

ABSTRACT

TREXLER, JONATHAN DAVID. Effects of Synthetic Chemicals and Bacteria on the Oviposition Behavior and Electroantennogram Responses of *Aedes albopictus* (DIPTERA: CULICIDAE). (Under the direction of Charles Smith Apperson.)

Five volatile synthetic chemicals (dimethyl disulfide, indole, 4-methylphenol, 3-methylindole, and trimethylamine) were tested as potential oviposition attractants of *Aedes albopictus* (Skuse) in laboratory and field experiments. In addition, the oviposition responses of *Ae. albopictus* to bacterially-enriched substrates were evaluated in behavioral and electrophysiological bioassays as sources of attractants and stimulants.

None of the five synthetic chemicals elicited a significant positive oviposition response. In laboratory bioassays that measured attraction of gravid females to olfactory stimuli, compounds were evaluated over a range of concentrations that spanned 4-5 logs. Three concentrations of 4-methylphenol and one concentration of 3-methylindole were significantly repellent. All other concentrations of the five chemicals tested did not attract more females than a water control.

The five synthetic compounds were loaded into controlled-release packets, which were used to bait water-filled ovitraps at five suburban residences. *Aedes albopictus* exhibited no oviposition preference for any of the baited traps versus adjacent traps containing only water. In addition, there was no

difference in the mean number of eggs laid per trap-day by *Ae. albopictus* among ovitraps treated with each of the five compounds.

Electroantennography indicated that *Ae. albopictus* did not exhibit a physiological response to any of the five chemicals at 0.025 mg/L.

The oviposition responses of *Ae. albopictus* to bacterially-enriched substrates were evaluated in laboratory bioassays. Gravid mosquitoes responded to volatiles from larval rearing water (LRW) and soil-contaminated cotton towels (T). Bacterial species were isolated from these substrates and from an organic infusion made with oak leaves (OLI). Through fatty acid-methyl ester analyses, 6 bacterial isolates from LRW, two isolates from T, and three isolates from OLI were identified to species. The response of gravid mosquitoes to these isolates was also evaluated in behavioral bioassays. Water containing *Psychrobacter immobilis* (from LRW), *Sphingobacterium multivorum* (from T) and an undetermined *Bacillus* species (from OLI) elicited significantly higher oviposition than control water without bacteria. Only volatiles collected from LRW elicited significant electroantennogram responses in females.

**EFFECTS OF SYNTHETIC CHEMICALS AND BACTERIA ON THE
OVIPOSITION BEHAVIOR AND ELECTROANTENNOGRAM RESPONSES
OF *Aedes albopictus* (DIPTERA: CULICIDAE)**

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CHAPTER 1.

MOSQUITO OVIPOSITION: FIELD AND LABORATORY EVALUATIONS OF ATTRACTANTS AND STIMULANTS

Introduction

Mosquito researchers have focused on a number of stages in the life cycle of various species: larval biology and ecology, mating, host-seeking, blood-feeding, and oviposition. By understanding these aspects of the mosquito life cycle, control measures can be developed and implemented to reduce the risk of pathogen transmission to humans and animals.

The chemical, physical, and behavioral factors that are involved in oviposition site selection by mosquitoes have been well studied. For a review of the literature before 1989 see Bentley and Day (1989). Since that review, a number of studies have been conducted in both the laboratory and in the field. We will not attempt to repeat the information that was presented in Bentley and Day (1989), but will focus on the research published since their review. We will discuss the work that has been conducted on isolating and identifying mosquito attractants and stimulants in the laboratory. We will also review field evaluations of attractants and stimulants, and discuss a number of effective bioassay techniques.

It is important to define the terms “attraction” and “stimulation” in regards to mosquito oviposition (Benzon and Apperson 1988). Following the definitions provided by Dethier et al. (1960), attraction refers to the process of drawing mosquitoes in from a distance to a chemical source. Attraction is an olfactory response to a chemical. Stimulation refers to the process whereby a chemical directly induces a mosquito to oviposit. Stimulation occurs when a

mosquito comes into direct contact with the chemical. We will discuss some bioassays developed to differentiate oviposition responses stemming from olfactory attraction or contact chemical stimulation.

Most studies of oviposition behavior use the endpoint of oviposition (the number of eggs laid) as a measure of the behavior. However, the units of the behavior should be defined as precisely, objectively, and as simply as possible (Harris and Foster 1995). Counting eggs is an over-simplification of oviposition behavior. The oviposition behavior of an insect results from the combination of a number of internal and external stimuli (Kennedy 1978). Factors that can act as stimuli include environmental conditions (e.g. temperature, photoperiod, humidity), physiological state of the insect, physical factors in the environment such as substrate texture and color, and chemical cues. Mosquitoes integrate a wide range of stimuli prior to the act of oviposition. Photoperiod, color and optical density of the water, oviposition substrate texture and moisture, temperature and reflectance, and volatile and contact chemical cues have all been shown to affect the choice of oviposition site by mosquitoes (Bentley and Day 1989). An additional factor affecting the endpoint of oviposition behavior is a behavior known as “skip oviposition” (Mogi and Mokry 1980), which occurs when females lay their eggs in several containers as opposed to laying their entire clutch in one container (Fay and Perry 1965, Rozeboom et al. 1973, Chadee and Corbet 1987, Apostol et al. 1994). This behavior increases the distribution of eggs in an area and may be

increased by the tendency of gravid females to avoid ovipositing in sites where eggs of conspecific females had been laid (Kitron et al. 1989, Chadee et al. 1990, Apostol et al. 1994).

Chemical cues that mediate the act of oviposition affect only part of the overall behavior of the mosquito. Much of the research on oviposition has focused on the responses of mosquitoes to crude infusions. Infusions are created by fermenting organic material such as leaves or hay in water for a specified period of time. However, relatively few attempts have been made to identify chemical stimulants and attractants of mosquitoes compared to the work done with crude infusions. Millar and Du (1997) identified three reasons for this discrepancy. First, the complex nature of organic infusions makes them difficult to fractionate and time-consuming to bioassay. Second, oviposition behavior is often mediated by chemical blends instead of single compounds. Finding the correct chemical blend can be difficult, and trial and error may be necessary. Third, bioassays of attractants and stimulants sometimes do not differentiate attraction to volatile chemicals from oviposition responses to contact chemical stimulants.

Laboratory Evaluations

Kentucky bluegrass sod increased the number of *Aedes albopictus* eggs that were laid in cups relative to water controls (Lampman and Novak 1996). In addition, females oriented to containers with sod infusion in experiments with an olfactometer.

Cups that contain the larval rearing water of *Ae. albopictus* or *Aedes aegypti* receive significantly more *Ae. albopictus* eggs than cups that contain water (Allan and Kline 1998). Cups that contain the eggs of *Ae. aegypti* or *Ae. albopictus* receive significantly more *Ae. aegypti* eggs than controls.

The attractiveness or repellence of an infusion can change over time (Kramer and Mulla 1979). Therefore, Isoe et al. (1995b) studied the effects of aged Bermuda grass infusions on the oviposition responses of *Culex quinquefasciatus* and *Culex tarsalis*. Using infusions aged from 0 to 63 days, they found that gravid *Cx. quinquefasciatus* were stimulated to oviposit by every age of infusion tested, whereas *Cx. tarsalis* was only stimulated by infusions aged from 5 to 25 days. Trexler et al. (1998) evaluated 7, 28, and 60 day-old oak leaf infusions on the oviposition responses of *Ae. albopictus* and *Ae. triseriatus* in the laboratory. They found that *Ae. albopictus* laid significantly more eggs in all ages of infusions versus water controls. *Aedes triseriatus* preferred 60 day-old infusions over the other infusion ages relative to water controls.

Allan and Kline (1995) examined the oviposition responses of *Ae. albopictus* and *Ae. aegypti* to both infusions and compounds that were identified as *Culex* oviposition attractants by Millar et al. (1992). Hay infusion and field-collected rearing water significantly increased the number of *Ae. albopictus* eggs that were laid in cups. 3-Methylindole and 4-ethylphenol were also shown to increase *Ae. albopictus* oviposition at two concentrations.

Phenol and 4-ethylphenol significantly increased *Ae. aegypti* oviposition at two concentrations. Allan and Kline (1995) demonstrated that the compounds that effectively increased oviposition by *Cx. quinquefasciatus* did not produce the same oviposition responses in *Ae. albopictus* or *Ae. aegypti*.

Copeland and Craig (1992) evaluated the oviposition responses of *Ae. hendersoni* and *Ae. triseriatus* to water collected from two types of tree holes. They found that *Ae. hendersoni* exhibited a preference for water in which their larvae are often found (maple rotholes). *Aedes triseriatus*, however, did not exhibit a preference for either the beech pans or the maple rotholes. The authors speculate that *Ae. hendersoni* increases its fitness by avoiding contact with *Ae. triseriatus* in larval habitats.

Toxorhynchites moctezuma and *Toxorhynchites amboinensis* both prefer to oviposit in containers that hold an ether extract of field-collected tire water (Collins and Blackwett 2002). Various concentrations of 4-methylcyclohexanol, 3-methylindole, 2-methylphenol, 3-methylphenol, and 4-methylindole also elicited significantly greater oviposition than water controls.

The water in which the larvae of *Culex quinquefasciatus* are raised affects the choice that a female makes when selecting an oviposition site (McCall and Eaton 2001). Females raised in water that contains 3-methylindole prefer to oviposit in containers that contain 3-methylindole as opposed to *p*-cresol. Similarly, females raised in water that contains *p*-cresol

prefer to oviposit in containers that hold *p*-cresol instead of 3-methylindole. This study provides evidence that a type of memory exists in this mosquito.

Interactions of Physical and Chemical Cues

Mosquitoes respond to both physical and chemical cues when locating an oviposition site (O’Gower 1963, Wilton 1968). Beehler et al. (1993) examined the interaction between chemical attractants of *Cx. quinquefasciatus* and visual cues in the laboratory. Mosquitoes laid significantly more egg rafts in cups that contained water that had been dyed with ink versus cups that contained water that had not been dyed. A chemical blend that had been identified from Bermuda hay infusions (Millar et al. 1992) was shown to receive a significantly greater number of egg rafts than controls. The dyed water and the chemical blend received approximately the same number of egg rafts compared to their respective controls. However, when the two treatments were combined, they acted to synergistically increase the number of egg rafts received, rather than act in an additive manner.

Isoe and Millar (1996) found that by changing the laboratory light levels and by changing the nutrition that was provided to *Cx. tarsalis*, they could alter the oviposition behavior of the mosquito. Females that had been starved for 24 h laid most of their egg rafts at dusk, as opposed to non-starved females which laid approximately equal numbers of egg rafts at dusk and dawn. Light levels affected the cues that mosquitoes used. When light levels were dim, females generally laid the same number of eggs in response to dyed water

and Bermuda grass infusion. However, when lights were turned off, mosquitoes did not use visual cues as much, and oviposited significantly more eggs in response to Bermuda grass infusion than dyed water.

Isolation and identification of chemical attractants/stimulants of gravid mosquitoes. Infusions are created by fermenting organic material in water.

Highly active infusions contain chemicals that increase mosquito oviposition.

Since infusions may exhibit variability in the quantity and quality of active ingredient(s), it is difficult to consistently produce infusions with the same characteristics (Millar et al. 1992). Therefore, identifying the chemical compound(s) attractive to ovipositing mosquitoes would not only increase our understanding of mosquito behavior, but can also provide a practical application in increasing the effectiveness of ovitraps.

A number of researchers have isolated and identified specific chemical oviposition attractants and stimulants. Bentley et al. (1979) isolated *p*-cresol (4-methylphenol) from the distillate of an infusion of decaying birch, which was found to be a volatile attractant in the laboratory for both male and female *Ae. triseriatus*. Synthetic analogs of *p*-cresol also elicited oviposition responses by *Ae. triseriatus*; some analogs acted as attractants, while others acted as stimulants (Bentley et al. 1981). Ikeshoji et al. (1979) reported that 7,11-dimethyloctadecane, produced as a metabolite by the bacterium *P. aeruginosa* on a capric acid substrate, was an oviposition attractant for *Ae. aegypti*. Hwang and Schultz (1983), however, were unable to duplicate the results of

these experiments using a synthesized compound. Beechwood creosote and *N*-butyl-*N*-ethyl-*o*-veratrylamine were the only compounds of 151 tested against gravid *Cx. quinquefasciatus* that were more attractive than water (Gjullin 1961).

Millar et al. (1992) identified five compounds (phenol, 4-methylphenol, 4-ethylphenol, indole, and 3-methylindole) from a fermented Bermuda grass infusion using bioassay-driven fractionation. They found that 3-methylindole, in concentrations spanning 5 orders of magnitude (0.01-100 µg/liter), caused significantly greater oviposition by *Cx. quinquefasciatus* when tested versus tap water. In addition, the activity of 3-methylindole was similar to that of the reconstituted blend of the five compounds.

Laboratory Evaluations of Bacteria

Bacteria are responsible for the production of attractants, stimulants, and repellents of gravid mosquitoes (Hazard et al. 1967, Wilton 1968, Maw 1970, Kramer and Mulla 1979, Ikeshoji et al. 1979). The isolation and identification of bacteria from various attractive or stimulatory substrates, and the subsequent evaluation of the bacteria in oviposition bioassays could lead to the discovery of new sources of attractants.

Benzon and Apperson (1988) examined the role of bacteria in the oviposition responses of *Ae. aegypti* to larval rearing water. A series of bioassays were conducted in the laboratory that conclusively demonstrated that attraction of gravid mosquitoes to larval rearing water was due to bacterial

activity, not to the production of an oviposition attractant by the larvae. They found that two species of bacteria were wholly responsible for the attraction of females to larval rearing water: *Enterobacter cloacae* and *Acinetobacter calcoaceticus*.

Aedes aegypti and *Ae. albopictus* oviposited more eggs in cups that contained drain water than in cups that contained rain, stream, well, or tap water (Vythilingam et al. 1999). *Pseudomonas aeruginosa* was isolated from the drain water, but was not found in the other treatments. This study provides circumstantial support to the work of Ikeshoji et al. (1975). Ikeshoji et al. (1975) demonstrated that *Pseudomonas aeruginosa* produced oviposition attractants and/or stimulants of *Ae. aegypti* and *Cx. pipiens molestus* when grown on a capric acid substrate.

Zahiri et al. (1997) reported about the production of an oviposition attractant by the larvae of *Ae. aegypti*. In their studies, however, they failed to demonstrate that bacteria did not produce the oviposition attractant, or that the attractant was not the product of microbial action. They filtered their larval rearing water with a 2 µm filter to remove bacteria, but this filter clearly did not remove metabolites produced by the bacteria.

Other recent work has focused on the isolation and identification of bacteria from various sources and the subsequent evaluation of those bacteria in laboratory bioassays. Trexler et al. (unpublished) isolated and identified bacteria from three separate sources of oviposition attractants and stimulants:

larval rearing water, oak leaf infusion, and soil-contaminated towels (Wallace 1996). They found that from larval rearing water, *Psychrobacter immobilis* elicited significantly higher oviposition than controls without bacteria.

Sphingobacterium multivorum from soil-contaminated cotton towels and an undetermined *Bacillus* species from oak leaf infusion also elicited significantly higher oviposition than controls without bacteria.

Field Research

One of Bentley and Day's (1989) criticisms of the state of research on oviposition attractants and stimulants was the lack of field studies. Since their review, a number of papers have been published that have evaluated not only crude infusions but also chemical attractants and stimulants in the field.

Field studies that monitor container-breeding mosquitoes usually use oviposition traps (ovitrap). Ovitrap were originally developed and used during the U.S. *Ae. aegypti* Eradication Program (Schliessmann 1964). Ovitrap typically consist of dark colored, water-filled containers to which balsa paddles or seed germination paper (Steinley et al. 1991) are attached as oviposition substrates.

In some studies, ovitrap that were used to monitor field populations of mosquitoes were enhanced by the addition of leaf material collected at the study site. This technique was used instead of using a particular fermented infusion or chemical. Loo and DeFoliart (1969) added oak leaves to ovitrap to detect the presence of *Ae. triseriatus* in Wisconsin. Hanson et al. (1988)

used approximately two grams of dry maple leaf litter added to ovitraps as an attractant in a study to determine the distribution of *Ae. triseriatus* in an urban area of northern Indiana. Szumlas et al. (1996) added dry oak leaf litter to ovitraps monitoring *Ae. triseriatus* in western North Carolina. Researchers also use fermented organic infusions to monitor populations of container breeding mosquitoes (Holck et al. 1988, Kitron et al. 1989).

Holck et al. (1988) evaluated the effects of adding either hay infusion (Reiter 1986), leaf litter, or a 1% emulsion of fish oil fertilizer to ovitraps in Louisiana, USA. They found that ovitraps that contained a hay infusion received significantly more *Ae. albopictus* eggs than ovitraps that contained distilled water, leaf litter and water, or fish oil emulsion. In addition, they found that *Ae. triseriatus* laid more eggs in ovitraps that contained fish oil emulsion than in ovitraps that contained hay infusion, leaf litter and water, or distilled water. This study prompted Beehler and DeFoliart (1990) to evaluate fish oil emulsion as an oviposition attractant or stimulant of *Ae. triseriatus* in Wisconsin, USA. They found that *Ae. triseriatus* was significantly repelled by both 1% and 5% fish oil emulsion infusions in both the laboratory and in the field.

A field and laboratory study evaluated eight potential oviposition attractants for *Cx. tarsalis* and *Cx. quinquefasciatus* (Reisen and Meyer 1990). The study examined the effects of tap water, larval rearing water, water from which pupae had emerged, field-collected larval rearing water, hay infusion,

hay infusion mixed with isopropyl alcohol, mulberry leaf infusion, and dried steer manure infusion on oviposition by these species. In the field, none of the potential attractants received significantly more *Cx. tarsalis* egg rafts than controls. However, hay infusion and steer manure infusion received a significant number of *Cx. quinquefasciatus* egg rafts compared to controls.

Reiter et al (1991) evaluated four potential attractants against field populations of *Ae. aegypti* in Puerto Rico. Undiluted hay infusion, 10% hay infusion, 0.5% methyl propionate in tap water and tap water were added to ovitrap pairs in the field. They found that ovitrap pairs that contained one ovitrap with 10% or undiluted hay infusion and one ovitrap with tap water received significantly more eggs than ovitrap pairs that contained two ovitraps with tap water. They also reported that an ovitrap pair that contained one ovitrap with 10% hay infusion and one ovitrap with undiluted infusion significantly increased the number of eggs collected by the pair versus control pairs or other combinations of treatments in the ovitrap pairs. A similar study in Trinidad found that ovitraps containing varying concentrations of hay infusion received approximately the same number of *Ae. aegypti* eggs (Chadee et al. 1993), and no enhancement of ovitraps was noted by the undiluted infusions.

Oak leaf infusion significantly increased the number of *Ae. albopictus* eggs laid in ovitraps relative to water controls in North Carolina, USA (Trexler et al. 1998). The three ages of oak leaf infusion that were tested against field

populations of the mosquito exhibited the same activity. In the same study, only ovitraps that contained infusion that was created with four times the usual amount of leaf material increased oviposition by *Ae. triseriatus*.

In a study of structure-activity relationships, five hexanoic acid derivatives were evaluated in experiments in the field in Kenya against populations of *Ae. aegypti* (Knight and Corbet 1991). Methyl hexanoic acid and 5-methyl-2-hexanone increased the number of eggs that were laid in ovitraps relative to controls. In addition, a dose-dependent reversal of response was noted in the oviposition responses to hexanoic acid. The study showed that at low concentrations, hexanoic acid increased oviposition by *Ae. aegypti*. However, as the concentration of the compound increased, the number of eggs that were laid in the test ovitrap decreased relative to the control. This study demonstrates the importance of concentration effects when evaluating oviposition attractants and stimulants.

The five-component chemical blend that was isolated and identified by Millar et al. (1992) spawned studies to evaluate the effectiveness of these compounds against field populations of mosquitoes. Beehler et al. (1994) used plastic tubs baited with either tap water or with tap water plus a blend of 3-methylindole, 4-methylphenol, 4-ethylphenol, phenol, and indole. They found that significantly more *Cx. quinquefasciatus* egg rafts were laid in the tubs that contained the five-component blend. In addition, they found that the blend attracted more adults in gravid female traps compared to tap water

controls. 3-Methylindole was also evaluated against the chemical blend and was found to be wholly responsible for the attraction to the blend.

Allan and Kline (1995) evaluated the compounds that were isolated and identified by Millar et al. (1992) against *Ae. aegypti* and *Ae. albopictus* in field cages. In the field cages, they compared the oviposition responses of the mosquitoes to well water, hay infusion, 3-methylindole, larval rearing water, and field-collected larval rearing water. The study reported that for both species, ovitraps with the test treatments received significantly more eggs than ovitraps with well water in one experiment. In a second experiment with the field cages, ovitraps with 3-methylindole did not receive more eggs than ovitraps with well water. In fact, for *Ae. albopictus*, hay infusion was more effective in receiving eggs than either 3-methylindole or the five component blend. In the second experiment, *Ae. aegypti* oviposited similar numbers of eggs in response to 3-methylindole, the five component blend and hay infusion relative to well water. *Aedes albopictus* and *Ae. aegypti* did not respond to either 3-methylindole or the five component blend as did *Cx. quinquefasciatus*.

Another study examined some of the components that were isolated and identified by Millar et al. (1992). Trexler et al. (unpublished) loaded chemicals into controlled-release packets, which consisted of a cellulose material sealed within a permeable plastic membrane, and evaluated the effects of the compounds on the number of *Ae. albopictus* eggs received in

ovitraps. Their study only examined the effects of compound odor — mosquitoes could not touch the compound at the substrate and were therefore not stimulated to oviposit by direct contact with the compound. They examined the effects of the odor of 3-methylindole, 4-methylphenol, trimethylamine, dimethyl disulfide and indole on *Ae. albopictus* oviposition. None of the compounds that were tested elicited significantly greater oviposition relative to untreated controls.

The oviposition pheromone of *Cx. quinquefasciatus* has been identified (Laurence and Pickett 1982) and has been shown to increase oviposition in the laboratory (Bruno and Laurence 1979, Laurence and Pickett 1982). A synthetic pheromone was produced and evaluated in the field in Kenya (Otieno et al. 1988). Oviposition sites that were baited with the synthetic pheromone received significantly more egg rafts than sites that were not baited. Otieno et al. (1988) also demonstrated that pairing the oviposition pheromone with an insect growth regulator did not affect the attractiveness of the pheromone.

Electrophysiological Investigations of Mosquito Attractants

Electrophysiological recording equipment such as electroantennograms (EAG) are additional tools that researchers can use in the search for oviposition attractants. By examining the response of an antenna to a test compound, the potential for the compound to be an oviposition attractant can be determined.

The activity of the *Cx. quinquefasciatus* oviposition pheromone and polluted water have been demonstrated in both laboratory bioassays and in EAG studies (Mordue et al. 1992). Oviposition and EAG responses both increased in a dose-dependent manner to the oviposition pheromone. The study used the EAG in support of the bioassay results. A combination of the oviposition pheromone and the polluted water increased oviposition by *Cx. quinquefasciatus* in an additive manner when compared to responses to the pheromone and polluted water alone. The EAG confirmed these results. In a related study, the EAG responses of *Cx. quinquefasciatus* were greater to 3-methylindole than to 4-methylphenol, 4-ethylphenol, or indole (Blackwell et al. 1993).

Du and Millar (1999) used coupled gas-chromatography electroantennogram detection (GC-EAD) to identify potential oviposition attractants of *Cx. quinquefasciatus* and *Cx. tarsalis* from Bermuda grass infusions. They found that phenol, *p*-cresol, 4-ethylphenol, indole, 3-methylindole, nonanal, 2-undecanone, 2-tridecanone, and naphthalene elicited significant antennal responses by both mosquito species. After identifying the compounds, they evaluated each compound individually (those that had not been bioassayed in Millar et al. (1992) and evaluated the blend in two types of bioassays. The first bioassay was an open-cup bioassay that used the number of eggs laid on the water surface as the measurement. The second bioassay was a sticky-screen bioassay (Isoe et al. 1995a) in which the number

of gravid females that were trapped on the screen was the endpoint. The sticky screen bioassay differentiates responses due to attraction from those due to stimulation. Their study indicated that the compounds that they identified from Bermuda grass infusion are more likely to be oviposition stimulants and arrestants than oviposition attractants.

Water samples from active breeding sites were examined in EAG studies with *An. gambiae* (Blackwell and Johnson 2000). Antennae showed significant responses to both the water samples and ether extracts of the water samples. In addition, they also evaluated compounds that had previously been implicated as oviposition attractants to other mosquito species. 3-Methylindole, indole, phenol, 4-methylcyclohexanol, *p*-cresol, *m*-cresol, and *o*-cresol elicited significant antennal responses from *An. gambiae*, indicating that the antennal receptors for these compounds exist.

Conclusion

The number of field evaluations of potential oviposition attractants and stimulants and infusions has greatly increased in the past fifteen years. We have a better understanding of how mosquitoes respond to potential attractants in their natural environments.

The use of coupled gas chromatography and electroantennogram detectors is an important development in the search for new oviposition attractants. This technique has the potential to isolate and identify potential attractants more quickly than time-consuming bioassay-driven chemical

fractionations of infusions. Additional research is needed using this technique to isolate and identify potential oviposition attractants of mosquitoes from a number of genera.

Studies of the behavior of mosquitoes prior to and during oviposition are lacking. There are a number of fundamental questions that need to be answered. How does a gravid mosquito approach an oviposition site? Wind tunnel studies using video cameras (Zanen and Cardé 1996) would provide insight. What behaviors occur when a gravid mosquito reaches the oviposition site? How do chemical arrestants affect behavior? Videos of females at the oviposition site would answer these questions.

References Cited

- Allan, S. A. and D. L. Kline. 1995. Evaluation of organic infusions and synthetic compounds mediating oviposition in *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae). *J. Chem. Ecol.* 21: 1847-1860.
- Allan, S. A. and D. L. Kline. 1998. Larval rearing water and preexisting eggs influence oviposition by *Aedes aegypti* and *Ae. albopictus* (Diptera: Culicidae). *J. Med. Entomol.* 35: 943-947.
- Apostol, B. L. W. C. Black, P. Reiter, and B. R. Miller. 1994. Use of randomly amplified polymorphic DNA amplified by polymerase chain reaction markers to estimate the number of *Aedes aegypti* families at oviposition sites in San Juan, Puerto Rico. *Am. J. Trop. Med. Hyg.* 51: 89-97.
- Beehler, J. W. and G. R. DeFoliart. 1990. A field evaluation of two suggested *Aedes triseriatus* oviposition attractants. *J. Am. Mosq. Control Assoc.* 6: 720-722.
- Beehler, J., S. Lohr, and G. DeFoliart. 1992. Factors influencing oviposition in *Aedes triseriatus* (Diptera: Culicidae). *Great Lakes Ent.* 25: 259-264.
- Beehler, J. W., J. G. Millar, and M. S. Mulla. 1993. Synergism between chemical attractants and visual cues influencing oviposition of the mosquito, *Culex quinquefasciatus* (DIPTERA: CULICIDAE). *J. Chem. Ecol.* 19: 635-644.

- Beehler, J. W., J. G. Millar and M. S. Mulla. 1994. Field evaluation of synthetic compounds mediating oviposition in *Culex* mosquitoes (Diptera: Culicidae). *J. Chem. Ecol.* 20: 281-291.
- Bentley, M. D. and J. F. Day. 1989. Chemical ecology and behavioral aspects of mosquito oviposition. *Ann. Rev. Entomol.* 34: 401-21.
- Bentley, M. D., I. N. McDaniel, M. Yatagai, H.-P. Lee and R. Maynard. 1979. *p*-Cresol: an oviposition attractant of *Aedes triseriatus*. *Environ. Entomol.* 8: 206-209.
- Bentley, M. D., I. N. McDaniel, M. Yatagai, H.-P. Lee and R. Maynard. 1981. Oviposition attractants and stimulants of *Aedes triseriatus* (Say) (Diptera: Culicidae). *Environ. Entomol.* 10: 186-189.
- Benzon, G. L. and C. S. Apperson. 1988. Reexamination of chemically mediated oviposition behavior in *Aedes aegypti* (L.) (Diptera: Culicidae). *J. Med. Entomol.* 25: 158-164.
- Blackwell, A., A. J. Mordue (Luntz), B. S. Hansson, L. J. Wadhams, and J. A. Pickett. 1993. A behavioral and electrophysiological study of oviposition cues for *Culex quinquefasciatus*. *Physiol. Entomol.* 18: 343-348.
- Blackwell, A. and S. N. Johnson. 2000. Electrophysiological investigation of larval water and potential oviposition chemo-attractants for *Anopheles gambiae* s. s. *Ann. Trop. Med. Parasitol.* 94: 389-398.

- Bruno, D. W. and B. R. Laurence. 1979. The influence of the apical droplet of *Culex* egg rafts on oviposition of *Culex pipiens fatigans* (Diptera: Culicidae). J. Med. Entomol. 16: 300-305.
- Canyon, D. V., J. L. K. Hii, and R Müller. 1999. Adaptation of *Aedes aegypti* (Diptera: Culicidae) oviposition behavior in response to humidity and diet. J. Insect Physiol. 45: 959-964.
- Chadee, D. D., A. Lakhan, W. R. Ramdath, and R. C. Persad. 1992. Oviposition response of *Aedes aegypti* mosquitoes to different concentrations of hay infusion in Trinidad, West Indies. J. Am. Mosq. Control Assoc. 9: 346-348.
- Chan, K. L. 1985. Methods and indices used in the surveillance of dengue vectors. Mosquito Borne Disease Bulletin 1: 79-88.
- Collins, L. E. and A. Blackwell. 2002. Olfactory cues for oviposition behavior in *Toxorhynchites moctezuma* and *Toxorhynchites amboinensis* (Diptera: Culicidae). J. Med. Entomol. 39: 121-126.
- Copeland, R. S. and G. B. Craig, Jr. 1992. Differential oviposition by *Aedes hendersoni* and *Aedes triseriatus* (Diptera: Culicidae) in response to chemical cues associated with treehole water. J. Med. Entomol. 29: 33-36.
- Corbet, P. S. and D. D. Chadee. 1993. An improved method for detecting substrate preferences shown by mosquitoes that exhibit 'skip oviposition.' Physiol. Entomol. 18: 114-118.

- Davis, E. E. 1985. Insect repellents: concepts of their mode of action relative to potential sensory mechanisms in mosquitoes (Diptera: Culicidae). *J. Med. Entomol.* 22: 237-243.
- Dawson, G. W., B. R. Laurence, J. A. Pickett, M. M. Pile, and L. J. Wadhams. 1989. A note on the mosquito oviposition pheromone. *Pestic. Sci.* 27: 277-280.
- Dethier, V. G., L. B. Browne, and C. N. Smith. 1960. The designation of chemicals in terms of the responses they elicit from insects. *J. Econ. Entomol.* 53: 134-136.
- Dhileepan, K. 1997. Physical factors and chemical cues in the oviposition behavior of arboviral vectors *Culex annulirostris* and *Culex molestus* (Diptera: Culicidae). *Environ. Entomol.* 26: 318-326.
- Du, Y.-J. and J. G. Millar. 1999. Electroantennogram and oviposition bioassay responses of *Culex quinquefasciatus* and *Culex tarsalis* (Diptera: Culicidae) to chemicals in odors from Bermuda grass infusions. *J. Med. Entomol.* 36: 158-166.
- Fay, R. W. and A. S. Perry. 1965. Laboratory studies of the ovipositional preferences of *Aedes aegypti*. *Mosq. News* 26: 531-535.
- George, N., K. D. Ramiah, C. H. Sujatha, M. Kalyanasundaram, and P. K. Das. 1986. Oviposition attractancy of some substituted esters and the pheromone extracted from egg rafts against *Culex quinquefasciatus*. *Current Sci.* 55: 1205-1207.

- Gerberg, E. G., D. R. Barnard and R. A. Ward. 1994. Manual for mosquito rearing and experimental techniques. American Mosquito Control Association Bull. 5.
- Gjullin, C. M. 1961. Oviposition responses of *Culex pipiens quinquefasciatus* Say to waters treated with various chemicals. Mosq. News 21: 109-113.
- Gjullin, C. M. and J. O. Johnsen. 1965. The oviposition responses of two species of *Culex* to waters treated with various chemicals. Mosq. News 25: 14-16.
- Gjullin, C. M., J. O. Johnsen, and F. W. Plapp, Jr. 1965. The effect of odors released by various waters on the oviposition sites selected by two species of *Culex*. Mosq. News 25: 268-271.
- Grimstad, P. R. 1988. California group virus disease, pp. 99-136. *In*: T. P. Monath (ed.) The arboviruses: epidemiology and ecology, vol. II. CRC Press, Boca Raton, FL.
- Grimstad, P. R., J. K. Kobayashi, M. Zhang and G. B. Craig, Jr. 1989. Recently introduced *Aedes albopictus* in the United States: potential vector of La Crosse virus (Bunyaviridae: California serogroup). J. Am. Mosq. Control Assoc. 5: 422-427.
- Gubler, D. J. 1971. Studies on the comparative oviposition behavior of *Aedes (Stegomyia) albopictus* and *Aedes (Stegomyia) polynesiensis* Marks. J. Med. Entomol. 8: 675-682.

- Hancock, R. G. and W. A. Foster. 1993. Effects of preblood-meal sugar on sugar seeking and upwind flight by gravid and parous *Aedes aegypti* (Diptera: Culicidae). J. Med. Entomol. 30: 353-359.
- Hancock, R. G. and W. A. Foster. 1997. Larval and adult nutrition effects on blood/nectar choice of *Culex nigripalpus* mosquitoes. Med. Vet. Entomol. 11: 112-122.
- Hanson, S. M., M. Song, and G. B. Craig, Jr. 1988. Urban distribution of *Aedes triseriatus* in northern Indiana. J. Am. Mosq. Control Assoc. 4: 15-19.
- Harrington, L. C. and J. D. Edman. 2001. Indirect evidence against delayed "skip-oviposition" behavior by *Aedes aegypti* (Diptera: Culicidae) in Thailand. J. Med. Entomol. 38: 641-645.
- Harris, M. O. and S. P. Foster. 1995. Behavior and integration. *In*: Chemical ecology of insects 2. (R. T. Cardé and W. J. Bell, eds.). New York: Chapman and Hall.
- Hazard, E. I., M. S. Mayer, and K. E. Savage. 1967. Attraction and oviposition stimulation of gravid female mosquitoes by bacteria isolated from hay infusions. Mosq. News 27: 133-136.
- Holck, A. R., C. L. Meek and J. C. Holck. 1988. Attractant enhanced ovitraps for the surveillance of container breeding mosquitoes. J. Am. Mosq. Control Assoc. 4: 97-98.

- Hwang, Y.-S. and G. W. Schultz. 1983. Is 7-11-dimethyloctadecane an ovipositional attractant for *Aedes aegypti*? J. Pesticide Sci. 8: 221-222.
- Hwang, Y.-S., W. L. Kramer, and M. S. Mulla. 1980. Oviposition attractants and repellents of mosquitoes: Isolation and identification of oviposition repellents for *Culex* mosquitoes. J. Chem. Ecol. 6: 71-80.
- Hwang, Y.-S., G. W. Schultz, H. Axelrod, W. L. Kramer, and M. S. Mulla. 1982. Ovipositional repellency of fatty acids and their derivatives against *Culex* and *Aedes* mosquitoes. Environ. Entomol. 11: 223-226.
- Ibáñez-Bernal, B. Briseño, J. P. Mutebi, E. Argot, G. Rodríguez, C. Martínez-Campos, R. Paz, P. de la Fuente-San Román, R. Tapia-Conyer and A. Flisser. 1997. First record in America of *Aedes albopictus* naturally infected with dengue virus during the 1995 outbreak at Reynosa, Mexico. Med. Vet. Entomol. 11: 305-309.
- Ikeshoji, T. 1975. Chemical analysis of woodcreosote for species-specific attraction of mosquito oviposition. Appl. Ent. Zool. 10: 302-308.
- Ikeshoji, T. and M. S. Mulla. 1970. Oviposition attractants for four species of mosquitoes in natural breeding waters. Ann. Ent. Soc. Am. 63: 1322-1327.
- Ikeshoji, T., K. Saito, and A. Yano. 1975. Bacterial production of the ovipositional attractants for mosquitoes on fatty acid substrates. App. Ent. Zool. 10: 239-242.
- Ikeshoji, T., I. Ichimoto, J. Konishi, Y. Naoshima and H. Ueda. 1979. 7,11-Dimethyloctadecane: an ovipositional attractant for *Aedes aegypti* produced

- by *Pseudomonas aeruginosa* on capric acid substrate. J. Pesticide Sci. 4: 187-194.
- Isoe, J. and J. G. Millar. 1995. Characterization of factors mediating oviposition site choice by *Culex tarsalis*. J. Am. Mosq. Control Assoc. 11: 21-28.
- Isoe, J., J. G. Millar, and J. W. Beehler. 1995a. Bioassays for *Culex* (Diptera: Culicidae) mosquito oviposition attractants and stimulants. J. Med. Entomol. 32: 475-483.
- Isoe, J., J. W. Beehler, J. G. Millar, and M. S. Mulla. 1995b. Oviposition responses of *Culex tarsalis* and *Culex quinquefasciatus* to aged Bermuda grass infusions. J. Am. Mosq. Control. Assoc. 11: 39-44.
- Isoe, J. and J. G. Millar. 1996. Water deprivation and light affect responses of *Culex tarsalis* (Diptera: Culicidae) to oviposition site cues. Environ. Entomol. 25: 1383-1390.
- Kennedy, J. S. 1978. The concepts of olfactory "arrestment" and "attraction". Physiol. Entomol. 3: 91-98.
- Kitron, U. D., D. W. Webb, and R. J. Novak. 1989. Oviposition behavior of *Aedes triseriatus* (Diptera: Culicidae): prevalence, intensity, and aggregation of eggs in oviposition traps. J. Med. Entomol. 26: 462-467.
- Knight, J. C. and S. A. Corbet. 1991. Compounds affecting mosquito oviposition: structure-activity relationships and concentration effects. J. Am. Mosq. Control. Assoc. 7: 37-41.

- Kramer, W. L. and M. S. Mulla. 1979. Oviposition attractants and repellents of mosquitoes: oviposition responses of *Culex* mosquitoes to organic infusions. *Environ. Entomol.* 8: 1111-1117.
- Kramer, W. L., Y.-S. Hwang, and M. S. Mulla. 1980. Oviposition repellents of mosquitoes: Negative responses elicited by lower aliphatic carboxylic acids. *J. Chem. Ecol.* 6: 415-424.
- Lampman, R. L. and R. J. Novak. 1996. Attraction of *Aedes albopictus* adults to sod infusion. *J. Am. Mosq. Control Assoc.* 12: 119-124.
- Laurence, B. R. and J. A. Pickett. 1982. *erythro*-6-Acetoxy-5-hexadecanolide, the major component of a mosquito oviposition attractant pheromone. *J. Chem. Soc., Chem. Commun.* 59-60.
- Laurence, B. R. and J. A. Pickett. 1985. An oviposition attractant pheromone in *Culex quinquefasciatus* Say (Diptera: Culicidae) *Bull. Ent. Res.* 75: 283-290.
- Laurence, B. R., K. Mori, T. Otsuka, J. A. Pickett and L. J. Wadhams. 1985. Absolute configuration of mosquito oviposition attractant pheromone, 6-acetoxy-5-hexadecanolide. *J. Chem. Ecol.* 11: 643-648.
- Loor, K. A. and G. R. DeFoliart. 1969. An oviposition trap for detecting the presence of *Aedes triseriatus* (Say). *Mosq. News* 29: 487.
- Maw, M. G. 1970. Capric acid as a larvicide and an oviposition stimulant for mosquitoes. *Nature* 227: 1154-1155.

- McCall, P. J. and G. Eaton. 2001. Olfactory memory in the mosquito *Culex quinquefasciatus*. *Med. Vet. Entomol.* 18: 197-203.
- McDaniel, I. N., M. D. Z Bentley, H.-P. Lee and M. Yatagai. 1976. Effects of color and larval-produced oviposition attractants on oviposition of *Aedes triseriatus*. *Environ. Entomol.* 5: 553-556.
- McDaniel, I. N., M. D. Bentley, H.-P. Lee and M. Yatagai. 1979. Studies of *Aedes triseriatus* (Diptera: Culicidae) oviposition attractants. Evidence for attractant production by kaolin-treated larvae. *Can. Ent.* 111: 143-147.
- Millar, J. G. and Y.-J. Du. 1997. Coupled gas chromatography-electroantennogram detection (GC-EAD) for identification of volatile chemicals mediating mosquito oviposition behavior. 1997 Proc. Calif. Vector Control Assoc. 29-40.
- Millar, J. G., J. D. Chaney and M. S. Mulla. 1992. Identification of oviposition attractants for *Culex quinquefasciatus* from fermented Bermuda grass infusions. *J. Am. Mosq. Control Assoc.* 8: 11-17.
- Millar, J. G., J. D. Chaney, J. W. Beehler and M. S. Mulla. 1994. Interaction of the *Culex quinquefasciatus* egg raft pheromone with a natural chemical associated with oviposition sites. *J. Am. Mosq. Control Assoc.* 10: 374-379.
- Mitchell, C. J., B. R. Miller and D. J. Gubler. 1987. Vector competence of *Aedes albopictus* from Houston, Texas for dengue serotypes 1 to 4, yellow fever and Ross River viruses. *J. Am. Mosq. Control Assoc.* 3: 460-465.

- Mitchell, C. J., M. L. Niebylski, G. C. Smith, N. Karabatsos, D. Martin, J.-P. Mutebi, G. B. Craig, Jr. and M. J. Mahler. 1992. Isolation of eastern encephalitis virus from *Aedes albopictus* in Florida. *Science* 257: 526-527.
- Mogi, M. and J. Mokry. 1980. Distribution of *Wyeomyia smithii* (Diptera: Culicidae) eggs in pitcher plants in Newfoundland, Canada. *Tropical Medicine* 22: 1-12.
- Mordue (Luntz), A. J., A. Blackwell, B. S. Hansson, L. J. Wadhams and J. A. Pickett. 1992. Behavioural and electrophysiological evaluation of oviposition attractants for *Culex quinquefasciatus* Say (Diptera: Culicidae). *Experientia* 48: 1109-1111.
- O'Gower, A. K. 1963. Environmental stimuli and the oviposition behaviour of *Aedes aegypti* var. *queenslandis* Theobald (Diptera, Culicidae). *Anim. Behav.* 11: 189-197.
- Osgood, C. E. 1971. An oviposition pheromone associated with the egg rafts of *Culex tarsalis*. *J. Econ. Entomol.* 64: 1038-1041.
- Otieno, W. A., T. O. Onyango, M. M. Pile, B. R. Laurence, G. W. Dawson, L. J. Wadhams and J. A. Pickett. 1988. A field trial of the synthetic oviposition pheromone with *Culex quinquefasciatus* Say (Diptera: Culicidae) in Kenya. *Bull. ent. Res.* 78: 463-470.
- Pickett, J. A. and C. M. Woodcock. 1996. The role of mosquito olfaction in oviposition site location and in the avoidance of unsuitable hosts. *In:*

- Olfaction in mosquito-host interactions, pp 109-123. Wiley: Chichester, England.
- Pile, M. M., M. S. J. Simmonds, and W. M. Blaney. 1991. Odour-mediated upwind flight of *Culex quinquefasciatus* mosquitoes elicited by a synthetic attractant. *Physiol. Entomol.* 16: 77-85.
- Reisen, W. K. and R. P. Meyer. 1990. Attractiveness of selected oviposition substrates for gravid *Culex tarsalis* and *Culex quinquefasciatus* in California. *J. Am. Mosq. Control Assoc.* 6: 244-250.
- Reiter, P. 1986. A standardized procedure for the quantitative surveillance of certain *Culex* mosquitoes by egg raft collection. *J. Am. Mosq. Control Assoc.* 2: 219-221.
- Reiter, P., M. A. Amador, and N. Colon. 1991. Enhancement of the CDC ovitrap with hay infusions for daily monitoring of *Aedes aegypti* populations. *J. Am. Mosq. Control Assoc.* 7: 52-55.
- Rockett, C. L. 1987. Bacteria as ovipositional attractants for *Culex pipiens* (Diptera: Culicidae). *Great Lakes Entomologist* 20: 151-155.
- Rozeboom, L. E., L. Rosen and J. Ikeda. 1973. Observations on oviposition by *Aedes (S.) albopictus* Skuse and *A. (S.) polynesienses* Marks in nature. *J. Med. Entomol.* 4: 397-399.
- Schliessmann, D. J. 1964. The *Aedes aegypti* eradication program of the U. S. *Mosq. News* 24: 124-132.

- Scott, T. W., L. H. Lorenz and S. C. Weaver. 1990. Susceptibility of *Aedes albopictus* to infection with eastern encephalitis virus. J. Am. Mosq. Control Assoc. 6: 274-278.
- Starratt. A. N. and C. E. Osgood. 1972. Oviposition pheromone of the mosquito *Culex tarsalis*: diglyceride composition of the active fraction. Biochim. Biophys. Acta 280: 187-193.
- Steinley, B. A., R. J. Novak, and D. W. Webb. 1991. A new method for monitoring mosquito oviposition in artificial and natural containers. J. Am. Mosq. Control Assoc. 7: 649-650.
- Sudia, W. D., V. F. Newhouse, C. H. Calisher and R. W. Chamberlain. 1971. California group arboviruses: isolations from mosquitoes in North America. Mosq. News 31: 576-600.
- Szumlas, D. E., C. S. Apperson and E. E. Powell. 1996. Seasonal occurrence and abundance of *Aedes triseriatus* and other mosquitoes in a La Crosse virus-endemic area in western North Carolina. J. Am. Mosq. Control Assoc. 12: 184-193.
- Trexler, J. D., C. S. Apperson and C. Schal. 1998. Laboratory and field evaluations of oviposition responses of *Aedes albopictus* and *Aedes triseriatus* (Diptera: Culicidae) to oak leaf infusions. J. Med. Entomol. 35: 967-977.

- Vythilingam, I., S. Novi, and P. Seleena. 1999. Oviposition response of *Aedes aegypti* and *Aedes albopictus* in different types of water. *Trop. Biomed.* 16: 23-28.
- Wallace, F. L. 1996. Construction of a field trap for initiating an ovipositional response in *Aedes taeniorhynchus*. *J. Am. Mosq. Control. Assoc.* 12: 491-493.
- Williams, R. E. 1962. Effect of coloring oviposition media with regard to the mosquito *Aedes triseriatus* (Say). *J. Parasitol.* 48: 919-925.
- Wilton, D. P. 1968. Oviposition site selection by the tree-hole mosquito, *Aedes triseriatus* (Say). *J. Med. Entomol.* 5: 189-194.
- Xue, R.-D., D. R. Barnard, and A. Ali. 2001. Laboratory and field evaluation of insect repellents as oviposition deterrents against the mosquito *Aedes albopictus*. *Med. Vet. Entomol.* 15: 126-131.
- Zahiri, N., M. E. Rau and D. J. Lewis. 1997. Oviposition responses of *Aedes aegypti* and *Ae. atropalpus* (Diptera: Culicidae) females to waters from conspecific and heterospecific normal larvae and from larvae infected with *Plagiorchis elegans* (Trematoda: Plagiorchiidae). *J. Med. Entomol.* 34: 565-568.
- Zanen, P. O. and R. T. Cardé. 1996. Effects of host-odour plume altitude and changing wind velocity on upwind flight manoeuvres of a specialist braconid parasitoid. *Physiol. Entomol.* 21: 329-338.

CHAPTER 2.

FIELD AND LABORATORY EVALUATIONS OF OVIPOSITION ATTRACTANTS FOR *AEDES ALBOPICTUS* (DIPTERA: CULICIDAE)

INTRODUCTION

Mosquitoes choose oviposition sites based on their physical characteristics such as color, substrate texture, and odorants and other chemicals (Bentley and Day 1989). In the aqueous milieu of an oviposition site, microbial degradation of organic material can produce volatile attractants or repellents, as well as nonvolatile arrestants and oviposition stimulants or deterrents. Of the biologically active compounds that have been isolated and the chemical structures identified, only a few have been tested both in the laboratory and in the field. Millar et al. (1992) identified five compounds from hay infusions (3-methylindole, 4-methylphenol, 4-ethylphenol, indole, and phenol) that increased oviposition by *Culex quinquefasciatus* (Say). One compound, 3-methylindole (skatole), was active at concentrations that spanned 5 orders of magnitude (0.01-100 pg/L). Experimental ponds treated with skatole received significantly more *Cx. quinquefasciatus* egg rafts than did adjacent untreated ponds (Beehler et al. 1994). In addition, 4-methylphenol, indole and 3-methylindole have been shown to elicit significant antennal responses from *Cx. quinquefasciatus* and *Culex tarsalis* Coquillett in electroantennogram (EAG) studies (Du and Millar 1999). Allan and Kline (1995) evaluated the five *Culex* oviposition chemicals with *Aedes albopictus* (Skuse) and *Aedes aegypti* L. in the laboratory and concluded that they were only weakly active. A dose-response relationship could not be established for any of the *Culex* oviposition chemicals. Only one concentration of 3-methylindole and one concentration of

4-methylphenol elicited a slightly greater oviposition response by *Ae. albopictus* relative to the well water control. Gravid *Ae. aegypti* did not discriminate when laying eggs between well water and 3-methylindole. Allan and Kline (1995) also evaluated the number of *Ae. albopictus* eggs laid in response to 3-methylindole in field cages. They found that 3-methylindole elicited a moderate oviposition response relative to well water. Their experimental design did not separate olfactory attraction from contact-mediated stimulation of oviposition.

Bentley et al. (1979) identified 4-methylphenol from birch bark infusions as an oviposition attractant of *Aedes* (= *Ochlerotatus*) *triseriatus* (Say). Under laboratory conditions, they determined that the compound acted as a contact chemical stimulant, but it also attracted gravid *Ae. triseriatus* from a distance.

The objective of our investigation was to determine whether several chemicals that have been implicated by other research as mosquito oviposition attractants can also attract *Ae. albopictus*. Three of the chemicals had been tested previously (Allan and Kline 1995), and two were tested for the first time (dimethyl disulfide and trimethylamine). Some candidate compounds were tested because they occur in organic infusions that were reported to increase the numbers of eggs laid in oviposition traps. Specifically, we ascertained whether 3-methylindole, indole, 4-methylphenol, trimethylamine, and dimethyl disulfide increased oviposition by *Ae. albopictus* under field and

laboratory conditions. In addition, we evaluated the EAG responses of *Ae. albopictus* to the test compounds.

MATERIALS AND METHODS

Mosquito colony origin and maintenance. *Aedes albopictus* eggs were collected from oviposition traps in Raleigh, NC in 1997. The colony was kept at ca. 26°C and at a relative humidity of ca. 75% under L:D 14:10. Included in the light phase were two 30-minute crepuscular periods simulated by a 40-watt incandescent bulb. Larvae were fed a 2:1 (wt.:wt.) mixture of liver powder:baker's yeast on a standardized schedule (Gerberg et al. 1994). Adults were kept in 30 x 30 x 30 cm Plexiglas cages fitted with cotton surgical stocking tops and were provided a 10% sucrose solution *ad libitum*. Four days prior to the initiation of a trial, adult mosquitoes were blood-fed on a human hand (Human Use Protocol IRB# 1388). The protocol for blood-feeding virus-free mosquitoes on a human was approved by the Institutional Review Board at North Carolina State University. Females not used in the experiments were allowed to oviposit on seed germination paper (Steinley et al. 1994). The F₄-F₇ generations were used in our experiments.

Laboratory experiments. Five synthetic compounds (dimethyl disulfide, indole, 4-methylphenol, 3-methylindole, trimethylamine) (Sigma Chemical Co., St. Louis, MO) were evaluated using the sticky screen bioassay method of Isoe et al. (1995) as modified by Trexler et al. (1998). The sticky-screen bioassay differentiates between responses due to attraction from contact chemical stimulation. In the bioassay, gravid female mosquitoes were presented a choice between a test and a control cup. Each cup was covered

with a sticky-screen that mosquitoes must first land upon before they could enter the cup and contact the substrate. The glue trapped females when they landed on the screen. Therefore, any positive or negative responses were due to attraction or repulsion, respectively, to volatile odorants. Galvanized hardware cloth screen (6 mm mesh, Gilbert and Bennet, Toccoa, GA) was cut into disks. Insect glue (Tanglefoot, Grand Rapids, MI) was dissolved in hexane and each disk was coated with the glue solution. The disks were placed in a fume hood for two hours to evaporate the hexane. Two 125 ml black polypropylene cups were placed in opposite corners of each oviposition cage (Trexler et al. 1998). Test solutions were made for each compound so that each experimental cup contained concentrations spanning four to five orders of magnitude (0.0083 mg/L to 83 mg/L). To achieve the desired final concentration, each experimental cup was filled with 29 ml of distilled water and 1 ml of the appropriate ethanolic stock solution of the test compound was added. Control cups were filled with 29 ml of distilled water and 1 ml of 75% ethanol. Each cup was covered with a glue-coated screen. Ten gravid females were placed in each cage. After a 24-h exposure period, the number of females trapped on each screen was counted.

The oviposition activity index (OAI) (Kramer and Mulla, 1979) was used to evaluate the responses of the females to each compound. 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 proportion of females trapped on the screen over the control cup. The OAI varies from -1 to 1 , with 0 indicating no response.

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 was severed and 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 L/min), which adapted the mechanoreceptors on the antenna. Each test solution (10 μ l) was applied to a filter paper strip and the solvent was allowed to evaporate. The filter paper was then inserted into a Pasteur pipette and attached to a glass syringe. A single, rapid 2 ml puff of test odorant was then introduced into the airstream. 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). To ensure that the equipment was functioning properly and antennal preparations were responsive, we used a

100% concentration of ethyl acetate (Fisher Scientific, Pittsburgh, PA) and three concentrations (1%, 10%, and 100%) of the insect repellent OFF[®] (S. C. Johnson, Racine, WI) as standard positive controls. Hexane was used as a negative control. All compounds were evaluated at a concentration of 0.025 mg/L.

Field experiments. Tested compounds were loaded onto controlled-release packets (Consep, Inc., Bend, OR). The packets were heat sealed plastic pouches that contained a permeable plastic membrane surrounded with an impermeable plastic backing material. The compounds (1 ml solution) were loaded onto a cellulose material within the pouch. The compounds were released in a controlled manner by passing through a hole punched in one side of the impermeable backing material. Dimethyl disulfide (250 mg), trimethylamine (27% water solution), indole (25 mg in 75% ethanol), 3-methylindole (25 mg in 75% ethanol) and 4-methylphenol (25 mg in 75% ethanol) were evaluated. Field experiments were conducted at five residences in the Raleigh, NC area where mosquito populations were known to be active (Trexler et al., 1997, 1998) from June to July, 1998. Five ovitrap pairs were placed around the perimeter of each residence in shaded locations on the ground. The ovitrap pairs were spaced at least 25 m apart, and the ovitraps within each pair were 1 m apart. Oviposition traps were black polypropylene cups (ca. 250 ml) with a drain hole drilled near the lip of each cup. Cups were filled with 100 ml of tap water. Red velour papers (2.5 cm x 11 cm) were

clipped to the inside of the ovitraps as oviposition substrates. The controlled-release packet was taped to the inside of one ovitrap of each pair. The other ovitrap contained only water. A sixth pair of ovitraps containing only water was placed as an additional control at each site. One ovitrap in this pair was designated permanently as the test ovitrap.

At each site, the location of the initial placement of each compound was randomly selected. Ovitrap pairs were serviced every two days, the ovistraps were collected, the cups were emptied, rinsed and scrubbed, and new ovistraps were placed in each ovitrap. Each ovitrap pair was systematically rotated to the next location at each residence so that each compound was evaluated at each location within a site. In addition, the position of each cup within the ovitrap pair was randomized for each new location. Eight 2-day trapping periods were completed over the 4-week duration of the study. After collection, the ovistraps were taken back to the laboratory where the eggs on each strip were identified to species (Linley 1989a,b) and counted.

Statistical procedures. Results of the field experiments were analyzed by analysis of variance (ANOVA) on $\sqrt{(\hat{y}+0.5)}$ transformed counts of eggs deposited in each trap (PROC GLM, SAS 1999b). Because our model contained both fixed and random effects, we used a mixed model. Experiments were replicated over time, and experimental sites were considered random effects, while trap treatment was a fixed effect. For the hypothesis of no site main effect, the F test was computed using the site MS

as the numerator, and the week (site) MS as the denominator. For the treatment main effect, the F test was computed with the treatment MS in the numerator, and the treatment * site interaction MS as the denominator. The F tests for the treatment * trap condition and treatment * site interactions used the treatment * condition * site interaction MS as the denominator. Trap condition indicates treatment versus control for the individual trap. Significantly different means were differentiated by the LSMEANS statement in PROC GLM (SAS 1999b) under the hypothesis $LSM_i = LSM_j$.

Laboratory sticky screen experiments were analyzed by a non-parametric signed-rank test (PROC UNIVARIATE, SAS 1999a) to determine if the mean OAI for each treatment was significantly different from zero.

EAG responses were determined by measuring the amplitude of the peak of the action potential produced by antennae. Peak heights produced by antennal responses to control substances were subtracted from peak heights produced in response to test solutions to form a data set of differences (PROC MEANS, SAS, 1999a). A t statistic generated from the data set was used to determine if mean differences were significantly different from zero.

RESULTS

Field studies. Analysis of the oviposition responses of *Ae. albopictus* females to the chemical-baited ovitraps indicated that there were no significant differences in the mean number of eggs laid in traps between sites (Site main effect; $df = 4, 15$; $F = 1.00$; $P = 0.44$). This demonstrates that oviposition activity was comparable among all sites that we used. Although the mean number of eggs laid in traps in response to each chemical lure varied among the sites (Fig. 1), the overall oviposition activity was not significantly different between sites when egg densities in the test ovitraps were averaged over the 4-week ovitrapping period (site * treatment effect; $df = 20, 148$; $F = 1, 20$; $P = 0.26$). Egg densities in ovitraps pairs that contained water only were similarly variable, but not significantly different ($P > 0.05$) within or between sites.

In general, ovitraps baited with dimethyl disulfide, 3-methylindole, trimethylamine, or 4-methylphenol received fewer eggs than matching control ovitraps that contained water (Fig. 2). Only ovitraps baited with indole stimulated more oviposition than untreated traps within an ovitrap pair. However, the differences between treated and control ovitraps within ovitrap pairs were not significant (treatment * condition; $df = 5, 20$; $F = 0.52$; $P = 0.75$). In addition, there were no significant differences between treated and control ovitraps when egg densities were averaged over all sites and over all treatments (condition main effect; $df = 1, 4$; $F = 1.23$; $P = 0.30$).

Laboratory studies. In laboratory experiments, no concentration of the compounds evaluated elicited a significantly positive OAI. *Aedes albopictus* females were either significantly repelled or had no preference for all concentrations of each compound (Table 1).

Aedes albopictus females were significantly repelled by three concentrations of 4-methylphenol. The greatest overall response to any concentration of any compound was to 4-methylphenol at 8.3 mg/l. The repellent effect at this concentration was highly significant (OAI = -0.64; $P < 0.0001$). Repellent effects of 4-methylphenol were also noted at 0.83 mg/l ($P < 0.05$) and at 0.083 mg/l ($P < 0.005$).

The highest concentration of 3-methylindole (83 mg/l) was significantly repellent ($P < 0.05$). The remaining concentrations of the rest of the chemicals did not have significant effects ($P > 0.05$) on the oviposition responses of gravid *Ae. albopictus*.

Electrophysiological Responses to Chemicals. The antennae of *Ae. albopictus* exhibited significant responses to compounds that were used as positive controls (Table 2). Significant ($P < 0.05$) EAG responses were obtained in response to 10% OFF and 100% isoamyl alcohol. In addition, the highest ratio value (2.59 ± 0.72) of any compound that we tested was in response to ethyl acetate, although this value was not significantly higher than controls ($P > 0.05$). None of the test compounds that we evaluated elicited a significant physiological response from *Ae. albopictus* antennae (Table 2).

Each compound was evaluated using antennae from six or seven gravid females. Indole, 4-methylphenol, and 3-methylindole elicited approximately the same response from antennae as the negative control. The EAG responses to dimethyl disulfide and trimethylamine were lower than the responses to the negative controls. However, there was no significant difference ($P > 0.05$) between the test compounds and the controls.

DISCUSSION

In both the laboratory and the field, *Ae. albopictus* exhibited either no preference or a negative oviposition response to all compounds tested. In addition, none of the compounds elicited an antennal response from *Ae. albopictus* in EAG studies. The range of concentrations (0.0083 – 83 mg/L) that we used in laboratory produced positive oviposition responses in two independent studies targeting *Ae. triseriatus* and *Cx. quinquefasciatus*. Bentley et al. (1979) demonstrated that a concentration of 10 mg/L of *p*-cresol attracted significantly more *Ae. triseriatus* than water controls. The highest concentrations that we tested in the laboratory were 83 mg/L (trimethylamine and dimethyl disulfide) and 8.3 mg/L (indole, 4-methylphenol, 3-methylindole). Millar et al. (1992) found that cups baited with 0.1 and 0.01 mg/L of 3-methylindole elicited significantly higher oviposition by *Cx. quinquefasciatus* than water controls. The concentrations evaluated in other studies show that the range of concentrations that we evaluated is likely to include a potential response threshold concentration.

Allan and Kline (1995) evaluated 4-methylphenol, 3-methylindole, and indole as oviposition chemicals of *Ae. albopictus*. The concentrations that we used in the laboratory were generally higher than those used in their study, but the two lowest concentrations that we used, 0.083 and 0.0083 mg/L, overlapped those used by Allan and Kline (1995). We obtained similar results in our evaluations of 3-methylindole and indole. Although only one

concentration of 3-methylindole was significantly repellent in our study, negative OAI values were observed in other experiments. However, whereas we found 4-methylphenol to be significantly repellent at the three highest concentrations, Allan and Kline (1995) showed that 4-methylphenol was not different from the control concentrations that were similar to those that we evaluated but they did not find a repellent effect by this compound. Because they used an open-cup bioassay, it is possible that 4-methylphenol acted as an oviposition stimulant, and masked the repellent effects that we noted.

Holck et al. (1988) reported evidence of increased oviposition by *Ae. triseriatus* in response to a 1% fish oil emulsion in Louisiana. In Wisconsin, however, Beehler and DeFoliart (1990) found that *Ae. triseriatus* was significantly repelled by 1% and 5% fish oil emulsion infusions in both the laboratory and in the field. Trimethylamine is a chemical component of fish odors (Milo and Grosch 1995). Our study shows that *Ae. albopictus* did not respond to four concentrations of trimethylamine in laboratory bioassays. In the field, fewer eggs were laid in ovitraps that were baited with trimethylamine. However, there was no significant difference between the baited and control ovitraps.

Dimethyl disulfide is a component of hog lagoon odors (Zahn et. al 2001). Du and Millar (1999) isolated the related compound dimethyl trisulfide as a volatile component of hay infusions. They found that the antennae of *Cx. quinquefasciatus* and *Cx. tarsalis* did not respond to the chemical, even

though the compound stimulated oviposition at a single concentration. Although pure dimethyl disulfide has not been previously evaluated as a mosquito oviposition attractant, *Cx. quinquefasciatus* is stimulated to oviposit by manure infusions (Kramer and Mulla 1979). In addition, hog lagoons and other animal waste repositories are common areas in which mosquitoes are produced (O'Meara and Evans 1983). We found that dimethyl disulfide did not elicit an oviposition response in *Ae. albopictus* in laboratory bioassays. In addition, fewer eggs were laid in ovitraps that were baited with dimethyl disulfide than in control ovitraps.

Blackwell et al. (1993) conducted electrophysiological studies of the responses of female *Cx. quinquefasciatus* to a number of chemicals. In experiments with an EAG, females were presented with a range of concentrations of 3-methylindole that had elicited oviposition responses. They found that the threshold for the antennae (1000 pg) was an order of magnitude greater than the behavioral threshold (100 pg). We conducted EAG experiments with *Ae. albopictus* using a single concentration (25 mg/L) of each of the five compounds. This concentration was 3x higher than the highest concentration of three of the compounds (indole, 4-methylphenol, and trimethylamine) and comparable to the highest concentrations of two of the compounds (dimethyl disulfide and 3-methylindole) that we evaluated in laboratory oviposition experiments. Because only two of the compounds

exhibited significant repellent effects, it is not surprising that we did not obtain significant EAG responses.

The antennae of *Ae. triseriatus* and *Ae. aegypti* produce “strong” responses to 4-methylphenol in EAG experiments (Bentley et al. 1982). Bentley et al. (1979) previously demonstrated that 4-methylphenol was a significant attractant and stimulant of *Ae. triseriatus*. *Aedes aegypti* was significantly repelled by 0.01 and 1.0 µg/L of 4-methylphenol (Allan and Kline 1995). Although we found that 4-methylphenol was significantly repellent to *Ae. albopictus* at concentrations that spanned three orders of magnitude, we did not obtain a significant EAG response. It is possible that we did not use a concentration that was high enough to elicit an antennal response.

Our experiments with known or potential mosquito oviposition attractants/stimulants failed to elicit a positive response from *Ae. albopictus*. The differential response exhibited to chemicals isolated from organic infusions reflects the adaptation of mosquito species to habitats that often vary substantially in physical and biological properties. Chemical attractants have the potential to enhance the response to oviposition traps or to increase the number of females trapped in gravid female traps. Laboratory bioassays coupled with electrophysiological investigations provides a means for screening candidate attractants (Du and Millar 1999). A controlled-release packet for delivery of oviposition attractants is an appealing concept. Relative to preparing organic infusions, a controlled-release packet would reduce the

time needed to set up and maintain oviposition traps. The packets would provide a standardized delivery system for oviposition attractants, so that the variability in quality of active ingredients often seen in organic infusions (Beehler et al. 1994) can be avoided.

REFERENCES CITED

- Allan, S. A. and D. L. Kline. 1995. Evaluation of organic infusions and synthetic compounds mediating oviposition in *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae). *J. Chem. Ecol.* 21: 1847-1860.
- Beehler, J. W. and G. R. DeFoliart. 1990. A field evaluation of two suggested *Aedes triseriatus* oviposition attractants. *J. Am. Mosq. Control Assoc.* 6: 720-722.
- Beehler, J. W., J. G. Millar, and M. S. Mulla. 1994. Field evaluation of synthetic compounds mediating oviposition in *Culex* mosquitoes (Diptera: Culicidae). *J. Chem. Ecol.* 20: 281-291.
- Bentley, M. D. and J. F. Day. 1989. Chemical ecology and behavioral aspects of mosquito oviposition. *Annu. Rev. Entomol.* 34: 401-421.
- Bentley, M.D., I. N. McDaniel, M. Yatagai, H.-P. Lee and R. Maynard. 1979. *p*-Cresol: an oviposition attractant of *Aedes triseriatus*. *Environ. Entomol.* 8: 206-209.
- Bentley, M. D., I. N. McDaniel and E. E. Davis. 1982. Studies of 4-methylcyclohexanol: an *Aedes triseriatus* (Diptera: Culicidae) oviposition attractant. *J. Med. Entomol.* 19: 589-592.
- Blackwell, A., A. J. Mordue (Luntz), B. S. Hansson, L. J. Wadhams and J. A. Pickett. 1993. A behavioral and electrophysiological study of oviposition cues for *Culex quinquefasciatus*. *Physiol. Entomol.* 18: 343-348.

- Du, Y.-J. and J. G. Millar. 1999. Electroantennogram and oviposition bioassay responses of *Culex quinquefasciatus* and *Culex tarsalis* (Diptera: Culicidae) to chemicals in odors from Bermuda grass infusions. *J. Med. Entomol.* 36: 158-166.
- Gerberg, E. G., D. R. Barnard and R. A. Ward. 1994. Manual for mosquito rearing and experimental techniques. *Am. Mosq. Control Assoc. Bull.* 5.
- Holck, A. R., C. L. Meek and J. C. Holck. 1988. Attractant enhanced ovitraps for the surveillance of container breeding mosquitoes. *J. Am. Mosq. Control Assoc.* 4: 97-98.
- Isoe, J., J. G. Millar and J. W. Beehler. 1995. Bioassays for *Culex* (Diptera: Culicidae) mosquito oviposition attractants and stimulants. *J. Med. Entomol.* 32: 475-483.
- Kramer, W. L. and M. S. Mulla. 1979. Oviposition attractants and repellents of mosquitoes: oviposition responses of *Culex* mosquitoes to organic infusions. *Environ. Entomol.* 8: 1111-1117.
- Kurtti, T. J. and M. A. Brooks. 1976. The dissociation of insect embryos for cell culture. *In Vitro* 12: 141-146.
- Linley, J. R. 1989a. Scanning electron microscopy of the egg of *Aedes (Protomacleaya) triseriatus* (Diptera: Culicidae). *J. Med. Entomol.* 26: 474-478.

- Linley, J. R. 1989b. Comparative fine structure of the eggs of *Aedes albopictus*, *Ae. aegypti*, and *Ae. bahamensis* (Diptera: Culicidae). *J. Med. Entomol.* 26: 510-521.
- Millar, J. G., J. D. Chaney and M. S. Mulla. 1992. Identification of oviposition attractants for *Culex quinquefasciatus* from fermented Bermuda grass infusions. *J. Am. Mosq. Control Assoc.* 8: 11-17.
- Milo, C. and W. Grosch. 1995. Detection of odor defects in boiled cod and trout by gas chromatography-olfactometry of headspace samples. *J. Agric. Food Chem.* 43: 459-462.
- O'Meara, G. F. and F. D. S. Evans. 1983. Seasonal patterns of abundance among three species of *Culex* mosquitoes in a South Florida wastewater lagoon. *Ann. Ent. Soc. Am.* 76: 130-133.
- SAS. 1999a. Base SAS Software. SAS OnlineDoc, Version 8. CD-ROM. SAS Institute Inc., Cary, NC.
- SAS. 1999b. SAS/STAT Software. SAS OnlineDoc, Version 8. CD-ROM. SAS Institute Inc., Cary, NC.
- Steinley, B. A., R. J. Novak, and D. W. Webb. 1994. A new method for monitoring mosquito oviposition in artificial and natural containers. *J. Am. Mosq. Control Assoc.* 7: 649-650.
- Trexler, J. D., C. S. Apperson, and C. Schal. 1997. Diel oviposition patterns of *Aedes albopictus* (Skuse) and *Aedes triseriatus* (Say) in the laboratory and in the field. *J. Vector Ecol.* 22: 64-70.

Trexler, J. D., C. S. Apperson and C. Schal. 1998. Laboratory and field evaluations of oviposition responses of *Aedes albopictus* and *Aedes triseriatus* (Diptera: Culicidae) to oak leaf infusions. *J. Med. Entomol.* 35: 967-976.

Zahn, J. A., A. A. DiSpirito, Y. S. Do, B. E. Brooks, E. E. Cooper and J. L. Hatfield. 2001. Correlation of human olfactory responses to airborne concentrations of malodorous volatile organic compounds emitted from swine effluent. *J. Environ. Qual.* 30: 624-634.

Table 2.1. Results of sticky screen bioassays to determine oviposition responses of *Aedes albopictus* to synthetic chemicals in the laboratory.

Compound	Conc. (mg/l)	n	Mean		
			OAI (SE) ^a	SR ^b	<i>P</i> > <i>t</i>
dimethyl disulfide	0.0083	15	0.04 (0.09)	3.5	0.65
	0.083	12	-0.03 (0.12)	-6.0	0.82
	0.83	12	0.10 (0.10)	8.5	0.36
	8.3	12	-0.04 (0.11)	-3.5	0.74
	83.0	12	-0.00 (0.13)	1.0	0.99
indole	0.0083	18	0.12 (0.08)	18.0	0.15
	0.083	12	-0.17 (0.12)	-15.5	0.21
	0.83	12	-0.19 (0.14)	-16.5	0.21
	8.3	12	-0.08 (0.17)	-7.0	0.62
4-methylphenol	0.0083	12	-0.07 (0.11)	-9.0	0.55
	0.083	12	-0.30 (0.09)	-21.5	0.005
	0.83	12	-0.21 (0.10)	-24.5	0.05
	8.3	12	-0.64 (0.07)	-33.0	0.0001
3-methylindole	0.0083	18	0.11 (0.09)	25.5	0.25
	0.083	12	-0.10 (0.08)	-11.0	0.24
	0.83	12	0.14 (0.18)	9.5	0.46
	8.3	12	-0.14 (0.11)	-16.0	0.21
	83.0	12	-0.24 (0.11)	-25.0	0.05

Table 2.1 (continued)

trimethylamine	0.0083	15	-0.10 (0.11)	-17.5	0.38
	0.083	12	-0.07 (0.11)	-4.0	0.52
	0.83	12	-0.14 (0.11)	-13.5	0.21
	8.3	12	-0.06 (0.09)	-6.5	0.52

^aOviposition Activity Index.

^bSigned-rank statistic derived through PROC UNIVARIATE (SAS 1999a).

Table 2.2. Electroantennogram responses of *Aedes albopictus* to synthetic chemicals that were candidate oviposition attractants or controls.

Compound	n	Mean Ratio ^a		
		(SE)	<i>t</i>	<i>P</i> > <i>t</i> ^b
Dimethyl disulfide	7	0.78 (0.19)	-1.17	0.28
Indole	6	1.04 (0.07)	0.60	0.58
4-Methylphenol	7	1.06 (0.14)	0.41	0.69
3-Methylindole	6	0.98 (0.14)	-0.12	0.91
Trimethylamine	7	0.72 (0.12)	-2.30	0.06

^aRatio of test substance and hexane (negative control).

^b*t*-tests conducted on the mean differences of antennal responses to the chemicals versus hexane controls.

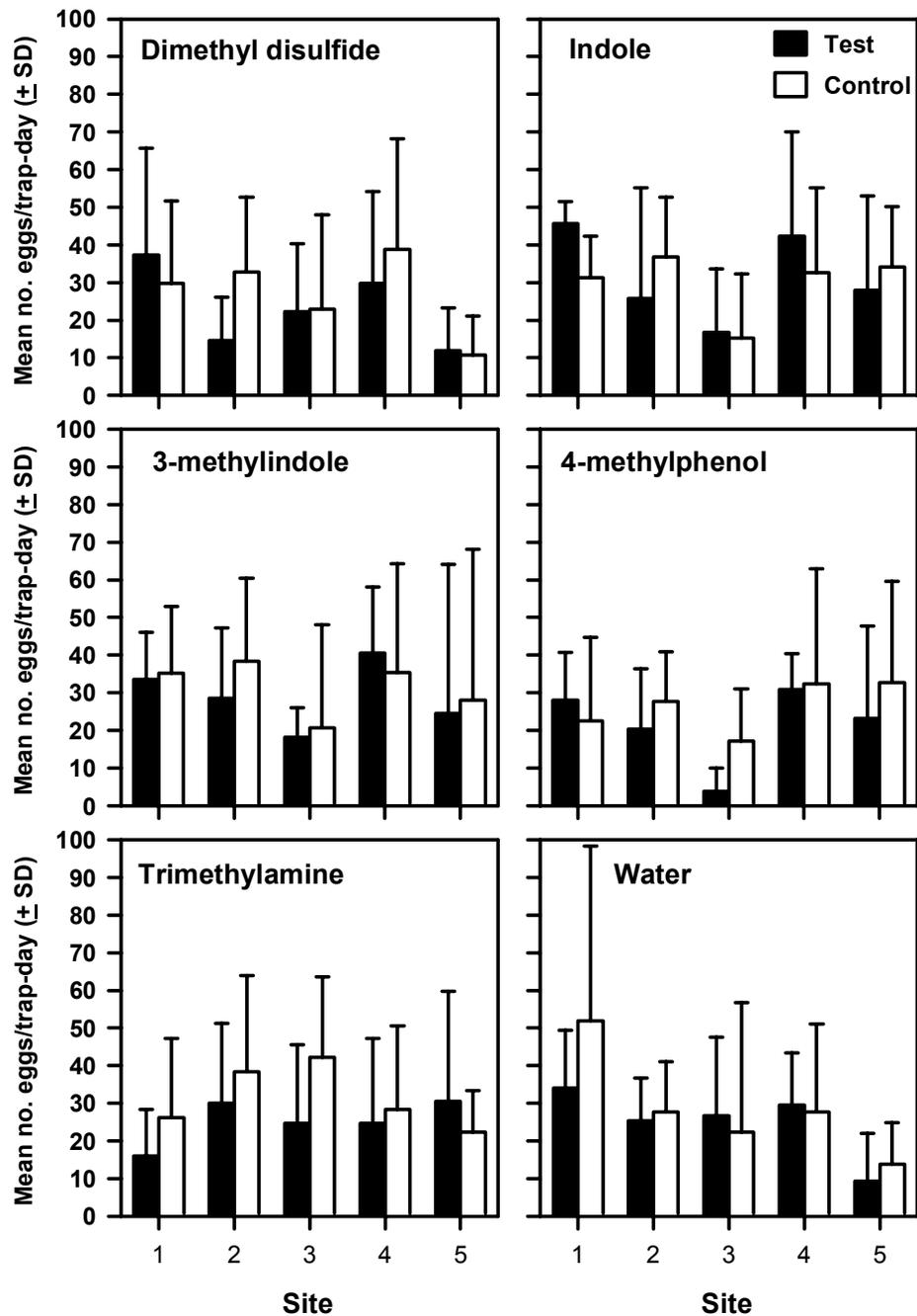


Figure 2.1. Oviposition responses of *Aedes albopictus* to pairs of water-filled ovitraps that were placed at five different field sites. One trap in each pair was baited with a candidate attractant (test) and the other trap contained water (control). Mean (\pm SD) egg counts per ovitrap are averaged over the 4 week ovitrapping period from June to July, 1998. N = 8.

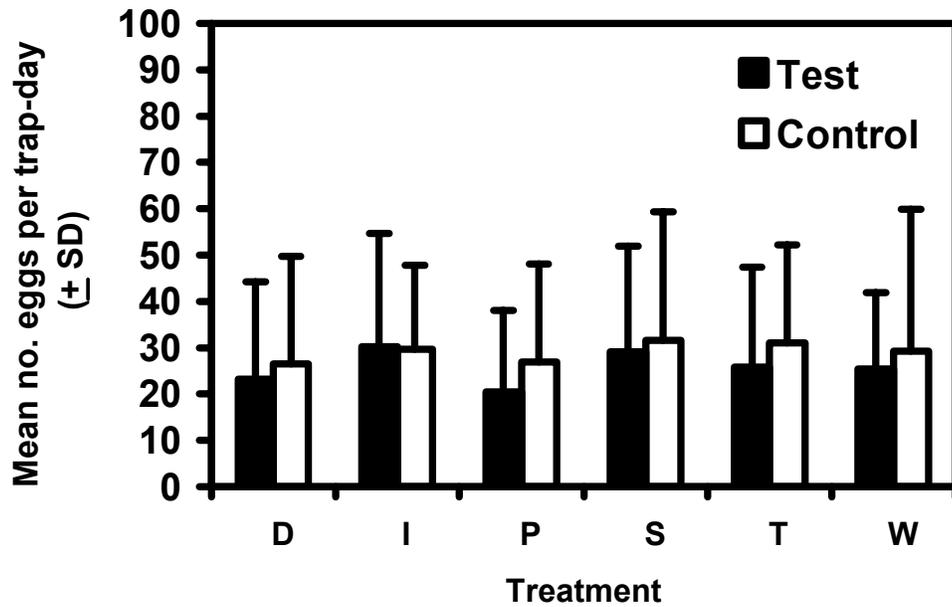


Figure 2.2. Mean (\pm SD) number of eggs laid by *Aedes albopictus* in treated ovitrap groups baited with 5 synthetic chemicals (D = dimethyl disulfide, I = indole, P = 4-methylphenol, S = 3-methylindole, T = trimethylamine) or in control ovitrap groups that contained water (W). Egg numbers are averaged over site over the 4 week ovitraping period from June to July, 1998.

CHAPTER 3.

ROLE OF BACTERIA IN MEDIATING THE
OVIPOSITION RESPONSES OF *AEDES ALBOPICTUS*
(DIPTERA: CULICIDAE)

INTRODUCTION

The oviposition behavior of insects results from integration of a number of internal and external stimuli (Kennedy 1978). Factors that can influence oviposition include environmental conditions (e.g. temperature, photoperiod, humidity), physiological state of the insect, physical factors in the environment such as substrate texture and color, and chemical cues (Bentley and Day 1989). In mosquitoes, the number of eggs laid in containers is also affected by a behavior known as “skip oviposition” behavior (Mogi and Mokry 1980), where females lay their eggs in several containers rather than as a single clutch in one container (Fay and Perry 1965, Rozeboom et al. 1973, Chadee and Corbet 1987, Apostol et al. 1994). Skip oviposition behavior increases the distribution of eggs in an area and may be amplified 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). Yet, studies of mosquito oviposition have typically assayed the endpoint of oviposition (the number of eggs laid) as a measure of oviposition site preference. Mosquito oviposition is therefore a good example of a case where the units of the behavior should be defined as precisely, objectively, and as simply as possible (Harris and Foster 1995). This may have confounded the interpretation of preferences because both olfactory and contact cues from the oviposition site are considered simultaneously.

Aedes albopictus (Skuse) is a container-inhabiting mosquito that oviposits in artificial and natural containers (Hawley 1988). In selecting a container for oviposition, females use volatile chemical attractants and contact chemical stimulants from fermenting organic material as semiochemical cues. Trexler et al. (1998) found that oak leaf infusions of varying ages and concentrations significantly increased oviposition by *Ae. albopictus*. In similar studies aqueous infusions made from leaves, grass sod, and hay increase the numbers of eggs laid by *Aedes* (*Stegomyia*) mosquitoes (Hazard et al. 1967; Gubler 1971; Reiter 1983; Lampman and Novak 1996).

Other substrates have been reported to significantly increase oviposition by mosquitoes. Conspecific larval rearing water increased oviposition by mosquitoes (Allan and Kline 1998; Bentley et al. 1976; Benzon and Apperson 1987). In addition, Wallace (1996) found that cotton towels, contaminated with soil from a dredge spoil site in South Carolina, significantly increased oviposition by *Ochlerotatus* (= *Aedes*) *taeniorhynchus* (Wiedemann).

Bacteria produce both volatile attractants and contact chemical stimulants as metabolites of organic material. Bacteria isolated from an infusion of alfalfa hay produced chemicals that stimulated *Aedes aegypti* L. and *Culex quinquefasciatus* (Say) to oviposit (Hazard et al. 1967). Hay infusion and *Enterobacter* (= *Aerobacter*) *aerogenes*, the primary bacteria isolated from the infusion, were more attractive to *Cx. quinquefasciatus* than distilled water in tests with an olfactometer. *Aedes aegypti* was not attracted to hay infusion

(Hazard et al. 1967) while its oviposition response to larval rearing water was wholly attributed to *Acinetobacter calcoaceticus* and *Enterobacter cloacae* (Benzon and Apperson 1988). *Culex quinquefasciatus* laid significantly more egg rafts in cups that contained agar washes of . *agglomerans*, *Pseudomonas maltophilia*, and *Bacillus cereus* than in control cups (Rocket 1987). In addition, Wallace (1996) postulated that the attractiveness of soil-contaminated cotton towels to *Oc. taenirohynchus* was due to the actions of bacteria and fungi.

The overall objective of our research was to isolate and identify bacterial species from some substrates, such as larval rearing water and plant infusions, that increased oviposition by *Ae. albopictus*. In addition, we wanted to determine whether the increased oviposition resulted from responses to the bacteria and whether specific bacteria produced volatile 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 kept at *ca.* 26°C and at a relative humidity of *ca.* 75% under L:D 14:10. Included in the light phase were two 30-minute 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 were kept in 30 x 30 x 30 cm Plexiglas cages fitted with cotton surgical stocking tops and were maintained on a continuously provided 10% sucrose solution. Four days prior to 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). Females not used in the experiments were allowed to oviposit on seed germination paper (Steinley et al. 1994). The F4-F7 generations were used in these experiments.

Sources of bacteria. Cotton towels were originally with soil from a dredge disposal island along the Atlantic Intracoastal Waterway near Charleston, South Carolina. Sterile towels were cross-contaminated by wrapping sterile towels with contaminated towels and placing the towels in a sterile plastic bag for one week. The newly contaminated towel was allowed to dry for *ca.* 48 h in a sterile fume hood, transferred to a sterile plastic bag, and stored at 4°C. Bacterial isolates were cultured from the towels prior to drying.

Oak leaf infusion (OLI) was prepared by fermenting approximately 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 the rearing dishes of *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), placed in plastic bottles, and stored at -20°C. LRW was allowed to thaw overnight prior to oviposition bioassays. Bacteria were cultured from LRW prior to freezing.

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 x 30 x 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. The oviposition bioassay chamber consisted of a large metal rack with three shelves. Six oviposition cages were placed on each shelf. 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 day length light cycle (16 h:8 h photophase:scotophase) was used during experimentation.

Bacterial isolates were bioassayed following the modified methods of Benzon and Apperson (1988). Isolates were inoculated (3.75×10^4 cells/ml) into dilute (12.5%) Bacto nutrient broth (NB) (Difco, Detroit, MI) and were allowed to grow at 26° C for 18 h. Cell densities were determined by direct count with 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 x 7.5 cm strips of towel were clipped to the insides of oviposition cups. Clean, sterile, 100% cotton towels (K-Mart, Troy, MI) were soaked in sterile distilled water and excess water was rung out. Wet towels were placed in sterile bags for one week at *ca.* 25° C. After the storage period, the sterile towels were allowed to dry in a sterile fume hood, and strips of these towels were used as control oviposition substrates. Each oviposition cup contained 30 ml of distilled water. Cups were placed in opposite corners of the bioassay cage and removed after 24 h. A single female was aspirated into each cage. The number of eggs deposited on control and test towels was counted. In the second bioassay method, 2.5 x 2.5 cm strips of contaminated and sterile 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. The number of eggs on the control and experimental ovistrips were counted after a 24 h exposure period. A single female was aspirated into each cage. In both methods, the number of eggs floating on the water surface and submerged in the cups were counted and added to the total number of eggs laid on the towel and ovistrip.

To differentiate oviposition responses elicited by odorants from those induced by contact chemo-stimulation, we used a modification of the sticky-screen bioassay of Isoe et al. (1995). In the 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 small enough that females were trapped when they land on the screen. Therefore, any positive or negative oviposition responses are due to attraction to volatile materials. 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 in Trexler et al. (1998). Two 125 ml black polypropylene cups 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. Each cup was covered with a glue-coated screen. Ten gravid females were placed in each cage. After a 24 h exposure period, the number of females trapped on each screen was counted.

The oviposition activity index (OAI) (Kramer and Mulla, 1979) was used to evaluate the responses of the females to test substrates. 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.

Isolation and Identification of Bacteria. Bacteria were isolated from the contaminated towels, oak leaf infusion, and larval rearing water by plating samples (100 μ l) on trypticase soy broth agar (TSBA) (Difco, Detroit, MI) and Eosin Methylene Blue Agar (EMB, Difco, Detroit, MI) plates and incubated aerobically at 27° C. Morphologically different colonies were isolated on TSBA 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, Pittsburg, PA), catalase test (using 3% hydrogen peroxide), oxidase test (BBL Oxidase, Becton Dickinson, Cockeysville, MD), and motility (examined on wet mount slide under microscope and by stabbing into SAM - soft agar medium: tryptose 10.0 g/liter, NaCl 5.0 g/liter, agar 5.0 g/liter) (Perry and Stanley,

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 1 indicate a close match to authentic standard values.

Volatiles Collection. Two methods were used to collect headspace samples. Volatiles were collected from crude LRW and crude towel using a closed collection system. Volatiles were trapped onto an adsorbent (Tenax TA 60/80 mesh) that was packed into a 6 mm OD by 120 mm long glass tube. Volatiles were collected over a 24 h period. The column was washed with hexane (3 x 25 ml) to remove the volatiles from the adsorbent, and the hexane eluent was evaporated under nitrogen to a volume of approximately 2 ml. In the second method, suspensions of LRW4 and T2 were grown 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 was severed and 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 L/min), which adapted the mechanoreceptors on the antenna. Each test solution (10 μ l) was applied to a filter paper strip and the solvent was allowed to evaporate. The filter paper was then inserted into a Pasteur pipette and attached to a glass syringe. A single, rapid 2 ml puff of test odorant was then introduced into the airstream. 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).

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 non-parametric signed-rank test (PROC UNIVARIATE, SAS Institute 1999a) to determine if

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 if the mean differences were significantly different from zero.

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* (Figure 1). Cups containing contaminated towels as oviposition substrates received a mean of $70.9 \pm 7.0\%$ (SEM) 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 $71.0 \pm 8.4\%$ 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 Towel 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 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 on cups that contained LRW.

Isolation and Identification of Bacterial Isolates. Eight 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 (Table 3). However, phenotypic characterization revealed that isolates T-1 and T-3 were identical. Isolates T-1 and T-3 were identified as non-pigmented *Serratia marcescens*, a common contaminant of insect colonies and a facultative pathogen of insects. The third isolate (T-2) was identified by FAME as *Sphingobacterium multivorum*.

Three isolates were cultured from OLI. All three isolates were Gram positive. We were able to determine the species level of isolate OLI-1 (*Bacillus cereus*) and isolate OLI-3 (*Paenibacillus pabuli*). 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). In response to cups containing LRW-5, *Ae. albopictus* laid a mean of 52.1 ± 4.1 eggs compared to 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 to control substances. However, eggs were laid in response to two bacterial isolates (LRW-7 and LRW-8) compared to 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, while 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 greater from 1. However, differences between antennal responses of test and control substances were not significantly different than 1. Volatiles collected from

isolate T-2 also did not elicit significant responses from *Ae. albopictus* antennae.

DISCUSSION

Oviposition bioassays. *Aedes albopictus* laid significantly more eggs in cups that were baited with either larval rearing water or microbially-contaminated cotton towels. Our results concur that *Ae. albopictus* females lay significantly more eggs in cups that contain larval rearing or holding water (Gubler 1971, Allan and Kline 1998). In our experiments with larval rearing water, approximately 75% of all eggs were laid in response to 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 to four other control and test containers. In a binary choice assay, *Ae. albopictus* laid about 60% of all eggs in containers that contained 50% larval rearing water versus containers with tap water (Allan and Kline 1998).

Wallace (1996) demonstrated that field populations of *Ae. taeniorhynchus* laid significantly more eggs in traps that contained microbially-contaminated cotton towels than in traps that contained control towels. Microbially-contaminated towels elicited more oviposition by natural populations of *Ae. taeniorhynchus* by approximately 100-fold compared to uncontaminated towels (Wallace 1996). Although we did not see 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 larval rearing water and contaminated cotton toweling produce oviposition attractants. In previous studies oak leaf infusions did not produce chemical attractants in sufficient quantities to influence the oviposition responses of *Ae. albopictus* (Trexler et al. 1998). Oak leaf infusion elicits an increased oviposition response 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 was obtained from larval rearing water. Benzon and Apperson (1988) reported that the two predominant bacterial species in larval rearing water of *Ae. aegypti* were *Enterobacter cloacae* and *Acinetobacter 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 species from *Ae. albopictus* larval rearing water in the laboratory. It is possible that these two species associate with *Ae. aegypti*, hence the very strong oviposition responses that they elicit from females, but

not with *Ae. albopictus*. In addition, *Ae. aegypti* females may inoculate additional oviposition sites with the bacteria.

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.

Bioassays of 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 bacteria population (Benzon and Apperson 1988). In bioassays of the isolates that were obtained from larval rearing water, 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 larval rearing water. In experiments with both 10% and 100% larval rearing water, 70% and 75% of all eggs were laid in test cups, respectively. It is likely that a combination of the bacterial species would elicit a stronger oviposition response, increasing the percentage of eggs that are deposited in test containers.

Electroantennogram experiments with volatiles 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 to 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.

We used only aerobic culturing techniques in this study. Microaerophiles, strict anaerobes and bacteria that were non-culturable on our artificial media were not isolated. Additional research should examine the roles that these bacterial groups play in mediating oviposition by *Ae. albopictus*.

Our studies indicate that the bacterial species that we isolated from larval rearing water, oak leaf infusions, and contaminated cotton towels significantly increase the number of eggs that are laid by *Ae. albopictus*. Future research should focus on the volatile compounds that these species produce as well as the isolation and identification of contact chemical oviposition stimulants.

REFERENCES CITED

- Allan, S. A. and D. L. Kline. 1995. Evaluation of organic infusions and synthetic compounds mediating oviposition in *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae). *J. Chem. Ecol.* 21: 1847-1860.
- Allan, S. A. and D. L. Kline. 1998. Larval rearing water and preexisting eggs influence oviposition by *Aedes aegypti* and *Ae. albopictus* (Diptera: Culicidae). *J. Med. Entomol.* 35: 943-947.
- Apostol, B. L. W. C. Black, P. Reiter, and B. R. Miller. 1994. Use of randomly amplified polymorphic DNA amplified by polymerase chain reaction markers to estimate the number of *Aedes aegypti* families at oviposition sites in San Juan, Puerto Rico. *Am. J. Trop. Med. Hyg.* 51: 89-97.
- Bentley, M. D. and J. F. Day. 1989. Chemical ecology and behavioral aspects of mosquito oviposition. *Annu. Rev. Entomol.* 34: 401-421.
- Benzon, G. L. and C. S. Apperson. 1988. Reexamination of chemically mediated oviposition behavior in *Aedes aegypti* (L.) (Diptera: Culicidae). *J. Med. Entomol.* 25: 158-164.
- Blackwell, A., A. J. Mordue (Luntz), B. S. Hansson, L. J. Wadhams, and J. A. Pickett. 1993. A behavioral and electrophysiological study of oviposition cues for *Culex quinquefasciatus*. *Physiol. Entomol.* 18: 343-348.
- Chadee, D. D. and P. S. Corbet. 1987. Seasonal incidence and diel patterns of oviposition in the field of the mosquito, *Aedes aegypti* (L.) (Diptera:

- Culicidae) in Trinidad, West Indies: a preliminary study. *Ann. Trop. Med. Parasitol.* 81: 151-161.
- Chadee, D. D., P. S. Corbet and J. J. D. Greenwood. 1990. Egg-laying yellow fever mosquitoes avoid sites containing eggs laid by themselves or by conspecifics. *Entomol. Exp. Appl.* 57: 295-298.
- Chadee, D. D., A. Lakhan, W. R. Ramdath, and R. C. Persad. 1993. Oviposition response of *Aedes aegypti* mosquitoes to different concentrations of hay infusion in Trinidad, West Indies. *J. Am. Mosq. Control Assoc.* 9: 346-348.
- Du, Y.-J. and J. G. Millar. 1999. Electroantennogram and oviposition bioassay responses of *Culex quinquefasciatus* and *Culex tarsalis* (Diptera: Culicidae) to chemicals in odors from Bermuda grass infusions. *J. Med. Entomol.* 36: 158-166.
- Fay, R. W. and A. S. Perry. 1965. Laboratory studies of the ovipositional preferences of *Aedes aegypti*. *Mosq. News* 26: 531-535.
- Gerberg, E. G., D. R. Barnard, and R. A. Ward. 1994. Manual for mosquito rearing and experimental techniques. *Am. Mosq. Control Assoc. Bull.* 5.
- Gubler, D. J. 1971. Studies on the comparative oviposition behavior of *Aedes (Stegomyia) albopictus* and *Aedes (Stegomyia) polynesiensis* Marks. *J. Med. Entomol.* 6: 675-682.

- Harris, M. O. and S. P. Foster. 1995. Behavior and integration. *In*: Chemical ecology of insects 2. (R. T. Carde and W. J. Bell, eds.). New York: Chapman and Hall.
- Hawley, W. A. 1988. The biology of *Aedes albopictus*. J. Am. Mosq. Control Assoc. 4 (Suppl. No. 1). 40 pp.
- Hazard, E. I., M. S. Mayer, and K. E. Savage. 1967. Attraction and oviposition stimulation of gravid female mosquitoes by bacteria isolated from hay infusions. Mosq. News 27: 133-136.
- Isoe, J., J. G. Millar, and J. W. Beehler. 1995. Bioassays for *Culex* (Diptera: Culicidae) mosquito oviposition attractants and stimulants. J. Med. Entomol. 32: 475-483.
- Janda, J. M. and S. L. Abbot. 1998. *The Enterobacteria*. New York: Lippincott-Raven, 387 pp.
- Kaufman, M. G., E. D. Walker, T. W. Smith, R. W. Merritt, and M. J. Klug. 1999. Effects of larval mosquitoes (*Aedes triseriatus*) and stemflow on microbial community dynamics in container habitats. Appl. Environ. Microbiol. 65: 2661-2673.
- Kennedy, J. S. 1978. The concepts of olfactory "arrestment" and "attraction". Physiol. Entomol. 3: 91-98.
- Kitron, U. D., D. W. Webb, and R. J. Novak. 1989. Oviposition behavior of *Aedes triseriatus* (Diptera: Culicidae): prevalence, intensity, and aggregation of eggs in oviposition traps. J. Med. Entomol. 26: 462-467.

- Kramer, W. L. and M. S. Mulla. 1979. Oviposition attractants and repellents of mosquitoes: oviposition responses of *Culex* mosquitoes to organic infusions. *Environ. Entomol.* 8: 1111-1117.
- Kurtti, T. J. and M. A. Brooks. 1976. The dissociation of insect embryos for cell culture. *In Vitro* 12: 141-146.
- Lampman, R. L. and R. J. Novak. 1996. Attraction of *Aedes albopictus* adults to sod infusion. *J. Am. Mosq. Control Assoc.* 12: 119-124.
- Mogi, M. and J. Mokry. 1980. Distribution of *Wyeomyia smithii* (Diptera: Culicidae) eggs in pitcher plants in Newfoundland, Canada. *Tropical Medicine* 22: 1-12.
- Perry, J. J., and J. T. Staley. 1997. Taxonomy of Eubacteria and Archaea, pp. 409-411. *In Microbiology: diversity and dynamic.* Saunders College Publishing, Orlando, FL.
- Reiter, P. 1983. A portable, battery-powered trap for collecting gravid *Culex* mosquitoes. *Mosq. News* 43: 496-498.
- Rockett, C. L. 1987. Bacteria as ovipositional attractants for *Culex pipiens* (Diptera: Culicidae). *Great Lakes Entomol.* 20: 151-155.
- Rozeboom, L. E., L. Rosen and J. Ikeda. 1973. Observations on oviposition by *Aedes (S.) albopictus* Skuse and *A. (S.) polynesienses* Marks in nature. *J. Med. Entomol.* 4: 397-399.
- SAS. 1999a. Base SAS Software. SAS OnlineDoc, Version 8. CD-ROM. SAS Institute Inc., Cary, NC.

- SAS. 1999b. SAS/STAT Software. SAS OnlineDoc, Version 8. CD-ROM.
SAS Institute Inc., Cary, NC.
- Steinley, B. A., R. J. Novak, and D. W. Webb. 1994. A new method for
monitoring mosquito oviposition in artificial and natural containers. *J. Am.
Mosq. Control Assoc.* 7: 649-650.
- Trexler, J. D., C. S. Apperson, and C. Schal. 1998. Laboratory and field
evaluations of oviposition responses of *Aedes albopictus* and *Aedes
triseriatus* (Diptera: Culicidae) to oak leaf infusions. *J. Med. Entomol.*
35: 967-976.
- Wallace, F. L. 1996. Construction of a field trap for initiating an ovipositional
response in *Aedes taeniorhynchus*. *J. Am. Mosq. Control Assoc.* 12:
491-493.

Table 3.1. Oviposition Responses of *Aedes albopictus* to Larval Rearing Water

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

Table 3.2. 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 ^c	P
Towel ^a	23	187	111	76	0.21	0.007
LRW ^b	23	178	120	58	0.36	0.0001

^aTowel was submerged in water.

^b100% concentration

^cOviposition Activity Index.

^dDifferences from zero were determined by a signed rank test (PROC UNIVARIATE, SAS, 1999a).

Table 3.3. Species identifications of bacteria isolated from three sources.

Isolate	FAME Library ID	Similarity Index
LRW-2	<i>Micrococcus luteus</i>	0.75
LRW-4	<i>Clavibacter michiganese</i>	0.81
LRW-5	<i>Psychrobacter immobilis</i>	0.65
LRW-6	<i>Bacillus brevis</i>	0.49
LRW-7	<i>Micrococcus kristiae</i>	0.76
LRW-8	<i>Rhodococcus sp.</i>	0.04
T-1	<i>Serratia marcescens</i>	phenotype
T-2	<i>Sphingobacterium multivorum</i>	0.75
T-3	<i>Serratia marcescens</i>	phenotype
OLI-1	<i>Bacillus cereus</i>	0.67
OLI-2	<i>Bacillus sp.</i>	0.42
OLI-3	<i>Paenibacillus pabuli</i>	0.72

Table 3.4. Oviposition responses of *Ae. albopictus* to bacterial isolates in binary assays.

Isolate ^a	n	Mean no. eggs in test cup (\pm SE)	Mean no. eggs in control cup (\pm 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	0.009
T-3	20	29.7 (6.8)	38.9 (7.5)	26	0.70	0.41

^aSee Table 3 to determine the corresponding bacterial species.

^bError df. The numerator df in each F test was 1.

Table 3.5. Oviposition responses of *Aedes albopictus* to bacterial isolates in sticky-screen bioassays.

Bacterial Isolate	No. cages	OAI ^a
LRW4	16	0.17
LRW5	18	-0.027
OLI2	18	0.15
T2	18	-0.01

^aOviposition Activity Index. OAI values are not significantly different from zero ($P > 0.05$) by PROC UNIVARIATE (SAS 1999a).

Table 3.6. Electroantennogram responses of *Aedes albopictus* to volatiles produced by bacteria.

Isolate	n	Mean ratio ^a	<i>t</i>	<i>P</i> > <i>t</i> ^b
LRW4	3	1.24 (0.38)	0.63	0.59
T2	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

^aRatio of test substance and hexane (negative control).

^b*t*-tests conducted on the mean differences of antennal responses to the chemicals versus hexane controls.

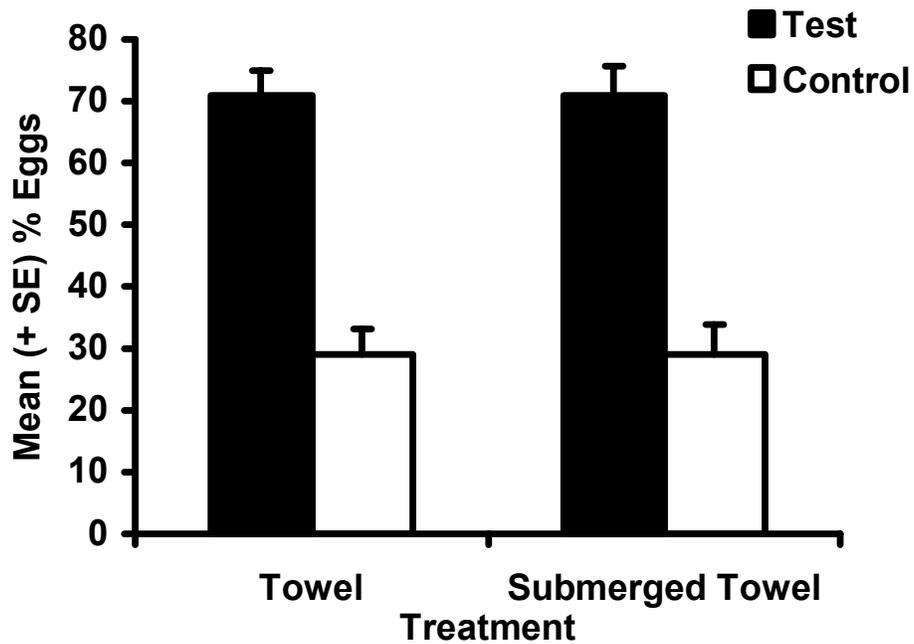


Figure 3.1. Oviposition responses of *Aedes albopictus* to microbially-contaminated cotton towels. Mosquitoes laid eggs directly on towels in the towel treatment. In the submerged towel treatment, mosquitoes were unable to contact the towels. Eggs were laid on seed germination paper.