Role of Bacteria in Mediating the Oviposition Responses of *Aedes albopictus* (Diptera: Culicidae)

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ABSTRACT The responses of *Aedes albopictus* to sources of oviposition attractants and stimulants were evaluated with a behavioral bioassay in which females attracted to odorants emanating from water were trapped on screens coated with an adhesive. Gravid mosquitoes were attracted to volatiles from larval-rearing water and soil-contaminated cotton towels. Bacteria were isolated from these substrates and from an organic infusion made with oak leaves. Through fatty acid-methyl ester analyses, six bacterial isolates from larval-rearing water, two isolates from soil-contaminated cotton towels, and three isolates from oak leaf infusion were identified to species. The response of gravid mosquitoes to these isolates was also evaluated in behavioral bioassays. Water containing *Psychrobacter immobilis* (from larval-rearing water), *Sphingobacterium multivorum* (from soil-contaminated cotton towels), and an undetermined *Bacillus* species (from oak leaf infusion) elicited significantly higher oviposition than control water without bacteria. Only volatiles collected from larval rearing water elicited significant electroantennogram responses in females.

KEY WORDS *Aedes albopictus*, bacteria, organic infusion, oviposition, attractants, electroantennogram

"SKIP OVIPOSITION" behavior (Mogi and Mokry 1980) of container-inhabiting mosquitoes results in females distributing their eggs in several containers rather than as a single clutch in one container (Fay and Perry 1965, Chadee and Corbet 1987, Apostol et al. 1994). Oviposition behavior requires integration of internal and external stimuli (Kennedy 1978), and skip oviposition in particular may be affected by the tendency of gravid females to avoid ovipositing in sites that already contain conspecific eggs (Kitron et al. 1989, Chadee et al. 1990, Apostol et al. 1994). Skip oviposition may also be affected by environmental conditions (e.g., temperature, humidity, photoperiod), by physical features in the oviposition site (such as substrate texture and color), and by chemical cues (Bentley and Day 1989).

In selecting a site for egg laying, female mosquitoes use various semiochemical cues including volatile attractants and repellents, as well as contact stimulants and deterrents from fermenting or decomposing organic material. Oviposition by *Aedes* (*Stegomyia*) mosquitoes in artificial and natural containers is increased in response to infusions made by fermenting leaves, grass sod, or hay in water (Hazard et al. 1967, Reiter et al.1991, Lampman and Novak 1996, Trexler et al. 1998). Similarly, an oviposition response is elicited when gravid mosquitoes are exposed to water in which conspecific larvae were reared (Allan and Kline 1998, Bentley et al. 1976, Benzon and Apperson 1987) or water from natural mosquito production sites (Ahmadi and McClelland 1983, Wilton 1968).

Semiochemicals in infusions are often metabolites of microbial decomposition of organic matter (Millar et al. 1992, reviewed in Clements 1999). Mosquitoes exhibit a differential response to oviposition media based on the composition of microbial species. Maw (1970) reported that bacteria of the family Pseudomonaceae in the presence of decanoic acid rendered rearing water attractive to *Culex restuans* Theobald. Based on this study, Ikeshoji et al. (1975) reported that *Pseudomonas aeruginosa* Hugh produced an oviposition attractant/stimulant for *Aedes aegypti* L. and *Cx. pipiens molestus* Forskål from decanoic acid. Hazard et al. (1967) reported isolating bacterial species from an infusion of alfalfa hay that produced chemical stimulants of oviposition in *Ae. aegypti* and *Cx. quinquefasciatus* (Say). *Enterobacter* (=*Aerobacter*) *aerogenes*

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(Hormaeche and Edwards), the primary bacteria isolated from the infusion, produced volatiles that attracted *Cx. quinquefasciatus,* but not *Ae. aegypti.* Similarly, from larval-rearing water, Benzon and Apperson (1988) isolated *Acinitobacter calcoaceticus* (Beijerinck) and *Enterobacter cloacae* (Jordan) that attracted gravid *Ae. aegypti.* Vythilingam et al. (1999) also observed that *Ae. aegypti* preferred to oviposit in well water that contained *Acinetobacter anitratus* (=*Ac. baumanni*) (Bouvet and Grimont). Hasselschwert and Rockett (1988) and Pavlovich and Rockett (2000) observed differential oviposition responses by *Ae. aegypti* and *Ae. albopictus* to several bacterial species. Rockett (1987) screened a variety of bacterial species against gravid *Cx. quinquefasciatus* and reported finding significantly more egg rafts in cups that contained agar washes of *Enterobacter agglomerans* (Beijerinck), *Pseudomonas maltophilia* (Hugh), and *Bacillus cereus* (Franklin) than in control cups, containing water only. Lastly, Wallace (1996) postulated that the attraction of *Ochlerotatus* (=*Aedes*) *taeniorhynchus* (Wiedemann) to cotton towels contaminated by soil from oviposition sites was caused by the actions of bacteria and/or fungi.

The objectives of our research were to: (1) determine whether crude substrates (larval rearing water, oak leaf infusion, and soil-contaminated towels) that were known to elicit an oviposition response from some *Aedes* or *Ochlerotatus* mosquitoes were also active with *Ae. albopictus;* (2) isolate and identify bacterial species from these substrates; (3) ascertain whether any increased oviposition resulted from responses to the presence of the bacteria; and (4) determine whether specific bacteria produced volatile oviposition attractants and/or contact chemical stimulants.

Materials and Methods

Mosquito Colony Origin and Maintenance. *Aedes albopictus* eggs were collected in oviposition traps in Raleigh, NC, in 1997. The colony was maintained at approximately 26°C and at a relative humidity of ≈75% under a photo regime of 14:10 h (L:D). Included in the light phase were two 30-min crepuscular periods provided by a 40-watt incandescent bulb. Larvae were fed a 2:1 mixture of liver powder:baker's yeast on a standardized schedule (Gerberg et al. 1994). Adults, kept in 30 × 30 × 30-cm Plexiglas cages fitted with cotton surgical stocking tops, were maintained on a 10% sucrose solution provided *ad libitum.* Females were fed via membrane on porcine blood that was obtained from a local slaughterhouse (Benzon and Apperson 1987).

Sources of Bacteria. Cotton towels, originally contaminated with soil from an *Oc. taeniorhynchus* production site on a dredge disposal island along the Atlantic Intracoastal Waterway near Charleston, SC, were used as a microbial source for experimentation. New cotton towels (K-Mart, Troy, MI) were cross-contaminated by first immersing them in sterile distilled water. The towels were wrung until free water could no longer be expressed. The moist towels were wrapped with contaminated towels and placed in a sterile plastic bag for 1 wk. The newly contaminated towels were allowed to dry for approximately 48 h in a sterile fume hood, transferred to a sterile plastic bag, and stored at 4°C. Bacterial isolates were cultured from the newly contaminated towels before drying.

Oak leaf infusion (OLI) was prepared by fermenting ≈126 g of white oak (*Quercus alba* L.) leaves in 15 liters of distilled water for seven days (Trexler et al. 1998). Bacterial isolates were cultured from the OLI at the end of the fermentation period.

Larval-rearing water (LRW) was collected from laboratory rearing pans containing *Ae. albopictus* larvae. Larvae were reared according to the standard techniques described above. When 50% of the larvae had pupated, all pupae and larvae were removed. LRW was filtered with a coarse grade filter paper (Whatman P8, Fisher, Pittsburgh, PA), transferred to plastic bottles fitted with tight-fitting screw cap lids, and stored at −20°C. LRW was allowed to thaw overnight before oviposition bioassays. Bacteria were cultured from LRW before freezing.

Isolation and Identification of Bacteria. Bacteria were isolated from the contaminated towels, OLI, and LRW by plating samples (100 μl) on trypticase soy broth agar (Difco, Detroit, MI) and eosin methylene blue agar (Difco, Detroit, MI) plates and incubated aerobically at 27°C. Morphologically different colonies were isolated on trypticase soy broth agar plates. Bacterial isolates were assigned alpha-numeric codes that corresponded to the source and an assigned number for each isolate.

Bacterial isolates were characterized phenotypically by cell morphology (size and shape) by phase-contrast microscopy, Gram-staining (Fisherbrand, Pittsburgh, PA), catalase test (using 3% hydrogen peroxide), oxidase test (BBL Oxidase, Becton Dickinson, Cockeysville, MD), and motility (examined on wet-mount slide under the microscope and by stabbing into SAM, soft agar medium, consisting of 10.0 g/liter tryptose, 5.0 g/liter NaCl, 5.0 g/liter agar) (Perry and Staley 1997). Members of Enterobacteriaceae were identified phenotypically by API 20E test (BioMerieux Vitek, Hazelwood, MO). Other bacterial species were identified by fatty acid methyl ester (FAME) analyses and comparing the FAME profiles to those of known bacterial species (Kaufman et al. 1999). For each species identified, a similarity index was calculated. The similarity index ranges from 0 to 1, and values closer to one indicate a close match to authentic standard values.

Oviposition Bioassays. Two-choice, open cup assays were used to determine the effect of bacteria on mosquito oviposition activity. Two polypropylene cups (120 ml), spray-painted black, were placed in opposite corners of a 30 × 30 × 30-cm Plexiglas oviposition cage. Each cup was lined with seed germination paper (Steinley et al. 1994) and filled with 30 ml of the test solution or an equivalent volume of water. Mosquitoes were supplied continuously with a 10% sucrose solution. Photophase light was provided by two 60-watt

fluorescent bulbs suspended over each shelf. Crepuscular light was provided at the beginning and end of the scotophase by a 40-watt incandescent bulb. A long daylength light cycle (16 h:8 h, L:D) was used during experimentation.

Four days before the initiation of a trial, adults were blood-fed on a human hand. The protocol that involved blood-feeding virus-free mosquitoes on a human was approved by the Institutional Review Board at North Carolina State University (Human Use Protocol IRB# 1388). Gravid females not used in bioassays were allowed to oviposit on seed germination paper, and these eggs were used to maintain the mosquito colony. The F_4-F_7 generations were used in experiments described below.

Bacterial isolates were bioassayed using the methods of Benzon and Apperson (1988). Isolates were inoculated (3.75×10^4 cells/ml) into 200 ml of dilute (12.5%) Bacto nutrient broth (NB) (Difco) and were allowed to grow at 26°C for 18 h. Cell densities were determined by direct count in a hemocytometer under a compound microscope. Test cups contained 30 ml of the inoculated NB and control cups contained the same volume of uninoculated NB.

Oviposition responses to contaminated towels were evaluated using two bioassay methods. In the first method, 2.5 × 7.5-cm strips of contaminated and control towels were clipped to the insides of separate oviposition cups. Control towels consisted of new 100% cotton towels that were autoclaved and then soaked in sterile distilled water. The excess water was wrung out, and the moist towels were placed in sterile bags for 1 wk at approximately 26°C. After the storage period, these towels were allowed to dry in a sterile hood, and strips of these towels were used as control oviposition substrates. Each oviposition cup contained 30 ml of distilled water, which moistened the towels. An experimental and a control cup were placed in opposite corners of the bioassay cage and a single gravid female was transferred into the cage. After 24 h, cups were removed and the eggs deposited on control and test ovistrips were counted.

In the second bioassay method, 2.5 × 2.5-cm strips of contaminated and control towels were placed in the bottom of oviposition cups. Each oviposition cup was lined with seed germination paper and filled with 30 ml of distilled water. The water volume was sufficient to completely submerge the towel so that the mosquitoes were unable to contact the towel surface. An experimental and a control oviposition cup were placed in opposite corners of a bioassay cage and a single gravid female was transferred into the cage. The eggs on the experimental and control substrates were counted after a 24-h exposure period. In both methods, the eggs floating on the water surface and submerged in the cups were counted and added to the total number of eggs laid on the oviposition substrates. The toweling experiments were each replicated 15 times.

To differentiate oviposition responses elicited by odorants from those induced by contact chemo-stimulation, we used the sticky-screen bioassay of Isoe et al. (1995) with modifications described in Trexler et al. (1998). In this bioassay, gravid mosquitoes were presented a choice between a test and a control cup. Each cup was covered with a sticky-screen with a mesh size that prevented females from entering without landing on the screen. Therefore, any positive or negative oviposition responses were caused by volatiles emanating from the test cup. Sticky screens were prepared using an insect glue (Tanglefoot, Grand Rapids, MI) and galvanized hardware cloth screen (6-mm mesh, Gilbert and Bennet, Toccoa, GA) as described previously (Trexler et al. 1998). Two 125-ml black polypropylene cups, covered with a glue-coated screen, were placed in opposite corners of each oviposition cage. Each cup was filled with either 30 ml of the test solution or an equivalent volume of control solution. Ten gravid females were placed in each cage, and after a 24-h exposure period, the females trapped on each screen were counted.

The oviposition activity index (OAI) (Kramer and Mulla 1979) was used to evaluate the responses of the females to test substances. The OAI was calculated for each experimental replicate as: $OAI = (N_t - N_c)/(N_t + N_c)$, in which N_t is the number of females trapped on the screen over the test cup, and N_c is the number of females trapped on the screen over the control cup. The OAI is a measure of the proportion of females trapped on the screen over the test cup after correcting for the number of females trapped on the screen over the control cup. The OAI varies from −1 to +1, with 0 indicating no response.

Volatiles Collection. Two methods were used to collect headspace samples of odorants. In the first method, volatiles were collected from crude LRW and contaminated towel using a closed collection system, consisting of adsorbent (Tenax TA 60/80 mesh) that was packed into a 6-mm OD × 120-mm long glass tube. To collect volatiles, air was drawn across the adsorbent over a 24-h period. The column was washed with hexane (3 × 25 ml) to remove the volatiles from the adsorbent, and the hexane elutent was evaporated under nitrogen to a volume of ≈2 ml. In the second method, suspensions of LRW4 and T2 were grown separately in trypticase soy broth over a 24-h period in Pyrex test tubes sealed with rubber septa. Volatiles were removed (2 ml) from the headspace above the bacterial suspensions with an airtight glass syringe.

Electrophysiology. Electroantennogram (EAG) recordings were made on excised heads of gravid female mosquitoes (Blackwell et al. 1993, Du and Millar 1999). Ag-AgCl wires, 0.5 mm in diameter, were inserted into glass capillary tubes that were filled with physiological saline (Kurtti and Brooks 1976). The end of one antenna, severed just below the penultimate segment, was inserted into a glass capillary tube that contained the recording electrode. The base of the head was placed into the glass capillary that contained the reference electrode. The antenna experienced a constant flow of humidified air (1.5 liters/min), which adapted the mechanoreceptors on the antenna. Each test solution (10 μl) was applied to a filter paper strip and the solvent was allowed to evaporate. The filter

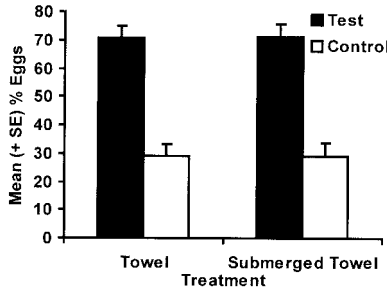

Fig. 1. Oviposition responses of *Aedes albopictus* to microbially contaminated cotton towels. Mosquitoes laid eggs directly on towels in the towel treatment ($n = 15$). In the submerged towel treatment ($n = 15$), mosquitoes were unable to contact the towels. Eggs were laid on seed germination paper.

paper was then inserted into a Pasteur pipette and attached to a 2-ml glass syringe. A single, rapid puff of test odorant was then introduced into the airstream by depressing the syringe's plunger. The signal was amplified by a variable DC amplifier (Grass P16, Astro-Med, West Warwick, RI). It was acquired through an A/D board installed in an HP5890 GC and recorded and analyzed with ChemStation software (Agilent Technologies, Palo Alto, CA). The response was quantified by measuring the peak amplitude.

Statistical Procedures. Results of binary open-cup bioassays were analyzed using a randomized complete block design. Experiments were blocked by shelf, and treatments were assigned randomly to each of the six cages on the shelf. Analysis of variance (ANOVA) tests were performed on untransformed counts of the numbers of eggs deposited in test and control cups using a generalized linear model procedure (PROC GLM, SAS Institute 1999b.).

Results of sticky-screen bioassays were analyzed by a nonparametric signed-rank test (PROC UNIVARIATE, SAS Institute 1999a) to determine whether the mean oviposition activity index for each treatment was significantly different from zero.

EAG recordings were analyzed using a *t*-test (PROC MEANS, SAS Institute, 1999a). A data set containing the differences between the responses to the control solutions and the responses to the test solutions was used to generate a *t* statistic. Differences in responses were tested to determine whether the mean differences were significantly different from zero.

Table 1. Oviposition responses of *Aedes albopictus* to larval rearing water

Concentration	*n*	Mean no. eggs in test cup (SE)	Mean no. eggs in control cup (SE)	$P > t^a$
10%	7	55.1 (8.0)	18.6 (5.7)	0.003
100%	7	53.3 (7.2)	20.0 (4.5)	0.002

[a] The mean numbers of eggs laid in the test and control cups are significantly different by a Student's *t*-test at $P < 0.05$.

Table 2. Responses of *Aedes albopictus* to oviposition substrates in sticky-screen bioassays

Substrate	No. cages	No. females responding	No. on test	No. on control	Mean OAI[a]	*P*
Towel[b]	23	187	111	76	0.21	0.007
LRW[c]	23	178	120	58	0.36	0.0001

[a] Oviposition activity index. Significance of differences from zero were determined by a signed rank test (PROC UNIVARIATE, SAS, 1999a).
[b] Towel was submerged in water.
[c] 100% concentration of larval rearing water (LRW).

Results

Oviposition Responses to Contaminated Towels. Towels presented either above or below the surface of the water significantly ($P < 0.01$) increased oviposition by *Ae. albopictus* (Fig. 1). Cups containing contaminated towels as oviposition substrates received a mean of 70.9 ± 7.0% (±SE) of the total number of eggs deposited (df = 1; $F = 17.4$; $P = 0.0003$). When toweling was submerged, *Ae. albopictus* females laid a mean of 71.0 ± 8.4% of the total eggs in cups that contained contaminated toweling (df = 1; $F = 18.9$; $P = 0.0002$).

Oviposition Responses to Larval-Rearing Water. In response to a 10% concentration of LRW, significantly (df = 1,6; $F = 17.4$; $P = 0.003$) more eggs were laid in cups containing LRW than in cups containing water (Table 1). Similarly, *Ae. albopictus* laid significantly (df = 1,6; $F = 15.2$; $P = 0.002$) more eggs in cups containing a 100% concentration of LRW than in cups containing distilled water.

Sticky-screen Bioassays of Contaminated Towels and Larval-Rearing Water. Significantly more females were trapped on screens that covered cups containing contaminated towels than in cups that contained control towels (Table 2). Of the 187 females responding in the bioassay, 111 were trapped over the test towel and 76 were trapped on screens over the control towel. The mean OAI of 0.21 was highly significant ($P = 0.007$). Similarly, significantly more females were trapped on screens over cups containing 100% LRW

Table 3. Species identifications of bacteria isolated from three sources

Isolate	FAME library ID	Similarity index
LRW-2	*Micrococcus luteus* (Schroeter)	0.75
LRW-4	*Clavibacter michiganese* (Smith)	0.81
LRW-5	*Psychrobacter immobilis* (Juni and Heym)	0.65
LRW-6	*Bacillus brevis* (Migula)	0.49
LRW-7	*Micrococcus kristiae* (Kloos)	0.76
LRW-8	*Rhodococcus* sp.	0.04
T-1	*Serratia marcescens* (Bizio)	phenotype
T-2	*Spingobacterium multivorum* (Yabuuchi)	0.75
T-3	*Serratia marcescens* (Bizio)	phenotype
OLI-1	*Bacillus cereus* (Frankland)	0.67
OLI-2	*Bacillus* sp.	0.42
OLI-3	*Paenibacillus pabuli* (Nakamura)	0.72

Table 4. Oviposition responses of *Ae. albopictus* to bacterial isolates in binary, open-cup bioassays

Isolate[a]	*n*	Mean no. eggs in test cup (±SE)	Mean no. eggs in control cup (±SE)	df[b]	*F*	$P > F$
LRW-2	29	42.7 (3.6)	41.1 (4.9)	39	0.07	0.80
LRW-4	28	43.3 (4.1)	34.5 (4.2)	38	2.23	0.14
LRW-5	27	52.1 (4.1)	36.9 (4.0)	37	5.50	0.025
LRW-6	20	34.9 (4.9)	29.2 (4.1)	25	0.90	0.35
LRW-7	15	28.3 (5.5)	42.2 (5.6)	20	3.51	0.076
LRW-8	26	39.1 (3.9)	45.4 (3.3)	33	1.50	0.23
OLI-1	23	40.3 (4.6)	30.4 (4.7)	30	1.74	0.19
OLI-2	17	49.0 (4.6)	33.1 (5.3)	21	4.41	0.048
OLI-3	19	43.0 (4.5)	41.3 (5.5)	25	0.05	0.83
T-1	31	47.2 (4.4)	48.8 (4.1)	43	0.06	0.80
T-2	29	49.2 (3.5)	32.9 (4.0)	40	7.44	0.0094
T-3	20	29.7 (6.8)	38.9 (7.5)	26	0.70	0.41

[a] Refer to Table 3 to determine the corresponding bacterial species.
[b] Error df. The numerator df in each *F* test was 1.

than on screens over cups that contained distilled water (OAI = 0.36; $P = 0.0001$). Of the 178 females that responded, 120 were trapped on screens over cups that contained LRW.

Isolation and Identification of Bacterial Isolates. Eight bacterial isolates were cultured from LRW on trypticase soy broth agar plates. We were not able to keep LRW-1 and LRW-3 viable. Five isolates were identified by FAME to the species level; one isolate (LRW-8) was identified to genus only (Table 3).

We cultured three bacterial isolates from the contaminated toweling. However, phenotypic characterization revealed that isolates T-1 and T-3 were nonpigmented *Serratia marcescens* (Bizio), a common contaminant of insect colonies and a facultative pathogen of insects. The third isolate (T-2) was identified by FAME as *Sphingobacterium multivorum* (Yabuuchi).

Three isolates were cultured from OLI. All three isolates were gram-positive. We were able to determine the species of isolate OLI-1 (*Bacillus cereus* [Frankland]) and isolate OLI-3 (*Paenibacillus pabuli* [Nakamura]). Isolate OLI-2 could only be determined as a *Bacillus* species.

Oviposition Responses to Bacterial Isolates. In binary, open-cup bioassays, *Ae. albopictus* laid significantly more eggs in cups that contained cultures of LRW-5 (*Psychrobacter immobilis*), OLI-2 (*Bacillus* sp.), and T-2 (*Sphingobacterium multivorum*) (Table 4). *Aedes albopictus* laid a mean of 52.1 ± 4.1 eggs in response to cups containing LRW-5, compared with 36.8 ± 4.0 eggs laid in cups that contained uninoculated bacto nutrient broth. Significantly more eggs were laid in cups that contained OLI-2 (49.0 ± 4.6 eggs) than in cups that contained uninoculated broth (33.1 ± 5.1 eggs). In response to T-2, significantly more eggs were laid in cups that contained the bacteria (47.7 ± 3.4) than in cups that contained the control broth (33.8 ± 3.4).

No other bacterial isolates elicited a significantly greater oviposition response compared with control substances. However, fewer eggs were laid in response to two bacterial isolates (LRW-7 and LRW-8) compared with controls, but the differences were not statistically significant ($P > 0.05$).

Sticky-screen Bioassays of Bacterial Isolates. Four isolates were evaluated to determine if volatile chemicals influenced oviposition responses of *Ae. albopictus.* None of the isolates tested were significantly attractive or repellent to gravid mosquitoes (Table 5). LRW-4 and OLI-2 elicited marginally positive OAI values, whereas LRW-5 and T-2 elicited marginally negative OAI values.

Electroantennogram Responses to Bacterial Isolates. Of the four collections of volatiles that were made, only collections from crude LRW elicited significant responses ($P < 0.05$) in EAG studies (Table 6). Collections from contaminated towels and LRW-4 also elicited EAG responses >1. However, differences between antennal responses to test and control substances were not significantly different than 0. Volatiles collected from isolate T-2 also did not elicit significant responses from *Ae. albopictus* antennae.

Table 5. Oviposition responses of *Aedes albopictus* to bacterial isolates in sticky-screen bioassays

Bacterial isolate	No. cages	OAI[a]
LRW-4	16	0.17
LRW-5	18	−0.027
OLI-2	18	0.15
T-2	18	−0.01

[a] OAI, Oviposition activity index. OAI values are not significantly different from zero ($P > 0.05$).

Table 6. Electroantennogram responses of *Aedes albopictus* to volatiles produced by bacteria

Isolate	*n*	Mean ratio[a]	t	$P > t$[b]
LRW-4	3	1.24 (0.38)	0.63	0.59
T-2	4	0.96 (0.16)	−0.24	0.82
LRW crude	7	1.26 (0.10)	2.50	0.046
Towel crude	3	1.13 (0.08)	1.67	0.24

[a] Ratio of test substance and hexane (negative control).
[b] Student's *t*-tests conducted on the mean differences of antennal responses to the chemicals versus hexane controls.

Discussion

Oviposition Bioassays. *Aedes albopictus* laid significantly more eggs in cups that were baited with either LRW or microbially-contaminated cotton towels. Our results agree with previous research reports (Gubler 1971, Allan and Kline 1998) that *Ae. albopictus* females lay significantly more eggs in cups that contain larval rearing or holding water. In our experiments with LRW, ≈75% of all eggs were laid in either 10% or 100% larval rearing water. Gubler (1971) found that *Ae. albopictus* preferred to oviposit in containers holding water that held conspecific larvae or water from oviposition traps in which conspecific eggs were laid. Approximately 67% of all eggs were laid in these two containers compared with four other control and test containers. Similarly, Allan and Kline (1998) reported that *Ae. albopictus*, in a binary choice assay, laid ≈60% of all eggs in containers of 50% LRW versus containers with tap water.

Wallace (1996) demonstrated that field populations of *Oc. taeniorhynchus* laid 100-fold more eggs in microbially contaminated towels compared with uncontaminated towels. Although we did not see a 100-fold difference in oviposition response between the treatment and control containers in laboratory bioassays, significantly more *Ae. albopictus* eggs were laid either on the contaminated towels or in cups that contained a submerged piece of the contaminated towel.

Results of sticky-screen bioassays indicate that both LRW and contaminated cotton toweling produce odorants that are marginally attractive to gravid *Ae. albopictus.* In previous studies (Trexler et al. 1998), oak leaf infusions did not produce chemical attractants in sufficient quantities to influence the oviposition responses of *Ae. albopictus.* Oak leaf infusion elicits increased egg laying through contact chemical stimulation.

Isolation and Identification of Bacteria. We isolated and identified eleven species of bacteria from the three oviposition substrates. The greatest number of isolates were obtained from LRW. Benzon and Apperson (1988) reported that the two predominant bacterial species in larval rearing water of *Ae. aegypti* were *Enterobacter cloacae* and *Acinitobacter calcoaceticus,* two gram-negative members of the Enterobacteriaceae that are commonly found in nature (Janda and Abbot 1998). Suspensions of both species were attractive to gravid *Ae. aegypti.* However, *A. calcoaceticus* elicited a significantly higher OAI than *E. cloacae,* and the oviposition responses of *Ae. aegypti* to suspensions of *A. calcoaceticus* were similar to those obtained from larval holding water (Benzon and Apperson 1988). We did not isolate either bacterial species from *Ae. albopictus* larval rearing water in the laboratory. It is possible that these two species associate with *Ae. aegypti,* but not with *Ae. albopictus,* hence these bacterial species elicit a very strong oviposition response from *Ae. aegypti* females. In addition, *Ae. aegypti* females may inoculate additional oviposition sites with the bacteria. It is also possible that these bacterial species were present in *Ae. Albopictus*-rearing water, but we were unable to isolate them.

Fungi were observed growing on the contaminated towels. Although we did not make any attempts to isolate and bioassay the fungi, future studies should examine the role of fungi in the production of volatile oviposition attractants.

Response to Bacteria. Oviposition responses to the bacterial species tested in open-cup bioassays were highly variable, possibly because we used dilute nutrient broth in our experiments to maintain a slow growth rate of the bacterial population (Benzon and Apperson 1988). In bioassays of the isolates that were obtained from LRW, only cups that contained suspensions of *Psychrobacter immobilis* elicited more oviposition than cups containing uninoculated nutrient broth. Approximately 60% of all of the eggs were laid in cups containing this bacterial species. However, the response of *Ae. albopictus* to *P. immobilis* alone does not account for the highly attractive nature of LRW. In experiments with both 10% and 100% LRW, 70% and 75% of all eggs were laid in test cups, respectively. It is possible that a combination of the bacterial species would elicit a stronger oviposition response, increasing the percentage of eggs that are deposited in test containers. Moreover, we used only aerobic culturing techniques in this study. Microaerophiles, strict anaerobes and bacteria that were noncultivable on our artificial media were not isolated. Additional research should examine the roles that these bacterial groups play in mediating oviposition by *Ae. albopictus.*

Hasselschwert and Rockett (1988) and Pavlovich and Rockett (2000) reported *Bacillus cereus* to elicit significant oviposition responses (relative to control substances) from *Ae. aegypti* and *Ae. albopictus,* respectively. In contrast, the *B. cereus* that we isolated from OLI did not elicit a significant response relative to distilled water. Why we obtained different results is unknown.

Electroantennography. Electroantennogram experiments with volatile collections from LRW-4, T-2, larval-rearing water, and soil-contaminated towels indicate that the antennae of *Ae. albopictus* can detect volatile compounds produced by bacteria. Volatiles collected from LRW elicited significant antennal responses compared with controls. However, ratio values of similar magnitude were obtained in evaluations of volatiles collected from LRW-4 and the crude microbially-contaminated towels. Because of the difficulty in preparing antennae that gave consistent responses to positive control substances, we were not able to complete additional replicate experiments with these bacterial isolates. Additional electroantennography experiments with LRW-4 and the crude soil-contaminated towels would likely show significant response by the antennae of gravid *Ae. albopictus.* Further experiments should be conducted using coupled gas chromatography-electroantennogram detection to determine which compounds in the volatiles caused electrophysiological responses.

Bacterial Mediation of Oviposition Behavior. Benzon and Apperson (1988) suggested that survival of

larvae might be increased if a gravid female could discriminate among oviposition sites based on bacterial quantity and species composition. Our studies indicate that some bacterial species isolated from larval rearing water, oak leaf infusions, and contaminated cotton towels significantly increase the number of eggs that are laid by *Ae. albopictus.* However, compared with their crude sources, such as LRW, the bacterial species that we isolated and tested in sticky-screen bioassays did not generate volatiles that attracted gravid mosquitoes. Oviposition attractants in LRW may be composed of a blend of several volatile metabolites; and therefore, more than one bacterial species may be involved in producing odorants that are biologically active.

A second explanation is that we failed to isolate bacterial species that produce metabolites that are active as oviposition attractants. A third explanation is that visual cues are more important in site finding for oviposition than are olfactory signals. Previous studies (Wilton 1968, Beehler et al. 1992, Pavlovich and Rockett 2000) of container-inhabiting mosquitoes have reported that container color and optical density of the water in containers significantly increased the numbers of eggs laid. These and other investigations of mosquito oviposition have typically used the endpoint of oviposition (i.e., the number of eggs laid) as a measure of oviposition site preference. Although the units of oviposition behavior should be defined precisely and simply (Harris and Foster 1995), the use of egg densities as a measure of choice or an index of adult abundance is an over-simplification of the oviposition process, and can be misleading. Under natural conditions there may not be a linear relationship between egg densities in containers and the number of females visiting and/or laying eggs in containers. High rates of egg-laying may be caused by nonvolatile chemotactile stimulants rather than by attraction of an increased number of females through volatile semiochemicals (Isoe et al. 1995).

Experiments conducted with oviposition cups in small cages and still air would be expected to measure short-range attraction. Olfactory mediation of oviposition by bacteria would require demonstration of long-range attraction through use of olfactometers and wind tunnels. Consequently, future research should focus on the isolation and identification of volatile compounds of bacterial origin, and the evaluation of the long-range activity of these odorants in wind tunnel experiments.

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