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Sandra Allan

USDA-ARS, sandy.allan@ars.usda.gov

Ulrich R. Bernier

University of Florida, ubernier@gainesville.usda.ufl.edu

David Kline

United States Department of Agriculture-ARS-Center for Medical

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EVALUATION OF OVIPOSITION SUBSTRATES AND ORGANIC INFUSIONS ON COLLECTION OF *CULEX* IN FLORIDA

SANDRA A. ALLAN, ULRICH R. BERNIER AND DANIEL L. KLINE

USDA/ARS, Center for Medical, Agricultural, and Veterinary Entomology, 1600/1700 SW 23rd Drive, Gainesville, FL 32608

ABSTRACT. Gravid mosquito traps are commonly used for both arbovirus surveillance and population surveillance of mosquitoes of the genus *Culex*. Oviposition substrates, used as baits in these traps, were tested against *Culex* under laboratory and field conditions. In the laboratory all substrates tested as 1% and 10% dilutions in 2-choice bioassays against female *Cx. quinquefasciatus* were significantly more effective than well water controls in eliciting oviposition. Strongest responses were to dilutions of dairy effluent, followed by larval water and infusions of alfalfa hay, alfalfa pellets, Bermuda hay, oak leaves, and *Typha* leaves, with lowest responses to cow manure infusion. In the field, few significant differences in collections were obtained between traps baited with different infusions. Significantly more *Cx. quinquefasciatus* and *Cx. nigripalpus* were collected in traps baited with cow manure infusion (highest) compared to alfalfa hay infusion (lowest). Responses of *Cx. quinquefasciatus* to dairy effluent and infusions of Bermuda hay, oak leaves, and *Typha* leaves were not significantly different from either cow manure infusion or alfalfa hay infusion. Responses of *Cx. nigripalpus* were highest to cow manure infusion and equally low to infusions of alfalfa hay and *Typha* leaves; moderate responses were observed to dairy effluent and infusions of Bermuda hay and oak leaves. Gravid females comprised 66.7–81.9% of the collections for each infusion type, with no significant difference among infusions in the proportion of gravid females collected.

KEY WORDS Mosquito, organic infusion, oviposition attractant, *Culex quinquefasciatus*, *Culex nigripalpus*

INTRODUCTION

Mosquitoes in the genus *Culex* have been implicated as the primary vectors of West Nile virus and St. Louis encephalitis in North America (Day 2001, Goddard et al. 2002). Effective monitoring of these species is essential as part of effective control programs, as well as population and disease surveillance. Although conventional light traps collect many *Culex* spp., they may underrepresent some of the vector species present (Sudia et al. 1967, Nayar et al. 2001). Moreover, collections from light traps consist primarily of unfed females, which are less likely to contain virus than females that have blood-fed or are gravid. Gravid mosquito traps offer another approach for surveillance because they are designed to collect gravid, bloodfed female mosquitoes (primarily *Culex* spp.) that are attracted to approach a potential oviposition site (Reiter 1983). Collections from these gravid traps may contain from 57% (Ritchie 1984) to 95% (Reiter 1987) gravid females and these traps are often incorporated into surveillance efforts (Savage et al. 1993, Nayar et al. 2001, Nasci et al. 2002).

Culex spp. generally oviposit in water high in organic matter and a wide array of oviposition substrates are reported as attractive to gravid females. These substrates may consist of naturally occurring materials such as water from field sites (natural or agricultural), rearing water, or organic infusions that consist of complex mixtures of compounds (Bentley and Day 1989). These compounds may be mosquito attractants, repellents, or both depending on concentration. Behaviorally active compounds are considered products of bacterial fermentation

(Ikeshoji et al. 1975, Beehler et al. 1994) and their composition and concentrations are dynamic through time. Sources of variability in composition of infusions may include source material (species of plant, age and quality of material, presence of weeds, and so on) and conditions of fermentation (temperature, darkness, duration, and so on) and this variability can confound comparisons between studies (Brust 1990, Lampman and Novak 1996). However, in the absence of standardized controlled-release lures of known composition, infusions remain the most practical means for baiting gravid female traps.

Infusions that have been reported as attractive to *Culex* spp. have been made by using materials such as hay (Reiter 1983), hay in conjunction with isopropyl alcohol (Ritchie 1984, Reisen and Meyer 1990), Bermuda hay (Isoe and Millar 1995), alfalfa hay (Reisen and Meyer 1990), grass and logs (Gjullin et al. 1965), sod (Brust 1990), alfalfa pellets (rabbit chow) (Lewis et al. 1974), laboratory animal chow (Kramer and Mulla 1979), bullrush (Du and Millar 1999, Reisen et al. 1999), chicken manure (Kramer and Mulla 1979), cow manure (Leiser and Beier 1982), horse manure (O'Gower 1963), and oak leaves (O'Meara et al. 1989). In trials in Illinois (Lampman and Novak 1996) and California (Reisen and Meyer 1990), significant differences were observed between local *Culex* spp. in responses to a range of infusions. In this study we compared the effectiveness of various oviposition substrates reported in the literature on collection of *Culex* spp. in gravid female traps in Florida.

MATERIALS AND METHODS

Culex quinquefasciatus Say was reared by using conventional methods with larvae at a standardized density and level of nutrition (1,000 immatures/3 liters of water and fed on a 1:1 mixture of liver powder and brewer's yeast) (Gerberg et al. 1994). Females were fed 5–7 days after emergence on manually defibrinated bovine blood and then held for 6 days at 27–29°C and 85–90% relative humidity under a photoperiod of 14:10 h light:dark. A 10% sugar solution was provided continuously.

Infusions: Age of infusion can significantly influence mosquito response (Kramer and Mulla 1979, Brust 1990, Du and Millar 1999), so published protocols for preparation of effective oviposition substrates were followed. Bermuda hay infusion was prepared following Reiter (1983) and Millar et al. (1992) by adding 225 g of Bermuda grass hay (*Cynodon dactylon*), 10 g of brewer's yeast, and 10 g of lactalbumen hydrolysate to 40 liters of well water (at room temperature 25–27°C), letting the mixture sit for 12 days. Bermuda grass hay was obtained from a local farm supplier. Alfalfa hay infusion was prepared similarly to the Bermuda hay infusion except dried alfalfa hay (Kaytee Products Inc., Chilton, WI) was used. Infusions of cow manure were prepared by following Leiser and Beirer (1982) by adding 1.2 kg of fresh moist manure to 20 liters of well water and steeping for 7 days. Oak leaf infusion was prepared by following O'Meara et al. (1989) by adding 675 g of dried oak leaves (combination of live oak [*Quercus virginiana*] and laurel oak [*Quercus laurifolia*]) to 19 liters of well water and steeping for 7 days. An infusion from alfalfa pellets was prepared by following Strickman (1988) by adding 32 g of alfalfa pellets (rabbit pellets, Kaytee Products Inc.) to 4 liters of well water and steeping for 11 days. An infusion was prepared similar to the bulrush infusion of Du and Millar (1999) except that we used cattail (*Typha latifolia*), a common local species. Leaves were collected, dried, and then 450 g was added to 20 liters of well water and steeped for 7 days. Water from an effluent lagoon at a commercial dairy that contained cow manure, egg rafts, and immatures (primarily *Cx. quinquefasciatus* with low numbers of *Culex nigripalpus* Theobald) also was included as a treatment. This and adjacent lagoons were considered the primary sources of the populations of *Cx. quinquefasciatus* at the commercial dairy. Water collected from the lagoon was sieved to remove particulate matter and immatures.

Larval water for use in laboratory bioassays was obtained from standard rearing practices for *Cx. quinquefasciatus* (Gerberg et al. 1994), with larvae reared at a density of about 1,000 per liter of water. Larval water was collected just before larvae pupated (ca. 6 days). Infusions were made and allowed to ferment for the appropriate times and then frozen (–20°C) in 50-ml and 1-liter aliquots for use

in laboratory and field studies. Frozen aliquots of infusions have been used successfully in previous studies to overcome some of the inherent variability from batch to batch of infusion (Kramer and Mulla 1979, Isoe et al. 1995, Trexler et al. 1998, Du and Millar 1999).

Laboratory bioassays: Two-choice bioassays were conducted with gravid mosquitoes held in a cage with 2 cups as choices for oviposition sites, including 1 control cup (well water) and 1 treatment cup (infusion). Bioassays were conducted in Plexiglas® bioassay cages (30 cm³) fitted with a cotton stockinette sleeve on one end and screening on the other end. Twenty female mosquitoes were verified gravid on a chill table, and then placed in bioassay cages. Sugar solution also was present in each bioassay cage. Infusions or water were placed in polypropylene cups (120 ml, Sweetheart Cup Co., Owings Mills, MD) that were previously spray painted with a flat-black paint. To remove possible contaminants and odors from the paint, paint was allowed to dry for several days and the cups were thoroughly rinsed in well water before use. Two cups were placed approximately 12 cm apart in the center of each bioassay cage. The control cup contained 50 ml of well water and the treatment cup contained 50 ml of the test infusion. The positions of treatment and control cups were random (through use of a random number table [Zar 1999]). Frozen infusions were thawed immediately before use and diluted with warm well water to reach a temperature of 25–27°C. Diluted infusions were used immediately for bioassays and not stored.

Bioassays were set up in late afternoon, treatments were placed in cages, and treatments were removed 22–24 h later. Bioassays were conducted at 26–29°C and a photoperiod of 14:10 h light:dark with 1 h of dusk. Bioassays were replicated 30 times with 6–10 replicates completed each day. Means of the replicates completed each day were compared by analysis of variance (ANOVA, SAS Institute 1999) to verify that no between-day differences existed before combining data. The positions of treatment and control cups (right or left) were noted and assigned randomly between the 2 positions. No position effect was present within any treatment (paired *t*-test, $P > 0.05$). After removal from cages, the numbers of egg rafts in the treatment and control cups were counted, the total number of egg rafts deposited in each cage determined, and the percentage of egg rafts deposited in the treatment and control cups in each cage was calculated.

Field study: The field study was conducted at a commercial dairy farm in Marion County, Florida (28°58'N, 82°24'W) at an effluent lagoon surrounded by grass (*Digitaria* spp.), sedges (*Cyperus* spp.), and herbaceous vegetation (primarily *Ambrosia artemisiifolia*, *Amaranthus hybridus*, and *Ipomoea trichocarpa*). Traps at the farm were placed adjacent to the effluent lagoon. Vegetation was cleared ad-

Table 1. Percentages (mean $\pm$ SE) of gravid *Culex quinquefasciatus* ovipositing in cups containing organic substrates or infusion (treatment) or well water (control) in a 2-choice laboratory assay ($n = 30$ replicates each with 20 mosquitoes).¹

	1% dilution			10% dilution		
	Treatment	Control	P	Treatment	Control	P
Alfalfa hay	76.4 (3.5)b	23.6 (3.5)	<0.001	74.1 (3.5)b	25.9 (3.5)	<0.001
Alfalfa pellets	80.4 (2.3)b	19.6 (2.3)	<0.001	75.8 (4.8)b	24.2 (4.8)	<0.001
Bermuda hay	82.6 (3.5)ab	17.4 (3.5)	<0.001	84.7 (3.6)b	15.3 (3.7)	<0.001
Cow manure	59.1 (4.2)c	40.9 (4.2)	<0.001	67.9 (3.5)c	32.1 (3.5)	<0.001
Dairy effluent	84.2 (2.1)a	15.8 (2.1)	<0.001	95.4 (1.1)a	4.6 (1.1)	<0.001
Larval water	83.9 (2.7)ab	16.1 (2.7)	<0.001	80.8 (2.5)b	19.2 (2.5)	<0.001
Oak leaves	74.3 (4.8)b	25.7 (4.8)	<0.001	84.2 (2.3)b	15.9 (2.3)	<0.001
<i>Typha</i> leaves	73.2 (3.0)b	26.8 (3.0)	<0.001	80.5 (3.5)b	15.9 (3.5)	<0.001
Water	50.5 (4.6)d	53.9 (4.7)	0.123	50.5 (4.6)d	53.9 (4.7)	0.123

¹ Means in a column followed by the same letter are not significantly different ($P < 0.05$, Tukey's standardized test [SAS Institute 1999]).

adjacent to traps and traps placed 0.25–0.5 m from the edge of lagoon.

The traps used for this study were Centers for Disease Control gravid traps (model 1712, John W. Hock Company, Gainesville, FL) supplied with a small green pan (ca. 22 cm wide $\times$ 34 cm long $\times$ 17 cm deep, Rubbermaid® 439, Rubbermaid® Commercial Products, Winchester, VA). Pans were pre-conditioned by filling with water and letting sit for at least 1 wk, then were scrubbed, rinsed, and dried before use. Traps were positioned at ca. 20-m intervals along the side of the dairy lagoon in Marion County and were moved daily among positions. Traps were not visible between positions. Each day batteries were replaced and traps were baited with 2 liters of freshly thawed infusion. Traps were evaluated for 12 nights and placed in the field at 1000–1200 h and picked up 22–24 h later. Mosquitoes were returned to the laboratory; sorted to species, sex, and gonotrophic condition; and counted. Gonotrophic condition was classified by following Edman et al. (1975). Females containing blood but no developed eggs were considered bloodfed, those with fully developed eggs were considered gravid, and those with partially digested blood were classified as bloodfed if they contained more blood than eggs or gravid if they contained more eggs than blood. Mosquitoes too damaged for species identification were classified by genus.

Statistical analysis: Means presented in tables are untransformed. Laboratory bioassay data (percentages) were arc-sine transformed and comparisons between treatments and controls were made with paired *t*-tests. Comparisons between treatments were made with PROC GLM (SAS Institute 1999) followed by means separations by using Tukey's standardized range test. Trap counts were transformed by $\log(x + 1)$ before analysis. Data analysis was conducted by using PROC GLM with treatment and position effects and interactions tested. The percentages of gravid females in each trap were arc-sine transformed before being analyzed by

PROC GLM and tested for means separation by using Tukey's standardized range test.

RESULTS

Laboratory bioassays

Results are summarized in Table 1. Oviposition responses of female *Cx. quinquefasciatus* in 2-choice bioassays were significantly greater to all of the infusions compared to the water controls ($P < 0.001$). Responses to water as a treatment were not different from those to water as a control ($P = 0.123$). For comparisons between infusions, significant differences were present with both 1% dilutions (ANOVA; $df = 8, 261$, $F = 51.97$, $P < 0.001$) and 10% dilutions (ANOVA; $df = 8, 261$, $F = 5.51$, $P < 0.001$). All infusions at both 1% and 10% dilutions elicited significantly more oviposition in the treatment cups than did the water controls (Table 1). The lowest oviposition responses to all infusions were to the 1% and 10% cow manure dilutions (59.1% and 67.9%, respectively). Responses were greatest at 1% dilution to the dairy effluent (84.2%), Bermuda hay infusion (82.6%), and larval water (83.9%). Lower responses that were not significantly different from the Bermuda hay infusions and larval water were obtained in response to infusions of alfalfa pellets (80.4%), alfalfa hay (76.4%), oak leaves (74.3%), and *Typha* leaves (73.2%). At 10% dilution, responses followed a similar pattern, with the greatest response to dairy effluent (95.4%). High responses also were obtained to Bermuda hay (84.7%), oak leaves (84.2%), larval water (80.8%), alfalfa pellets (75.8%), and alfalfa hay (74.1%). The lowest response to infusions was to the cow manure infusion (67.9%).

Field study

Results are summarized in Table 2. In the field comparison of the effect of infusions on trap col-

Table 2. Effect of infusion type on average daily collection (mean (SE)) of mosquitoes in Centers for Disease Control gravid traps placed on commercial dairy farm in Marion County, Florida.¹

	Alfalfa hay	Bermuda hay	Cow manure	Dairy effluent	Oak leaves	<i>Typha</i> leaves
Total female <i>Culex</i>	68.3 (8.6)b	146.3 (19.6)ab	178.7 (35.2)a	133.2 (29.8)ab	143.1 (30.7)ab	135.0 (30.3)ab
<i>Cx. quinquefasciatus</i>	40.6 (5.1)b	98.6 (16.9)ab	117.4 (30.3)a	85.0 (19.5)ab	90.7 (20.1)ab	107.0 (27.2)ab
<i>Cx. nigripalpus</i>	27.2 (4.3)b	47.6 (5.9)ab	70.8 (13.8)a	48.2 (11.8)ab	52.5 (11.4)ab	30.0 (3.5)b
Total gravid female						
<i>Culex</i>	54.8 (8.3)b	104.8 (14.2)a	123.8 (27.5)a	103.4 (23.1)a	109.2 (22.9)a	114.8 (25.5)a
<i>Cx. quinquefasciatus</i>	31.0 (5.6)b	72.6 (13.0)ab	98.6 (25.5)a	68.9 (15.2)ab	68.9 (13.8)ab	90.4 (22.7)a
<i>Cx. nigripalpus</i>	24.8 (3.4)b	32.2 (3.0)ab	43.4 (9.7)a	34.6 (9.2)ab	37.5 (8.9)ab	24.4 (3.4)b
Bloodfed females	1.0 (0.4)a	1.8 (0.6)a	0.8 (0.3)a	0.6 (0.4)a	0.8 (0.4)a	1.8 (0.6)a
Male <i>Culex</i>	7.6 (1.5)a	12.4 (2.9)a	20.5 (5.8)a	11.4 (2.5)a	16.7 (6.9)a	9.4 (3.3)a
<i>Culex</i> spp.	0.1 (0.1)a	0.6 (0.5)a	2.7 (0.5)a	2.8 (0.7)a	5.7 (4.2)a	2.8 (0.5)a

¹ Means in a row followed by the same letter are not significantly different ($P < 0.05$, Tukey's standardized test [SAS Institute 1999]).

lections, a total of 11,604 *Culex* were collected over 72 trap nights. The most common mosquitoes collected were female *Cx. quinquefasciatus* (58.7%) and *Cx. nigripalpus* (31.9%); however, low numbers (<40) of *Uranotaenia lowii* Theobald, *Aedes albopictus* (Skuse), and *Culex salinarius* Coquillett also were present. The remaining collection consisted of males (7.2%) and *Culex* that could not be identified (1.6%).

Significant differences in trap collections existed between infusions for number of females *Cx. quinquefasciatus*, female *Cx. Nigripalpus*, total female *Culex*, gravid *Cx. quinquefasciatus*, gravid *Cx. Nigripalpus*, and total gravid females ($P < 0.01$), but not for male *Culex*, or *Culex* spp. Relatively few bloodfed mosquitoes were collected in traps and no difference was found in numbers collected between traps with different infusions. Traps with cow manure infusions collected significantly more female *Culex* than those with alfalfa hay infusion. However, no difference was found in collections from traps with cow manure, *Typha* leaves, Bermuda hay, dairy effluent, and oak leaves. Collections of female *Cx. quinquefasciatus* showed a similar trend. However, fewer female *Cx. nigripalpus* were collected in traps with alfalfa hay and *Typha* leaves compared to cow manure. Significantly fewer gravid females were collected in traps baited with alfalfa hay compared to the other infusions. For *Cx. quinquefasciatus*, significantly more gravid females were collected in traps baited with cow manure and *Typha* leaves compared to alfalfa hay. However, no differences were found between cow manure and *Typha* leaves and Bermuda hay, dairy effluent, and oak leaves. Significantly fewer gravid *Cx. nigripalpus* were collected in traps with alfalfa hay and *Typha* leaf infusions than with cow manure infusions. No difference was found in collections between traps with Bermuda hay, dairy effluent, oak leaves, or cow manure infusions. The portion of trap collections that represented gravid females ranged from 66.7% to 81.9%, with no difference between infusions (PROC GLM; $df = 5,66$, $F = 0.86$, $P = 0.51$). No differences were found in col-

lections of male *Culex* and bloodfed *Culex* between traps baited with different infusions.

DISCUSSION

In our laboratory studies, oviposition responses by *Cx. quinquefasciatus* were high to all of the infusions except the cow manure infusion. The greatest responses were to the dairy effluent, with stronger responses at the 10% dilution compared to the 1% dilution. Effluent water likely contained a composite of potential oviposition attractants from several sources including cow manure, and high numbers of immatures and egg rafts. Cow manure provides a substrate for bacterial activity that could result in emission of attractant compounds. Attraction to water that contained larvae appeared to be associated with the presence of immature mosquitoes (Wilmot et al. 1987) and their associated bacterial fauna (Benzon and Apperson 1988). This has been reported in numerous species of *Culex*, such as *Culex tarsalis* Coquillett (Hudson and McLintock 1967), *Culex pipiens molestus* Forskal and *Cx. tri-taeniorhynchus summorosus* Day (Nakamura 1978), *Cx. quinquefasciatus* Say (Suleman and Shirin 1981), and *Culex annulirostris* Skuse and *Culex molestus* Forskal (Dhileepan 1997). An oviposition pheromone identified from egg rafts of *Cx. quinquefasciatus* also is an oviposition attractant for several species of *Culex* (Laurence and Pickett 1985). Reisen and Meyer (1990) evaluated water from a natural field site in laboratory assays with mixed results and reported that it was only as attractive as tap water for oviposition by *Cx. quinquefasciatus* and *Cx. tarsalis*. Subsequently, Isoe and Millar (1995) evaluated field water from 2 sites and obtained significant oviposition by *Cx. tarsalis* to water from only 1 of the sites. In their study, responses to the field water were similar to those to Bermuda grass infusion. In contrast, we obtained the highest responses to the dairy effluent from the lagoon that was also a field site for immatures. Differences between these studies may reflect differing levels of organic matter present at the study sites.

Responses in our study to larval water were high but not significantly different from those to Bermuda hay infusion, which is generally considered a good oviposition attractant (Isoe and Millar 1995, Isoe et al. 1995). Although attractant compounds have been identified from Bermuda hay infusion (Millar et al. 1992), it is not known if the same compounds are responsible for the attraction to larval water. Attraction of gravid females to manure and the other infusions is likely due to compounds produced by the bacterial decomposition of the organic matter. Presumably differences between infusions result from the composition and density of bacterial flora in conjunction with richness in organic matter as substrate. The lowest responses to infusions in our laboratory study occurred in response to cow manure with a slight increase in response (<10%) with increase in concentration (from 1% to 10%). In contrast, Reisen and Meyer (1990) reported steer manure as the most attractive infusion for both *Cx. quinquefasciatus* and *Cx. tarsalis*. The difference in these results may be related to the difference in methods for preparation of the manure infusion because our infusion was made with moist rather than the dry manure and fermented for 1 wk rather than 3 wk.

In laboratory studies, infusions were diluted and tested at 1% and 10% concentrations to reduce repellent effects at high concentration in small bioassay chambers. Testing at these dilutions generally provides representative comparisons of infusions (Kramer and Mulla 1979, Allan and Kline 1995, Isoe and Millar 1995, Du and Millar 1999).

Most of the infusions tested were highly effective for collection of both *Cx. quinquefasciatus* and *Cx. nigripalpus* in gravid female traps. In general, laboratory results with *Cx. quinquefasciatus* supported the results in the field study except for the cow manure infusion. In laboratory assays, responses to this infusion were significantly lower than to the other infusions; however, under field conditions, traps with this infusion collected the greatest number of mosquitoes. One possible reason for the difference in responses could be due to concentration because the field tests were conducted with undiluted infusion and the laboratory tests were conducted with 1% and 10% dilutions of the infusions. The increased oviposition from 59.1% response (1% cow manure infusion) to 67.9% response (10% cow manure infusion) appears to support this. Many of the infusions were equally effective for collection of both *Cx. quinquefasciatus* and *Cx. nigripalpus*, with the lowest responses consistently to alfalfa hay infusions. A similar report of the lower responses to alfalfa hay infusion compared to Bermuda hay infusion was reported by Isoe and Millar (1995).

Responses of gravid *Cx. quinquefasciatus* and *Cx. nigripalpus* to the infusions tested seemed relatively similar with the exception of the lower response of *Cx. nigripalpus* to the *Typha* infusion.

Although *Cx. quinquefasciatus* and *Cx. nigripalpus* may be present in the same larval habitat (as they were in this study), *Cx. quinquefasciatus* generally is present in larval habitats that are higher in organic matter than those of *Cx. nigripalpus* (Provost 1969). In studies by Reisen and Meyer (1990), responses by *Cx. tarsalis*, a species associated with habitats with cleaner water than *Cx. quinquefasciatus*, to infusions were very low compared to *Cx. quinquefasciatus*. In field trials conducted by Lampman and Novak (1996) on attraction to infusions, appreciably lower responses were reported for *Culex restuans* Theobald, a species associated with less polluted larval habitats, than *Cx. pipiens*. In our study, collections of immatures and eggtraps made in the adjacent dairy lagoon throughout the trapping period consisted of both *Cx. quinquefasciatus* and *Cx. nigripalpus* with *Cx. nigripalpus* comprising one third or less of the collections (unpublished data). Trap collections of both species of *Culex* appeared to be representative of what was produced in the lagoon, with both species responding to the range of infusions tested.

In summary, a range of infusions was effective as baits in gravid female traps for collection of both *Cx. quinquefasciatus* and *Cx. nigripalpus*. Infusions made with cow manure, oak leaves, and Bermuda hay were consistently attractive to both species and should serve as effective baits for gravid female traps for these species.

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