northern house mosquito). A specific objective of this work was to gain additional insight into the relative, independent attractant values for oviposition, among selected species of bacteria (pure cultures) commonly found in natural breeding waters for *Cx. pipiens*.

MATERIALS AND METHODS

Egg rafts and larvae of *Cx. pipiens pipiens* were collected in the vicinity of Bowling Green, Ohio. The mosquitoes were subsequently maintained in the laboratory until molting to adult had occurred. Adults (100–200) were maintained in screen cages (80 × 40 × 40 cm) and initially fed on sucrose-soaked cotton pads and raisins. Maintenance temperature, Rh, and photoperiod were approximately 26°C, 80%, and 15L:9D, respectively. Approximately 3 days after molting to adult had occurred, adults were provided with a blood meal, normally a domestic chicken or house sparrow. Oviposition containers were placed in the stock cages 3–4 days after the females had been provided with a blood meal. In conducting replicates of individual experiments, placement sites for the specified ovipositional containers were varied in an attempt to negate any attractant effect on oviposition from extraneous environmental variables. The containers were left overnight and the resultant egg rafts were collected and counted the following day.

Ovipositional media containing pure cultures of individual bacterial species were varied according to the experiment being conducted. Except as noted, all selected bacteria utilized were commonly obtained from natural breeding areas for *Cx. pipiens*. No attempt was made to directly cross compare all of the different bacterial species commonly found in breeding waters. A series of six experiments was conducted. Basic procedural methodology for all experiments was similar; any variations in methodology for an individual experiment are noted.

1. Pure culture cells of *Pseudomonas maltophilia* (Hugh and Ryschenkow) and *Escherichia coli* (Migula) were washed from agar plates with 50 ml of distilled water by agitation with a bent glass rod. The resultant "wash" waters of each bacterial species were then placed in separate 250 ml beakers for use as an ovipositional container. Each of the two beakers was placed in one end of two separate cages containing gravid female mosquitoes. A beaker containing only distilled water (50 ml) was placed on the other end

of each cage. The number of egg rafts was counted the following day and used as an indicator of bacterial attractiveness over distilled water. Two replicates were done for each bacteria-distilled water combination in the two cages.

2. A bacterial wash of *Enterobacter agglomerans* (Beijerinck) was placed in one end of a cage and compared with an agar wash placed at the other end of the cage. The procedure was repeated in another cage by using *P. maltophilia* instead of *E. agglomerans*. An agar wash was prepared by simply washing a plain agar plate with 50 ml of distilled water. The agar medium was the same as that used for culturing the bacteria. This procedure allowed standardization of the bacterial and distilled water washes. Two replicates were done with both cages.

3. Washes of *Bacillus cereus* (Frankland), *P. maltophilia*, and agar were placed in a single cage. Four replicates were done.

4. Separate washes of *P. maltophilia* and *B. cereus* were placed on separate ends of a single cage. The agar wash was excluded. Five replicates were done.

5. The relative attractiveness of *B. cereus* versus *E. agglomerans* was compared. Three replicates were done.

6. The relative attractiveness of *E. aerogenes* Hormache and Edwards, *P. maltophilia*, and *Staphylococcus epidermidis* (Winslow and Winslow) were compared by placing individual washes of the three species in a single cage. *S. epidermidis* was chosen because, unlike the other bacterial species used in this work, *S. epidermidis* is a common bacterium on human skin and not in breeding waters for *C. pipiens*. Three replicates were done.

Statistical determinations for all experiments, when utilized, were obtained by using the Chi-Square Test.

RESULTS

The following results were obtained for the series of experiments.

1. There was a highly significant difference ($P < 0.001$) in the degree of attractiveness for gravid mosquitoes in comparing the combined washes of *P. maltophilia* and *E. coli* over distilled water. *P. maltophilia* and *E. coli* had a combined total of 72 egg rafts (61 and 11 respectively) as compared to six rafts in the distilled water. Four of the six rafts noted in the distilled water containers, were in the cage containing the *P. maltophilia* wash.

2. *P. maltophilia* and *E. agglomerans* washes had a combined total of 68 egg rafts (34 and 34 respectively) in comparison with 21 egg rafts for the agar washes. Fifteen of the 21 egg rafts noted in the plain agar washes, were in the cage containing the *P. maltophilia* wash. The difference between the combined bacterial washes versus the agar washes were highly significant ($P < 0.001$).

3. Utilization of *P. maltophilia*, *B. cereus* and an agar wash in a single cage also resulted in significant difference ($P < 0.001$) in attractiveness for ovipositing females. A total of 139, 117, and 43 egg rafts were observed in the *P. maltophilia*, *B. cereus*, and agar wash waters, respectively. It was again very obvious the bacterial washes were much more attractive than the agar wash. The difference in attractiveness between the two bacterial species was not appreciable. Also, there was considerable variation in individual replicate results (Table 1).

4. In the direct comparison between *P. maltophila* and *B. cereus*, *P. maltophila* was not significantly more attractive than *B. cereus* with 102 and 80 egg rafts respectively.

5. In the direct comparison between *B. cereus* and *E. agglomerans*, *B. cereus* with 80 egg rafts counted was significantly ($P < 0.001$) more attractive to gravid mosquitoes than *E. agglomerans* with 18 egg rafts counted.

6. Comparison of three bacterial species in a single cage resulted in 31 egg rafts, for *P. maltophilia*, 13 for *Staphylococcus epidermidis*, and only six rafts for *E. aerogenes*. There was a significant difference in attractiveness ($P < 0.001$).

Table 1. A comparison of *P. maltophilia*, *B. cereus*, and agar washes as ovipositional attractants for *Cx. pipiens*.

Replicate	Number of egg rafts		
	<i>Pseudomonas maltophilia</i>	<i>Bacillus cereus</i>	Agar wash
1	15	20	3
2	22	19	5
3	30	50	17
4	72	28	18
Totals	139	117	43

DISCUSSION

The results of this work further substantiate the idea that complex discriminating factors are involved in the selection of oviposition sites. From this set of preliminary investigations on bacteria as ovipositional attractants, the conclusion is that bacteria do act as oviposition attractants for at least some mosquitoes, notably *Cx. pipiens*. Even though bacteria obviously served as an attractant for oviposition, the bacterial attractancy was not sufficient to ensure 100% placement of egg rafts in the bacterial washes as compared to the distilled water or distilled water-agar washes.

Gerhardt (1959) noted that lab experiments on oviposition behavior are difficult to do because of the reactions of mosquitoes in a restricted environment such as cages. He further noted that, in the laboratory, mosquitoes will often oviposit in anything that contains water, even solutions lethal to eggs. The actual reason as to why a few mosquitoes will oviposit in distilled water or agar wash-water over bacterial washes is not known. As stated previously, eggs themselves may serve as an ovipositional attractant. If only a few (or even one) gravid mosquitoes would display unusual oviposition behavior and deposit a few eggs in the distilled water, this act would conceivably make the distilled water slightly more attractive for oviposition.

The major objective of this work was to determine if house mosquitoes could actually select one species of bacteria over another when ovipositing. With three replicates being performed, there was a highly significant difference in the ovipositional attractiveness of *B. cereus* versus *E. agglomerans*. These results would tend to provide additional evidence that *Cx. pipiens* can detect difference in at least some bacteria. While both *B. cereus* and *E. agglomerans* are commonly found in breeding waters of *Cx. pipiens* in northwest Ohio, these two bacterial species do not appear to share equal values of attractiveness.

Other species did appear to share somewhat equal values as ovipositional attractants. *P. maltophilia* was not significantly more attractive to ovipositing mosquitoes than *B. cereus*. With the experiment involving a direct comparison (single cage) between *P. maltophilia*, *E. aerogenes*, and *Staphylococcus epidermidis*, washes utilizing *S. epidermidis* yielded over twice as many egg rafts as washes of *E. aerogenes*.

The relatively low oviposition attractiveness of *E. aerogenes* in this work tends to conform with the results of Steelman and Colmer (1970) who found that in comparing the ovipositional attractiveness of various coliform bacteria, *Escherichia coli* washes were more attractive as an oviposition site for *Cx. pipiens quinquefasciatus* than washes of *E. aerogenes*. *E. aerogenes* is considered as being low in attractiveness only if compared with more attractive bacteria. Steelman et al. (1970) found washes of *E. aerogenes* to be considerably more attractive than sterile water with 95% of the egg rafts being placed in *E. aerogenes* washes over sterile water. Hazard et al. (1967) did indicate that *E. aerogenes* was an ovipositional attractant; however, the bacterial washes utilized were

obtained from hay infusions in which the author stated that *E. aerogenes* was simply the primary species. The presence of other bacterial species was not mentioned.

It should be stressed that, in this work, all reported comparisons on bacterial attractiveness for oviposition involved fresh wash waters that were used only overnight and then discarded. The degree of ovipositional attractiveness exhibited by the various bacteria in fresh wash waters has not been necessarily the same if bacterial washes are left undisturbed for 2 or 3 days. It is tempting to speculate that attractant variables other than bacteria are coming into play. For example, egg and larval pheromones are undoubtedly important.

This was intended to be a preliminary work. Additional work in the laboratory and field must be conducted to fully delineate the role of bacteria as ovipositional attractants for gravid mosquitoes. It is tempting to speculate that, in the future, bacteria might be utilized as ovipositional "lures" for mosquito control purposes.

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