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INTERACTION OF THE *CULEX QUINQUEFASCIATUS* EGG RAFT PHEROMONE WITH A NATURAL CHEMICAL ASSOCIATED WITH OVIPOSITION SITES[1]

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ABSTRACT. In laboratory bioassays, gravid *Culex quinquefasciatus* mosquitoes were strongly attracted and/or stimulated to oviposit by a habitat-derived chemical cue, 3-methylindole, at several concentrations ranging from 0.01 to 1 μgram/liter in water. At concentrations above 10 μgrams/liter, 3-methylindole became repellent or deterrent. Responses to the known egg raft pheromone, 6-acetoxy-5-hexadecanolide, were much weaker and were relatively constant above a threshold dosage of about 0.1 μgram. Responses to blends of a fixed amount of the pheromone with variable doses of 3-methylindole were shown to be additive rather than synergistic.

INTRODUCTION

Culex mosquitoes vector a number of diseases such as St. Louis encephalitis, western equine encephalitis, and Bancroftian filariasis. Public health and vector control agencies maintain active surveillance programs to monitor populations and infection levels of mosquito species known to transmit these diseases. Oviposition traps are useful for virus surveillance programs because they attract a much higher proportion of gravid, bloodfed mosquitoes that may have acquired disease organisms from a blood meal, than do CO_2, light, or vertebrate-baited traps, which attract predominantly host-seeking females, many of which will not have taken a blood meal. Oviposition traps are usually baited with fermented infusions of organic materials such as grass cuttings or sod (Reiter 1983, Brust 1990). These materials are cumbersome to work with, and their attractiveness changes fairly rapidly so that they must be replenished frequently (Reiter et al. 1991). Infusions are also impossible to standardize, because each infusion is a dynamic system, with constantly changing microbial fauna and chemical constitution. The identification of oviposition attractants for *Culex* species, and the consequent development of standardized baits containing a known blend of attractants would be of considerable benefit to mosquito surveillance programs.

It is well known that gravid female mosquitoes use a combination of physical factors (e.g., temperature, light, humidity) and chemical cues (e.g., attractants, arrestants, and oviposition stimulants) to locate suitable oviposition sites (Benzon and Apperson 1988, Bentley and Day 1989, Beehler et al. 1993). Each species uses cues characteristic to its preferred oviposition sites to locate and colonize those sites, reflecting the variety of niches occupied by different species in aquatic habitats. Chemical cues can be positive, in the form of attractants and oviposition stimulants, or negative, in the form of repellents and deterrents. Some chemical cues, such as egg-raft pheromones, are insect derived, and other chemical cues are produced as a result of microbial decomposition of organic matter in larval habitats.

The egg raft pheromone for *Culex quinquefasciatus* Say was identified as (5*R*, 6*S*)-erythro-6-acetoxy-5-hexadecanolide by Laurence and Pickett (1982). This discovery gave rise to a number of ingenious syntheses of the pheromone, and laboratory and field tests have been conducted to evaluate the pheromone as an attractant in monitoring programs (Laurence and Pickett 1982, 1985; Laurence et al. 1985; Hwang et al. 1987; Otieno et al. 1988a, 1988b; Dawson et al. 1990). However, the relative importance of the pheromone in comparison to chemical cues derived from the oviposition site itself has not been elucidated. We initiated the present studies to determine dose–response relationships for the egg raft pheromone and for 3-methylindole, which we have recently identified as a *Culex quinquefasciatus, Culex tarsalis* Coq., and *Culex stigmatosoma* Dyar oviposition attractant from grass infusions (Millar et al. 1992, Beehler et al. 1994). Our 2nd objective was to determine the nature of the interaction between the insect-derived egg raft pheromone and the habitat-derived 3-methylindole.

MATERIALS AND METHODS

Insects: Gravid adult female *Cx. quinquefasciatus* were obtained from a colony originating

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from and supplemented periodically with egg rafts collected from natural oviposition sites in Orange and Riverside counties, California. The colony was maintained in the laboratory as previously described (Kramer and Mulla 1979). Five-day-old mated adult females were bloodfed on chicks (University of California Animal Use Protocol A 3439-01) approximately 8 days before being used in bioassays. Gravid females were used only once and discarded.

For collection of egg rafts, gravid bloodfed females were allowed overnight access to 125-ml (5.1-cm-diam) paper oviposition cups filled with distilled water. Egg rafts were collected the next morning, and used for analysis or bioassays the same afternoon (see below).

Synthetic chemicals: 3-Methylindole (>99% purity) was obtained from Eastman Kodak Co. (Rochester, NY). The egg raft pheromone was synthesized as a mixture of 4 stereoisomers (2 enantiomeric pairs of diastereomers), as previously described (Dawson et al. 1990). It has been previously shown that the unnatural stereoisomers are neither attractive nor repellent (Sakakibara et al. 1984, Laurence and Pickett 1985, Laurence et al. 1985, Hwang et al. 1987). Spectra of synthetic intermediates and of the final stereoisomeric mixture matched the literature data (Dawson et al. 1990). The purified mixture of stereoisomers contained less than 0.5% of other impurities.

Natural egg raft pheromone: Egg raft pheromone was collected by absorption of apical droplets from 6–12-h-old eggs onto filter paper points; the amount of pheromone/egg raft has been shown to remain unchanged for 24 h after eggs are laid (Laurence and Pickett 1985). The filter papers were rinsed with 0.1 ml of hexane, and the resulting extract was analyzed by coupled gas chromatography–mass spectrometry (0.2 mm × 20 m Ultra-2 column, 50–250°C at 15°C/min) using a Hewlett-Packard 5890 GC coupled to an H.-P. 5970A mass selective detector. The pheromone component comprised 72% of the extracted volatiles, and the retention time and mass spectrum (EI, 70 eV) corresponded exactly with the earlier eluting peak in the synthetic egg raft pheromone mixture, synthesized as described above.

Bioassays: Oviposition bioassays with egg rafts were conducted in 23 × 23 × 30.5-cm wooden frame cages covered with plastic screen, with glass tops and muslin sleeves for access. Each cage contained 2 125-ml (5.1-cm-diam) waxed paper cups, placed in the back corners of the cage, and filled with 80 ml of distilled water. Varying numbers of egg rafts were placed randomly in one of the 2 cups. Cages were placed in a well-ventilated room, with a 14:8 light cycle, and 1 h simulated dawn and dusk periods. Temperature was maintained at 27 ± 2°C, with a relative humidity of 40–60%. Six replicate cages of 20 gravid females each (7–8 days after bloodfeeding) were run simultaneously, and each test was repeated at least twice on different dates. Bioassays were started at 1700 h, and evaluated the next morning at 0830 h by counting the numbers of egg rafts in each cup (subtracting the number of egg rafts placed in the treatment cups at the start of the bioassay from the total number of egg rafts). Collated results were analyzed with paired *t*-tests.

Oviposition bioassays of synthetic chemicals were conducted in 45-cm cubic screen cages constructed of PVC plastic tubing frames covered with white Dacron® screen, with a sleeve on the front for access. Two 125-ml waxed paper cups, one containing 100 ml of distilled water spiked with the test treatment and one containing distilled water alone, were placed in each of the back corners of the cage. Dose–response curves for the egg raft pheromone and 3-methylindole were generated by testing each compound versus a distilled water control. 3-Methylindole was added to cups as an ethanol solution (0.1 ml ethanol) and controls were treated with 0.1 ml pure ethanol. Because the egg raft pheromone is highly lipophilic and virtually insoluble in water, the hexane solution of the egg raft pheromone was pipetted onto thin glass microscope cover slips (2.2 cm diam), the hexane was allowed to evaporate, and the cover slip was floated on the water surface in the test cups. Control cups had a glass coverslip treated with hexane alone. The positions of the cups were alternated between the different replicates, with cages placed side by side and separated by 10 cm. Mosquitoes were introduced into cages at 1700 h and bioassays were allowed to run overnight at ambient temperatures in a well-ventilated room, under natural light conditions, with cages placed in a row along a bench by the windows. Four replicate cages of 50 gravid females per cage were run for each bioassay, and each bioassay was repeated at least twice on different dates.

The responses of gravid females to 3-methylindole or the synthetic egg raft pheromone alone or in blends were tested in multiple choice assays using a factorial design. The concentration of pheromone was held constant at 0.1 mg (loaded on a 2.2-cm-diam glass cover slip floated on the water surface) per 100 ml water (1 mg/liter), while the concentration of 3-methylindole was varied from 10 ng to 0.1 mg/100 ml water (100 ng to 1 mg/liter). A treatment or control cup (9.5 cm diam glass crystallizing dish with 100 ml distilled water plus a glass cover slip) was placed in each of the 4 corners of the cages, with the placement of all cups being randomized between replicates.

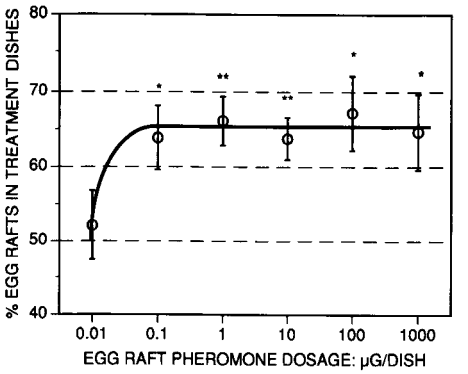

Fig. 1. Oviposition responses of gravid female *Culex quinquefasciatus* to different doses of synthetic egg raft pheromone presented on floating glass discs. Circles indicate means, error bars included. Asterisks indicate significant differences between treatment and control cups using 2-tailed paired *t*-tests (* = $P < 0.05$, ** = $P < 0.01$).

All assays were scored the following morning at 0900 h by counting the number of egg rafts laid in each of the oviposition cups. Raw data were transformed (ln[x + 1]) and treatments were compared to controls by paired *t*-tests. Means in factorial experiments were separated by ANOVA-protected Fisher's least significant differences tests. To determine whether the treatment effects were additive or synergistic in the factorial experiments, an orthogonal contrasts test was used, testing the null hypothesis that the sum of the responses to the 3-methylindole and pheromone treatments was equal to the response to the blend (Snedecor and Cochran 1980).

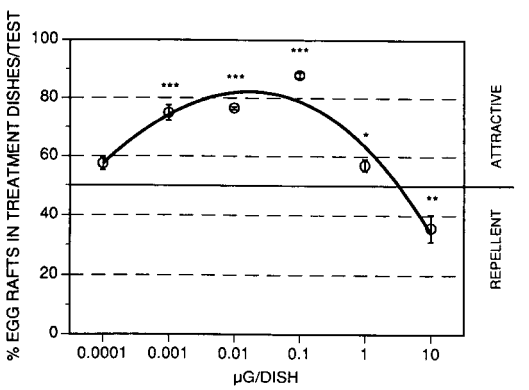

Fig. 2. Oviposition responses of gravid female *Culex quinquefasciatus* to different doses of the habitat-derived compound 3-methylindole in water. Circles indicate means, error bars included. Asterisks indicate significant differences between treatment and control cups using 2-tailed paired *t*-tests (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

Table 1. Effect of 12- to 24-h-old conspecific egg rafts on oviposition behavior of gravid *Culex quinquefasciatus* females.

Rafts/ dish	No. trials[1]	Mean number of egg rafts deposited: Distilled water + egg rafts	Mean number of egg rafts deposited: Distilled water
2	4	29.4	27.4 NS
5	4	37.6	30.1 NS
10	4	38.9	40.5 NS
15	6	44.5	41.4 NS

[1] Each trial consisted of 6 cages with 20 mosquitoes/cage.

RESULTS

Effect of conspecific egg rafts: Attempts to demonstrate that conspecific egg rafts influenced *Cx. quinquefasciatus* oviposition failed to elicit responses from gravid females. Oviposition cups treated with 2, 5, or 15 12- to 24-h old egg rafts were no more attractive than the distilled water controls (Table 1). To corroborate these results, each test was multiply replicated over several days (4–6 repetions, with 4 replicates per repetition).

Response to synthetic egg raft pheromone: Synthetic egg raft pheromone was tested as an oviposition attractant for gravid *Cx. quinquefasciatus* females at doses from 0.01 µg to 1 mg/100 ml water (Fig. 1). Significantly more egg rafts were laid in the treated dishes than in the distilled water controls for all except the lowest dose (0.01 µg). All doses between 0.1 µg and 1 mg received approximately equal levels of oviposition (63–67% of total oviposition). Thus, although the synthetic egg raft pheromone did have some biological activity, the activity was weak.

Response to 3-methylindole: Mosquitoes were acutely sensitive to 3-methylindole (Fig. 2), with the threshold dose for response being between 0.0001 and 0.001 µg/100 ml water (between 1 and 10 parts per trillion). The response increased with increasing dosage up to 0.1 µg, and then decreased, with the 1 µg dosage being no more stimulatory than the distilled water control, and the 10 µg dosage being significantly repellent or inhibitory. Thus, the range of doses eliciting a positive response covers about 3 orders of magnitude; beyond this range, 3-methylindole becomes repellent or deterrent.

Effect of blends of pheromone and 3-methyl-

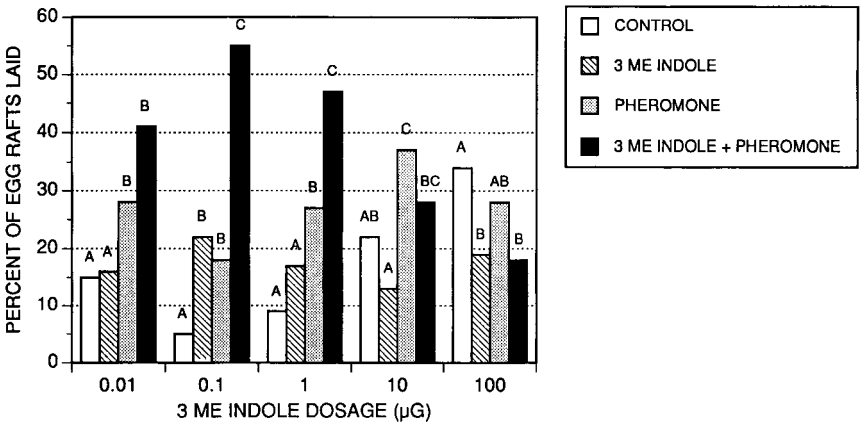

Fig. 3. Oviposition responses of gravid female *Culex quinquefasciatus* in multifactorial experiments to a distilled water control, egg raft pheromone alone, 3-methylindole, and blends of egg raft pheromone with 3-methylindole. The dose of egg raft pheromone was held constant at 100 μg, while the dose of 3-methylindole was varied. The number of egg rafts deposited for each category and dosage are given as a percent of the total number of egg rafts laid in that experiment. The letters signify differences of site selection within a particular dose, not comparisons between doses.

indole: The responses of gravid females to 3-methylindole and the synthetic egg raft pheromone, alone or in blends, were tested in multiple choice assays using a factorial design (Fig. 3). At the lowest dose of 3-methylindole (0.01 μg), the 3-methylindole treatment and the distilled water controls were not significantly different. The blend of 3-methylindole with the pheromone produced a significantly higher response than the response to 3-methylindole alone, which was however, not significantly different than the response to the pheromone alone.

Increasing the dose of 3-methylindole to 0.1 μg resulted in increased percentages of egg rafts being laid in the 3-methylindole and blend treatments, with less than 5% of the egg rafts being laid in the control. As the dose of 3-methylindole was increased further to 1 and 10 μg, into the repellent–deterrent concentration range (*vide supra,* Fig. 2) the percentage of egg rafts laid in the 3-methylindole and the blend cups decreased, whereas the percentages in the pheromone and control cups increased. At the highest dose of 3-methylindole tested (100 μg), the 3-methylindole alone and the blend treatments received significantly fewer egg rafts than the distilled water control, demonstrating the repellent–deterrent effect of the superoptimal dose.

For the 3 lowest doses of 3-methylindole (0.01–1 μg), orthogonal contrasts tests in all cases failed to reject the null hypothesis that the sum of the responses to the individual treatments was equal to the response to the blend of the 2 components, suggesting that the responses seen were additive rather than synergistic. The highest 2 doses were not subjected to this test, as the responses to the blends were equal to or less than the distilled water controls.

DISCUSSION

In the first experiment, testing the attractive effects of conspecific egg rafts gave the unexpected result that the presence of 2–15 egg rafts apparently had no significant effect on oviposition. It has been previously reported that as little as one egg raft can significantly influence oviposition in bioassays very similar to those used in this study (Bruno and Laurence 1979). However, several authors have reported considerable variability in the sensitivity and responses of different strains of *Cx. quinquefasciatus* to conspecific egg rafts and to synthetic egg raft pheromone (Laurence and Pickett 1985, Otieno et al. 1988a, Dawson et al. 1990). The preference for the pheromone is often significant but not overwhelming. For example, reported ratios of oviposition in pheromone treatments versus distilled water controls may be as low as 1.5:1 (Dawson et al. 1990). Thus, literature reports corroborate the data reported here that the response to the natural pheromone may be weak and variable. Similar results have been reported for the congeneric *Cx. tarsalis,* with one strain responding well to 10 egg rafts (Bruno and Laurence 1979), whereas a different strain required 200–2,000 egg rafts in order to demonstrate an effect (Osgood 1971).

Initial experiments to verify the biological activity of the synthetic egg raft pheromone were carried out using the "direct" approach of Hwang

et al. (1987), in which 1,2-dichloroethane solutions of the pheromone were added directly to the water surface in the oviposition cup. Results obtained with this bioassay were inconsistent. More detailed examination revealed a possible source of the inconsistency, because it was found that the drops of dichloroethane solution, which were denser than water, would often sink to the bottom of the cup instead of dispersing over the water surface. Consequently, this bioassay method was modified to alleviate this problem. Consistent results were obtained with the floating disc method, in which the pheromone solution was pipetted onto the surface of a glass cover slip, which was then floated on the water surface in the oviposition cup. With this improved bioassay method, the responses obtained to the synthetic pheromone were significant but not overwhelming, with the synthetic pheromone treatments consistently receiving approximately 60–70% of the total oviposition. The doses used (0.01–1,000 μg of the mixture of 4 stereoisomers) bracketed the amount obtained from individual egg rafts (0.3 μg) (Laurence and Pickett 1982).

Responses also did not increase regularly with increasing dosage, possibly due to the low volatility of the pheromone. With a low vapor pressure and a limited area for release, increasing the dose of pheromone applied to the small glass cover slips may only result in minimal increases in the concentration of the pheromone in the vapor phase, and little increase in the active space of the pheromone.

The response to the habitat-derived oviposition attractant 3-methylindole was more straightforward, with a response threshhold in the range of 0.0001–0.001 μg per 100 ml water, and a steady increase in response up to a critical concentration, beyond which the 3-methylindole became repellent or inhibitory. This dose–response relationship is in agreement with preliminary work from our laboratory (Millar et al. 1992), and with the 0.0002 μg response threshold reported in an independent study with a different *Cx. quinquefasciatus* strain (Mordue et al. 1992). The repellent effect seen at higher concentrations suggests that although mosquitoes may respond positively to low levels of metabolic products from degradation of organic matter, higher concentrations of the same cues deter mosquitoes from ovipositing in waters that may be too polluted, or otherwise detrimental to development of their offspring.

Responses to blends of the synthetic pheromone and 3-methylindole proved to be additive rather than synergistic. Analogous results have been recently reported in a multifactorial experiment testing the biological activity of pheromone, of fermented rabbit pellet infusion, and of a blend of the pheromone with infusion (Mordue et al. 1992). Interestingly, 3-methylindole was not detected in the rabbit pellet infusion, despite careful analysis, suggesting that the suite of chemicals influencing *Cx. quinquefasciatus* behavior is flexible, with mosquitoes able to respond to a range of different chemical constituents, in keeping with the presumably variable chemical constitution of natural oviposition sites. Field studies by Otieno et al. (1988b) also suggested an interaction between habitat-derived cues and the egg raft pheromone, because it was found that freshly prepared tap water solutions of the pheromone in aluminum trays on a veranda did not become attractive until they began to collect wind-blown debris.

The mechanism, or combinations of mechanisms, by which the pheromone and 3-methylindole or other habitat-derived cues exert their effects is unclear. Electrophysiological studies have demonstrated that both the pheromone and 3-methylindole are perceived by antennal receptors (Mordue et al. 1992), and this result has been corroborated for the pheromone by wind tunnel behavioral studies (Pile et al. 1991). The results of our floating-disc type bioassays in which the pheromone is placed on a water-impermeable disc on the water surface also suggest an olfactory perception mechanism. Furthermore, the pheromone is produced in the apical droplets of egg rafts, rather than being released into the water, and it has been demonstrated that removal of egg rafts from the water eliminates the oviposition response, again suggesting an olfactory mechanism (Bruno and Laurence 1979). However, other tests in which the pheromone is dispersed into the oviposition waters, either by effervescent tablets (Otieno et al. 1988a, 1988b) or with the surfactant Triton X-100 (Sakakibara et al. 1984) have generally resulted in much higher response levels than are seen when the pheromone is released only from a floating disc, suggesting that the pheromone may also act as a contact oviposition stimulant.

In summary, both 3-methylindole and the egg raft pheromone have been shown to influence *Cx. quinquefasciatus* oviposition behavior in both laboratory (Millar et al. 1992, Mordue et al. 1992) and field bioassays (Otieno et al. 1988a, 1988b; Beehler et al. 1994). The additive effect of the 2 compounds suggests that blends of the 2 components may provide a useful attractive mixture for use in mosquito surveillance and control programs.

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